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Effects of nutrient limitation on cell growth, TEP production and aggregate formation of marine *Synechococcus*

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ABSTRACT: Pico-cyanobacteria are abundant primary producers in most of the global ocean but their role in the export of organic carbon to depth remains a matter of debate. A recent laboratory study using roller tanks showed that marine *Synechococcus* cells can form visible (>1 mm) aggregates that sink at velocities of more than 400 m d^{-1} in seawater. The present study aimed to investigate the mechanism behind such aggregation by exploring 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 the lowered growth rates, cells in the nutrient-limited cultures had higher cell-normalized TEP production, and formed a larger total volume of aggregates that had higher settling velocities compared to aggregates formed from cells in the nutrient-replete cultures. This study contributes to our understanding of the physiology of marine *Synechococcus* as well as their role in the ecology and biogeochemistry in the oceans.

KEY WORDS: Nutrient limitation · Marine *Synechococcus* · TEP production · Aggregate formation

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 slime (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 bacterial cells and clay particles via cation bridging (Kiørboe & Hansen 1993, Bhaskar & Bhosle 2005), and are also resistant to hydrolysis by bacteria (Aluwihare & Repeta 1999).

In seawater, APS form larger colloids within hours to days, and eventually form 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). Therefore, they are especially important during phytoplankton blooms in

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which they facilitate the aggregation and subsequent sedimentation of phytoplankton cells that otherwise would be too small to sink (Passow et al. 2001), thus enhancing 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, distinct from conventional microbial uptake/growth (Alldredge et al. 1993, Engel et al. 2004). In the field, the length of individual TEP can range from 3 to 5 µm up to several 100 µm (Passow & Alldredge 1994), and their concentration in different oceanic environments ranges from 10 to 900 µg l^{-1} xanthan gum equivalent (Passow & Alldredge, 1995) depending on season, depth, and plankton community composition (Wurl et al. 2011). During phytoplankton blooms, the potential importance of TEP is actually 2-fold; 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 an intracellular 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, Radi 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).

Kiørboe 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 was observed to increase in the cases of nitrogen, phosphorus, iron and silicon limitation in batch cultures (Corzo et al. 2000, Berman-Frank et al. 2007) and mesocosm experiments (Mari et al. 2005). *In situ* analyses (Mari & Burd 1998) have also shown higher TEP abundance during periods of low inorganic N:P ratios in the surface mixed layer. Nutrient limitation can also affect the interaction between heterotrophic bacteria and phytoplankton in TEP production (Gärdes 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 fixation in autotrophs, which has to be excreted to maintain the autotrophs' elemental homeostasis (Elser et al. 2000, Hessen et al. 2004). In this scenario, the proportion of excreted carbon that cannot be used for cell growth will increase due to nutrient limitation, and excreted polysaccharides will serve as the precursors of TEP (Passow 2000).

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 their small size and lack of natural ballasting minerals, can still form aggregates that sink at measureable velocities (>100 m d^{-1}) 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 suggestions from previous studies (Corzo et al. 2000, Mari et al. 2005, Berman-Frank et al. 2007), we formulated the following 3 hypotheses: (1) TEP play an important role in *Synechococcus* aggregation; (2) when cells become nutrient limited, they have higher cell-normalized TEP production and thus form more aggregates; and (3) these aggregates result in higher settling velocities compared to cells grown under nutrient-replete conditions.

