Effects of salinity on cellular growth and exopolysaccharide production of freshwater *Synechococcus* strain CCAP1405

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Abstract

The picocyanobacterium *Synechococcus* is ubiquitous in freshwater and high saline marine ecosystems. The cellular response of freshwater *Synechococcus* to salinity variations is not well understood. Herein, we compare cellular physiology, cell division and exopolysaccharide production of freshwater *Synechococcus* CCAP1405 when grown under different salinity conditions. A comparable growth rate between salinity 0 and 10 indicates that this strain is capable of adapting to such moderate salinity change. The photosynthetic efficiency and growth were significantly lower at salinity 20 and 30 suggesting that cells experienced stress above salinity 10. For the first time, we record bimodal DNA distribution pattern for a freshwater *Synechococcus* strain with cell division synchronized to light-dark cycle. Pre-replication (G1) and synthesis (S) phase durations were significantly longer at high salinity, pointing to slower growth. However, post-replication (G2) phase duration was not influenced by a change in salinity. The soluble and attached exopolysaccharide production increased with salinity and age of the culture. Increased exopolysaccharide production in the vicinity of cell-boundary facilitated its survival and microaggregates formation under salinity stress conditions. These inferences indicate that flux of freshwater *Synechococcus* into estuarine and marine conditions would influence its growth, exopolysaccharide production and picophytoplankton carbon flow in the food web.

**Key words:** *Synechococcus*, Salinity, Photosynthetic efficiency, Exopolysaccharide production, Cell cycle
Introduction

The picophytoplankton (0.2 – 2.0 µm in diameter) group consists of different genera of picocyanobacteria (*Synechococcus* and *Prochlorococcus*) and photosynthetic picoeukaryotes (*Micromonas*, *Ostreococcus*, and *Bathycoccus*) (Olson et al., 1990; Veldhuis et al., 1993; Vaulot et al., 2008). The picocyanobacterium *Synechococcus* is distributed worldwide, ranging from tropics to polar regions, and ubiquitous in variety of aquatic ecosystems; freshwater lake, brackish water estuary, coastal and Open Ocean waters (Paerl, 1977; Callieri et al., 1996; Callieri, 2008; Flombaum et al., 2013; Bemal and Anil, 2016a). Based on spectral emission, the *Synechococcus* strains are grouped into two types; 1) yellow autofluorescing phycoerythrin-rich (PE-rich; $\lambda_{\text{max}} = 570$ nm) and 2) red autofluorescing phycocyanin-rich (PC-rich; $\lambda_{\text{max}} = 630$ nm) types. The PE-rich *Synechococcus* strains are ecologically adaptive to blue-green light and predominate in clear open-ocean waters. On the other hand, the PC-rich strains are abundant in estuarine and coastal turbid waters in which red light prevails (Calleiri et al., 1996; Stomp et al., 2007). Several observations around the world confirm that the freshwater *Synechococcus* strains can be advected via riverine discharge into the estuaries. Higher freshwater *Synechococcus* abundance is reported in Hong Kong estuary owing to heavy discharge from Pearl River during summer season (Xia et al., 2015). Waleron et al., (2007) have estimated a large concentration of picocyanobacteria ($10^{18}$ cells year$^{-1}$) carried into the Beaufort shelf region via freshwater discharge (330 km$^3$ year$^{-1}$) from the Mackenzie River. The phylogenetic analysis of six *Synechococcus* strains, isolated from Portuguese Atlantic estuaries, revealed two strains (LEGE06316 and LEGE06322) clustered with freshwater congeners suggesting estuarine strains can be derived from upstream sources (Lopes et al., 2012).