MATERIALS AND METHODS

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 to 100 µmol photons m^{-2} s^{-1} in a 14:10 h light:dark cycle, and on rocking platforms to better simulate the natural open water condition. Autoclaved artificial seawater (Sigma Seasalt, at 30‰ salinity) enriched with nutrients according to Eppley et al. (1967) was used as the replete growth medium (Table 1). *Synechococcus* were grown in 3 treatments: nutrient-replete, N-limited and P-limited, based on the results of preliminary experiments to determine the reduced nitrogen and phosphorus concentrations that resulted

Table 1. Nutrient concentrations (µM) in the growth media used in batch culture treatments of marine *Synechococcus*

Nutrient treatment	N	P	N:P
Nutrient-replete	500	35	14
N-limitation	5	35	0.14
P-limitation	500	0.35	1400

in significantly lowered growth rates compared to nutrient-replete conditions (Table 1). Growth medium (1 l) was inoculated with 5 to 10% of culture and sampled every 2 to 3 d until their late stationary and decline phases were reached. Replicated cultures under nitrogen and phosphorus limitation were compared to the replicated nutrient-replete treatment, and cell abundance, aggregate formation, and chlorophyll *a* (chl *a*) content were measured throughout the growth period (11 d).

The experimental cultures were axenic. The liquid medium 'f/2_PM' (with bacto-peptone and methylamine-hydrochloride, as suggested by the NCMA) was used to regularly test for potential contamination by heterotrophic bacteria and fungi, and only cultures that had negative results were considered axenic and were used to inoculate experimental treatments.

Cells and aggregates

Synechococcus cells and aggregates were analyzed after being fixed with glutaraldehyde (1%) and filtered onto Poretics Polycarbonate filters (black, 25 mm, 0.2 µm pore size). Cells were counted using an epifluorescence microscope (Zeiss Axioscope) with blue-light excitation to visualize phycoerythrin fluorescence as in Amacher et al. (2009), and the concentration (number ml^{-1}) was calculated. Aggregates from preliminary experiments were observed using a scanning electron microscope (SEM, JEOL 6300) to depict aggregate matrices, as well as a combination of epifluorescence and brightfield microscopy (Logan et al. 1994) to investigate the association between TEP and *Synechococcus* cells.

Aggregates were analyzed using a Multisizer 3 particle counter (Beckman Coulter). Samples in duplicate were diluted to a 1 to 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 ($µm^3$ ml^{-1}) was calculated within the size range of 5 to 60 µm (equivalent spherical diameter).

TEP and chlorophyll *a*

TEP were determined using a dye-binding assay for the spectrophotometric measurement as in Passow & Alldredge (1995). Duplicate 10 ml samples were filtered at low, constant vacuum (10 cm Hg) onto Poretics Polycarbonate filters (25 mm, 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 to 3 h (gently agitated 2 or 3 times). Absorption at 787 nm was measured using a spectrophotometer (Shimadzu UV-1601) against distilled water, and the mean absorption of filter blanks was subtracted from the absorption of samples. The absorption values that were used to calculate the cell-normalized TEP production rates during the exponential growth phase fall into the linear range (<0.4) of the regression between the amount of TEP (as xanthan gum equivalents) and absorption as shown in Passow & Alldredge (1995). The same batch of dye was used for the analyses of all experimental treatments to be able to compare the results of the different nutrient treatments. TEP production rates were calculated according to Fukao et al. (2012).

The 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 h at 4°C. Fluorescence was measured using a fluorometer (Turner Designs TD700) as in Welschmeyer (1994), which was calibrated using chl *a* standards (Sigma; µg l^{-1}).

Settling velocity

The settling velocity was determined using the SETCOL method as described in Bienfang (1981) for cells and aggregates collected on the last sampling day. Uniformly mixed culture medium in each nutrient treatment was settled in 1 l cylinders (33 cm in height) for 0.5 h, 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 then calculated based on the change of biomass in vertical distribution over the settling time and converted to velocities in centimeters per day (Bienfang

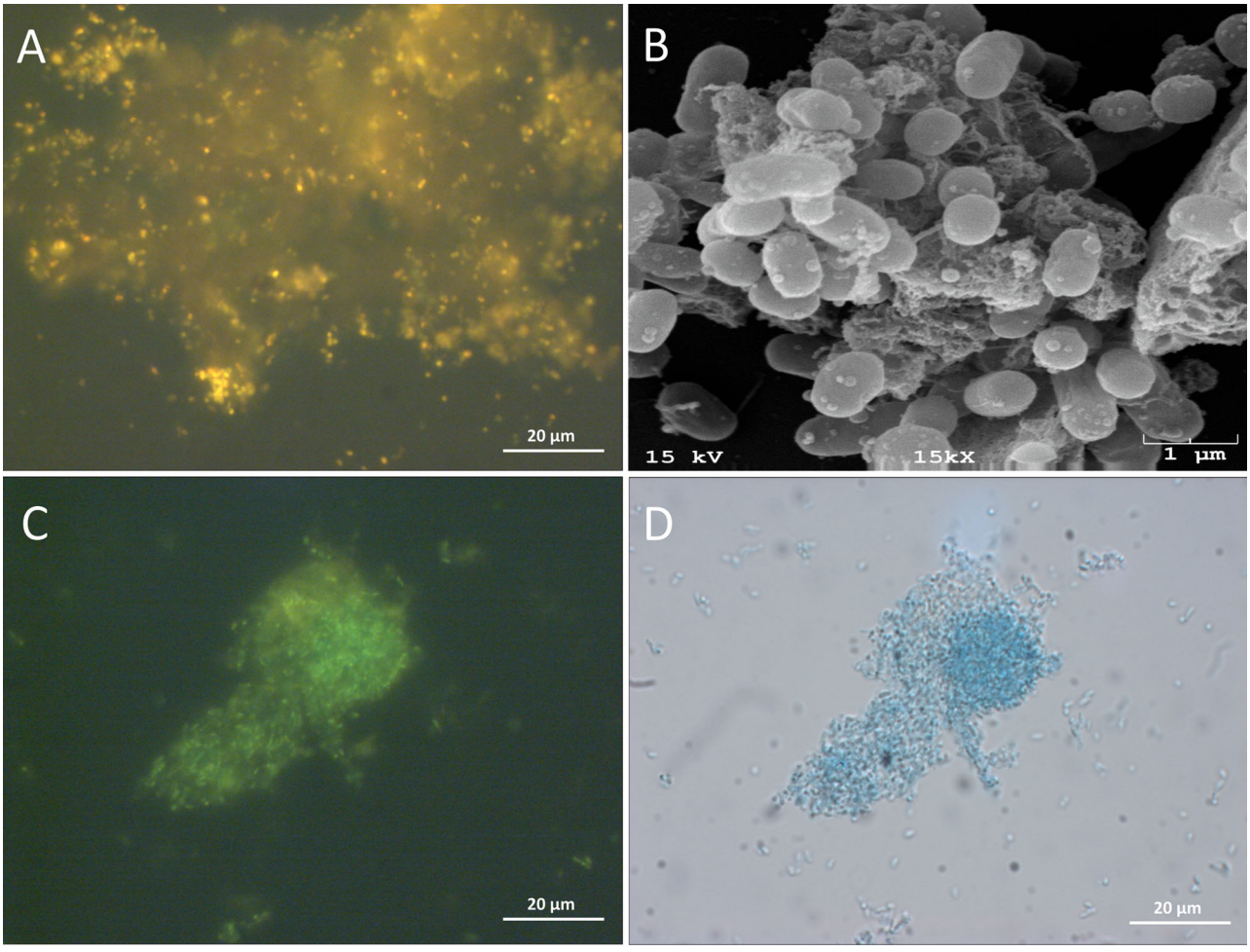

Fig. 1. (A) Epifluorescence microscope image of a *Synechococcus* aggregate under blue-light excitation. (B) Scanning electron microscope image of cells attached to an aggregate matrix. (C) Epifluorescence image and (D) the corresponding brightfield image of an Alcian Blue stained aggregate. All images were taken from cells growing in nutrient-replete cultures in preliminary experiments

1981). Changes in chl *a* concentration and aggregate volume concentration (5 to 60 µm, using the Multisizer) were measured in the settling columns.