The estuaries form transition zones linking the freshwater and marine biomes, are characterized by a strong gradient of different physicochemical components (light availability, salinity, nutrients and dissolved oxygen concentration) (McLusky, 1993). The growth rate of different cyanobacterial strains decreases with increasing salt concentration, and the extent of growth inhibition varies among the strains (Blumwald and Tel-Or, 1982; Vonshak et al., 1988; Lehtimäki et al., 1997). The enhanced photoinhibition restricts the photosystem II activities of cyanobacteria *Spirulina platensis* under salinity stress conditions (Zeng and Vonshak, 1998). The nitrogen-fixing estuarine cyanobacterial isolates (*Nodularia* spp., *Anabaena aphanizomenoides*, *Anabaenopsis* sp., and *Cylindropermopsis raciborskii*) adjust the photosynthesis process in higher salinity levels by increasing the maximum photosynthetic rates (Moisander et al., 2002).

The freshwater and brackish water *Synechococcus* strains have developed intracellular (accumulation of compatible solutes) as well as extracellular (deposition of exopolymeric substance) adaptations to
overcome the salinity stress (Brown, 1976; Blumwald and Tel-Or, 1984; Scanlan et al., 2009; Qui et al., 2012). Production of exopolysaccharide is an energy expensive process which benefits the producers for continuing cellular activities in adverse conditions (Poli et al., 2010). These extracellular polymeric substances (EPS) have been broadly grouped into two fractions: dissolved form and cell-attached particulate form. The dissolved fraction of EPS is known as soluble extracellular polymeric substances (S-EPS) and contributes a large portion of total cell exudates. The attached fraction of EPS forms a sticky, discrete gel-like matrix in which phytoplankton cells or other particles are embedded. The cell-bound EPS around algae and bacteria is often divided into tightly-bound EPS and loosely-bound EPS (Liu et al., 2010; Shao et al., 2009; Jenkinson et al., 2015), or a closely adhering capsule EPS and loosely adhering slime EPS (Wotton, 1996). This cell-bound EPS consists largely of acidic polysaccharides and is termed Transparent Exopolymer Particles (TEP) (Passow and Alldredge, 1995; Passow, 2002a, b). The formation of TEP by coagulation and gelation from the dissolved polysaccharide depends on the type and concentration of precursor as well as environmental parameters (turbulence, ion density and inorganic colloids concentration) (Passow, 2002b). A significant amount of S-EPS production by different Synechococcus strains was observed under adverse growth conditions; elevated salt concentration (Roux, 1996; Rosales et al., 2005; Qiu et al., 2012), nutrients limited conditions (Sanger and Dugan, 1972) and carbon dioxide enriched environments (Phlips et al., 1989). Deng et al., (2016) showed that the formation of TEP-microaggregate of Synechococcus cell increased in nutrient limited condition, resulting in higher settling velocity of cell aggregates.

The organic carbon from the exopolysaccharide secretion significantly contributes to the food chain as well as in deep water carbon transport. A major fraction (< 3% to > 90%) of primary production is released as S-EPS and contributes to the dissolved organic matter (DOM) pool and is available to the microbial food web system. On the other hand, the cell-bound TEP and particulate form of EPS play significant role in the production of particulate organic matter (POM) pool and its transfer through various trophic levels of the classical food web (Passow, 2002b).

The Synechococcus population dynamics depends on two interdependent physiological processes; cell growth (increase in biomass) and cell division (generation of a new cell). The generation time is average time between two successive cell divisions and measured by two ways: (1) measuring the change in cell density with time and (2) addition of time fractions of different cell cycle phases (White, 1991; Yamaguchi et al., 2007). Studies regarding the influence of salinity on growth and exopolysaccharide production of freshwater Synechococcus are limited (Roux, 1996; Rosales et al., 2005; Qiu et al., 2012; Deng et al., 2016). The influence of salinity on the freshwater Synechococcus
strains is not understood and has implications determining the fate of these strains in estuarine and marine conditions. In this context, laboratory experiments were carried out with freshwater *Synechococcus* strain to answer the following questions: What is the effect of salinity on the growth, photophysiology and cell division of a freshwater *Synechococcus* strain? How do different fractions (dissolved and particulate) of exopolysaccharide production vary with salinity? How does the relationship between exopolysaccharide secretion and cellular growth vary with salinity? The answers to these questions on physiological adaptation and growth characteristics of freshwater *Synechococcus* strain under salinity conditions can help in furthering our understanding of picophytoplankton survival strategy when transported to marine conditions and its influence in the food web dynamics.