RESULTS

Synechococcus aggregates and TEP

Using different microscopic observations we could confirm the aggregation of *Synechococcus* cells in our culture flasks in preliminary experiments. The size of aggregates observed under epifluorescence microscopy ranged from 3 to 5 µm to over 100 µm (Fig. 1A). The SEM image (Fig. 1B) depicts an aggregate matrix, which apparently consists mainly of 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. 1C) appeared stained with Alcian Blue dye in brightfield (Fig. 1D), 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 centrifugation tests (data not shown) showing that in the final supernatant, the TEP concentration correlated with the decreasing cell abundance as the centrifugation time/speed increased, despite the difference in excess density between TEP and cells.

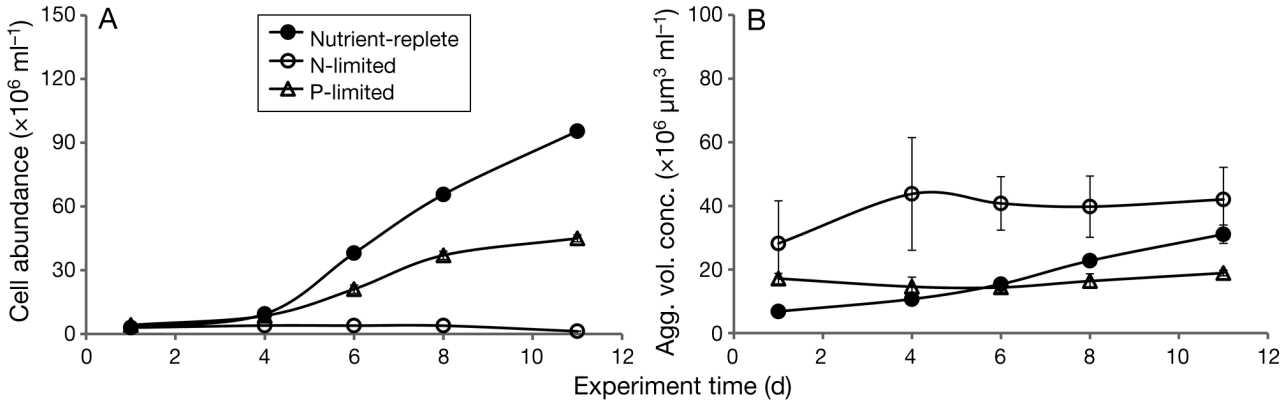

Fig. 2. Results of batch culture experiments on marine *Synechococcus*: (A) *Synechococcus* cell abundance and (B) changes of aggregate volume concentration (5 to 60 µm) in nutrient-replete, nitrogen-limited and phosphorus-limited cultures. Error bars represent mean ± SE (in some cases, the error bar falls within the symbol size)

Cell growth and aggregate formation

In general, *Synechococcus* cells in the nutrient-replete cultures were more abundant and reached the maximum cell number and growth rate a few days later compared to the nutrient-limited cultures (Fig. 2A). 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 nutrient-limited cultures (*t*-tests, Table 2), confirming the growth-limiting effect of the reduced nutrient concentrations in our experimental treatments (Table 1).

Around 90% of the aggregates analyzed using the Multisizer were in the size range of 5 to 10 µm, and the nutrient-limited cultures had a larger total aggregate volume concentration (5 to 60 µm) compared to the nutrient-replete cultures, especially over the lag and early exponential growth periods (Fig. 2B). We calculated the total aggregate to cell volume ratio (Table 2), assuming the single cell volume to be 1 μm^3, at the end of time intervals when cells grew at the maximum rate in each nutrient treatment (Table 2). Ratios in the phosphorus-limited (0.68 ± 0.01) cultures were significantly higher (*t*-tests, Table 2) compared to the nutrient-replete cultures (0.38 ± 0.04). The ratios in the nitrogen-limited cultures (11 ± 5), were also higher (but not significantly, due to high variability between replicates, Table 2) compared to the nutrient-replete cultures.

TEP production and chl *a* content

In general, the cell-normalized TEP concentration was higher in the nutrient-limited compared to the nutrient-replete cultures over the lag and early exponential growth periods (Fig. 3A). The nitrogen-limited cultures consistently showed a high cell-normalized TEP concentration throughout the 11 d sampling period (Fig. 3A). During the exponential

Table 2. Results of batch culture experiments on marine *Synechococcus*: maximum cell abundance at time since inoculation, maximum growth rate and time interval when this occurred, cell-normalized chl *a* content, and ratio of total aggregate to cell volume at end of the time interval when cells grew at the maximum rate. Times are shown as number of days since the start of the experiment (d). Results (p-values) of *t*-tests compare nutrient-replete with nutrient-limited cultures. Significant results ($\alpha = 0.05$) are shown in bold

Nutrient treatment	Max. cell abundance ($\times 10^6$ cells ml^{-1})		Max. growth rate (d^{-1})			Chl *a* (fg $cell^{-1}$)		Aggregate:cell volume ratio	
	Value (mean ± SE)	Time (d)	Value (mean ± SE)	Time interval (d)	p	Value (mean ± SE)	p	Value (mean ± SE)	p
Nutrient-replete	95[a]	11	0.70 ± 0.01	4–6	N/A	2.6 ± 0.04	N/A	0.38 ± 0.04	N/A
N-limited	4.0 ± 0.1	8	0.09 ± 0.02	1–4	**<0.005**	2.2 ± 0.2	0.20	11 ± 5	0.28
P-limited	45 ± 2	11	0.45 ± 0.005	4–6	**<0.005**	1.0 ± 0.08	**<0.005**	0.68 ± 0.01	**0.02**

[a]Lack of replication due to loss of one replicate on Day 8

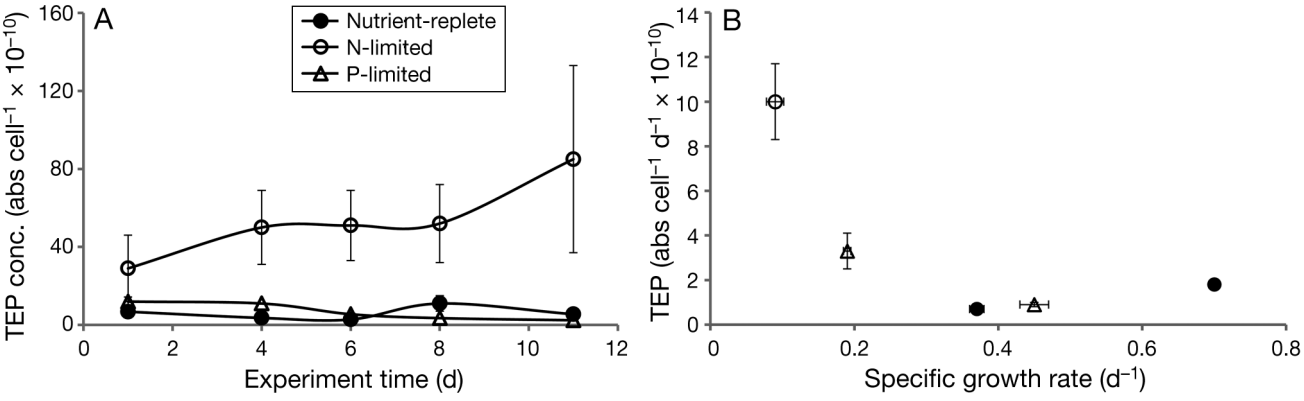

Fig. 3. Results of batch culture experiments on marine *Synechococcus*: (A) cell-normalized TEP concentration and (B) cell-normalized TEP production in absorption (abs) units at specific growth rates in nutrient-replete, nitrogen-limited and phosphorus-limited cultures. Error bars represent mean ± SE (in some cases, the error bar falls within the symbol size)

growth phase, a negative relationship between growth rates and the corresponding cell-normalized TEP production was observed in the nutrient-limited treatments (Fig. 3B). At the time intervals when cells grew at the maximum rate (Table 2), cell-normalized TEP production (absorption cell^{-1} d^{-1} ×10^{-10}) was significantly greater (*t*-test, $p < 0.05$) in the nitrogen-limited cultures (10 ± 1.7) compared to the nutrient-replete cultures (1.8 ± 0.1).

At the end of the time intervals when cells grew at the maximum rate (Table 2), the cell-normalized chl *a* content in the phosphorus-limited cultures (1.0 ± 0.08 fg cell^{-1}) was significantly lower (*t*-tests, Table 2) compared to the nutrient-replete cultures (2.6 ± 0.04 fg cell^{-1}). The cell-normalized chl *a* content of nitrogen-limited cultures (2.2 ± 0.2 fg cell^{-1}) was also smaller (but not significantly) compared to the nutrient-replete cultures.

Settling velocity

We calculated settling velocities based on the changes of both chl *a* concentration and aggregate volume concentration (5 to 60 µm, analyzed using the Multisizer) in the settling columns on the last day in each nutrient treatment (Fig. 4). As expected, settling velocities based on the sinking aggregates were much higher than the chl *a* based values (Fig. 4) that represent the bulk biomass including single cells. Settling velocities in the nitrogen-limited (52 ± 19 cm d^{-1}, chl *a* based) and phosphorus-limited cultures (341 ± 13 cm d^{-1}, aggregate based) were significantly higher (*t*-test) compared to the nutrient-replete cultures (0.02 ± 1.92 cm d^{-1}, chl *a* based; 183 ± 14 cm d^{-1}, aggregate based) (Fig. 4). This is consistent with the greater proportion of cells involved in the aggregate formation, in addition to the higher total aggregate to cell volume ratio in the nutrient-limited treatments presented earlier.

DISCUSSION

TEP production and *Synechococcus* aggregation

Studies have shown that different phytoplankton groups, including many cyanobacteria such as *Trichodesmium* (Berman-Frank et al. 2007), *Anabaena* (Bittar & Vieira 2010), and *Crocosphaera* (Sohm et al. 2011) can generate TEP abundantly. This study adds important information on the TEP production by marine *Synechococcus,* one of the most abundant and widespread unicellular pico-cyanobacteria.

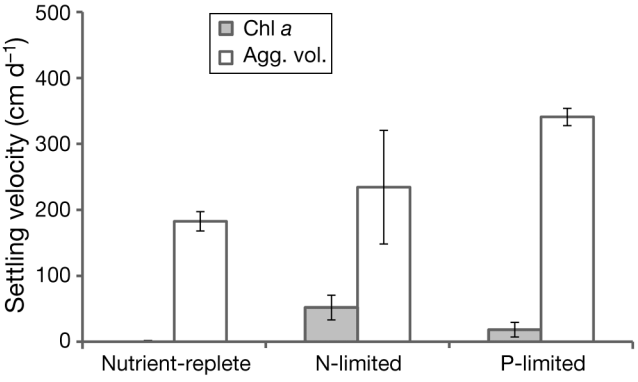

Fig. 4. Results of batch culture experiments on marine *Synechococcus*: settling velocities calculated based on the changes of chl *a* and aggregate volume (5 to 60 µm) concentrations in the settling columns, on the last day in nutrient-replete, nitrogen-limited and phosphorus-limited cultures. Error bars represent the mean ± SE

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 Alcian Blue stainable cell coatings was also observed in large-celled strains of *Crocosphaera watsonii* (Sohm et al. 2011) and some diatoms such as *Nitzschia* (Crocker & Passow 1995, Passow 2002). These TEP coatings likely resulted in high cell stickiness, facilitating the aggregation of *Synechococcus* in our study. Furthermore, the changes in total aggregate volume concentration (5 to 60 µm, Fig. 2B) were positively associated with the TEP concentration (Fig. 3A) of cultures in different nutrient treatments over the growth periods. Specifically, at the end of time intervals when cells grew at the maximum rate (Table 2), cell-normalized total aggregate volume was positively correlated with the cell-normalized TEP concentration (Fig. 5).

Effects of nutrient limitation

In general, despite the lowered growth rates (Table 2), nutrient-limited cultures formed a greater total volume of aggregates (Table 2, Fig. 2B), had larger cell-normalized TEP concentrations and higher TEP production (Fig. 3). This resulted in higher settling velocities (Fig. 4) compared to the nutrient-replete cultures, supporting our second and third hypotheses. *Synechococcus* cells in our study had smaller cellular chl *a* content (Table 2) in the nutrient-limited cultures; moreover they were more sensitive to nitrogen compared to phosphorus-limitation (Fig. 2A). Most marine *Synechococcus* (including the strain used in this study) 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 demand 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 increasing their affinity, which allows for relative high uptake rates at low concentrations (Lomas et al. 2014) by using non-phosphorus lipids like sulfolipids (Van Mooy et al. 2009) and lowering their maximum growth rates with reduced allocation to the P-rich ribosomal RNA (the 'growth rate hypothesis'; Elser et al. 2003, Mouginot et al. 2015). This low phosphorus requirement of *Synechococcus* should clearly impart a competitive advantage over other phytoplankton in the oligotrophic oceans.

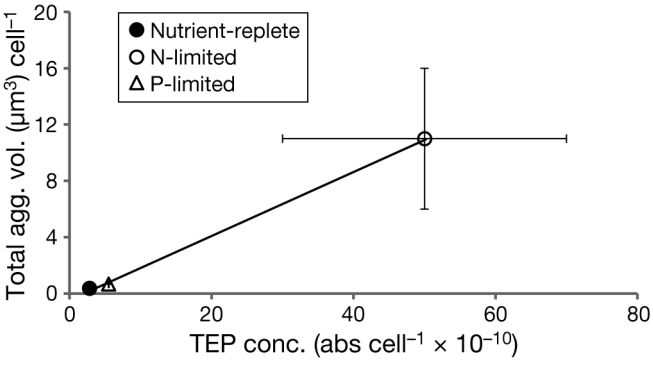

Fig. 5. Cell-normalized TEP concentration in absorption (abs) units and the corresponding total aggregate volume (5 to 60 µm) of cultures in nutrient-replete, nitrogen-limited and phosphorus-limited cultures at the end of time intervals when cells grew at the maximum rate (Table 2). Error bars represent mean ± SE (in some cases, the error bar falls within the symbol size) and line indicates the positive relationship between total aggregate volume and TEP concentration

It can be noted that the total aggregate volume (5 to 60 µm) in the nutrient-replete cultures exceeded those in the phosphorus-limited cultures (Fig. 2B) after the end of time intervals when cells grew at the maximum rate (Table 2), mainly due to the much larger number of total cells under nutrient-replete conditions (though cell-normalized values were smaller). When cultures reached the late stationary and decline phases, other 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 limitation of nutrients. 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 TEP production as a function of growth rate, we focused on time intervals within the exponential growth phase (as in Corzo et al. 2000 and Fukao et al. 2010, 2012).

We also calculated settling velocities using the epifluorescence microscopy analysis of aggregates larger than 100 µm in preliminary experiments. These aggregates (>100 µm) were much less abundant (ca. 10 to 100 ml^{-1}) compared to the smaller aggregates (5 to 60 µm) analyzed using the Multisizer (ca. 10^4 to 10^5 ml^{-1}), but their settling velocities reached over 50 m d^{-1} (data not shown). However, these settling velocities may still not be enough for aggregates to sink out of the ocean mixed layer. By using roller tanks, Deng et al. (2015) found that *Synechococcus*

can form visible aggregates with sizes and sinking velocities (ca. 1.4 mm and 440 m d^{-1}, respectively) comparable to marine snow in the ocean (Alldredge & Gotschalk 1988, Peterson et al. 2005). This indicates that the small, 'suspended aggregates' (De La Rocha et al. 2008, Deng et al. 2015) formed in our culture experiments have the potential of being 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).

Implications for the oligotrophic oceans

An important conclusion from our study is that a reduction of growth rate will increase the cell-normalized TEP production and aggregation of *Synechococcus* (Table 2, Fig. 3B), which is consistent with many previous studies on various phytoplankton groups. This growth rate reduction can result from (1) the reduced nutrients (or altered ratios) in the starting growth medium (Corzo et al. 2000, Mari et al. 2005) as in our case; (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^{-1} in the spring mixed layer of the Sargasso Sea; DuRand et al. 2001) is much lower compared to our laboratory cultures (ca. 10^7 cells ml^{-1} in the replete growth medium). However, our results indicate that *Synechococcus* cells in a nutrient-limited environment, representative of the vast oligotrophic ocean gyres, would have a greater aggregation potential due to their higher cell-normalized TEP production than nutrient-replete cells. Furthermore, we consider growing *Synechococcus* in batch culture and measuring TEP production dynamics during the exponential growth phase to be representative of its growth dynamics *in situ*, where cells experience high grazing loss (Worden & Binder 2003) and a continuous, albeit low, nutrient supply due to nutrient regeneration.

In addition to nutrient availability and other factors influencing growth in the field, the TEP production by *Synechococcus* could be influenced by other organisms, especially heterotrophic bacteria. Though phytoplankton appear to be the most significant source of TEP, heterotrophic bacteria can generate TEP themselves (Radi et al. 2006) or possibly enhance the TEP production by phytoplankton (Passow et al. 2001, Passow 2002, Grossart et al. 2006, Gärdes et al. 2012), in addition to the degradation of the organic matter in aggregates (Ploug & Grossart 2000, Iversen & Ploug 2010). Despite all of the above, the quantification of TEP produced by phytoplankton in axenic culture conditions is rarely seen in the literature (Corzo et al. 2000, Passow 2002, Mari et al. 2005, Berman-Frank et al. 2007, Claquin et al. 2008, Sohm et al. 2011). Using axenic cultures in our experiments allowed us to attribute the observed changes in TEP production solely to *Synechococcus*.

Aggregation as observed in our study will have different ecological consequences for the *Synechococcus*. As an example, aggregation increases the functional size of pico-cyanobacteria, making them better at avoiding microzooplankton grazing but more susceptible to grazing by larger zooplankton (Pernthaler 2005, Jezberová & Komárková 2007). *Synechococcus* are observed abundantly in mesozooplankton guts and fecal pellets (Wilson & Steinberg 2010, Stukel et al. 2013). In addition, the enhanced TEP production and aggregation can benefit phytoplankton in environments that result in suboptimal 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 of nutrients, e.g. at the end of phytoplankton blooms. Via the formation and settling of aggregates, *Synechococcus* cells that are nutrient limited will be able to reach deeper depths with higher nutrient availability.

Pico-cyanobacteria like *Synechococcus* have also been found in the deep sea (sediments or sediment traps at several thousand meters depth) in earlier studies (Pfannkuche & Lochte 1993, Turley & Mackie 1995). Richardson & Jackson (2007) 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. Specifically, at the Bermuda Atlantic Time-series Study (BATS) site located in the Sargasso Sea, *Synechococcus* have been found in particle traps below the euphotic zone (Amacher et al. 2013), and are estimated to contribute 2 to 13% of the total particulate organic carbon flux in the upper 500 m (Lomas & Moran 2011). Together with recent laboratory experiments on the aggregation and sinking of

marine *Synechococcus* (Deng et al. 2015), we can now contribute information on the mechanism behind such aggregation and *Synechococcus*'s contribution to the carbon export in the oligotrophic oceans. Our results show that despite their small size and lack of natural ballasting minerals, *Synechococcus* can form sinking aggregates by producing TEP and thus sink out of the euphotic zone to the deep sea.

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