**Materials and Methods**

*Culture condition and experimental set-up*

Freshwater PC-rich strain *Synechococcus leopoliensis* CCAP1405 was procured from the Culture Collection of Algae and Protozoa, UK (www.ccap.ac.uk). This strain is maintained in batch-culture of f/2 growth medium, prepared from aged (> 50 days) freshwater (0 salinity; passed through 0.22-μm pore size filter), for the last three years in the laboratory of Biofouling and Bioinvasion Division, CSIR-National Institute of Oceanography, Goa, India.

To evaluate the effect of salinity on *Synechococcus*, the strain CCAP1405 was grown in batch-culture of four different salinity treatments: 0.09, 10.11, 20.13 and 30.38 (referred henceforth as treatments 0, 10, 20 and 30, respectively). Prior to the experiment, the strain was acclimatized (> 6 months) growth medium of the three salinity (10, 20 and 30). Salinity was adjusted by mixing of different ratio of aged seawater (35 salinity) and freshwater. The seawater was collected from a coastal site near Calangute (15°32'35.49"N 73°45'17.17"E), Goa, India, and freshwater from the running water facility system at the institute (CSIR-National Institute of Oceanography). A 12:12 h light-dark (LD) cycle with irradiance fixed at 80 μmol m⁻² s⁻¹ (fluorescent light sources; Toshiba) was provided to all four salinity conditions (0, 10, 20 and 30). The salinity-influence experiment was carried out in Erlenmeyer flasks (2 L) in three replicates, and each replicate contained 1000 mL of f/2 growth media. Initial cell concentration (~10⁶ cells mL⁻¹) was kept constant in all the treatments and maintained at a fixed temperature (23±1°C) throughout the experimental period. Gentle stirring was carried out four times in a day and before each sampling of the cell cycle analysis.

*Growth rate (µ) and photosynthetic efficiency (Fv/Fm) measurement*
One milliliter of culture sample was pipetted out every alternate day to determine the cell concentration change in the experimental treatments. The cellular growth was monitored by cell count using a BD FACSM™ II flow cytometer equipped with 480 and 633 nm laser sources. The doubling time \((T_d)\) was calculated using the formula \(24\times (0.6931/\mu)\); where specific growth rate, \(\mu\) (day\(^{-1}\)), was measured from cell concentration variation during the experimental period.

\[
\mu = \frac{\ln[N(t_2)/N(t_1)]}{(t_2 - t_1)}
\]

Where, \(N_t_1\) and \(N_t_2\) correspond to the cell concentration (cells mL\(^{-1}\)) at time \(t_1\) and \(t_2\) (day\(^{-1}\)), respectively.

Samples in triplicate (3 mL) were collected during the light period and incubated in dark conditions for 15 min. Subsequently, samples were analyzed in a fluorescence induction and relaxation fluorometer (FIRe-Fluorometer, Satlantic, Halifax, Canada) to measure the photosynthetic efficiency. The analyses of all experimental samples were completed sequentially in dark conditions and took around 30 min. Maximum photosynthetic efficiency \((F_v/F_m)\) was calculated according to the following equation (Schreiber, 1986):

\[
F_v/F_m = (Fm - F_0) / F_m
\]

Where, \(F_0\) is the dark-adapted minimum chlorophyll fluorescence, and \(F_m\) is the maximum fluorescence after application of a single turnover flash with a duration of 120 \(\mu\)s (Hung et al., 2013).

The early stationary phase of these batch cultures was assumed when cell concentration remained stable during two successive sampling dates along with a decrease in the maximum photosynthetic efficiency value.

**Cell size measurement**

The cellular equivalent spherical diameter of plankton can be roughly estimated from light scattering normalized to size calibration beads. According to Mie theory, the volume of small particle can be described as a power-law function of light scattering (Morel, 1991). An empirical power-law equation between bead diameter and mean forward-scattered light (FSC) value has been obtained for 1, 2, 3 and 6-micron size calibration beads (Bangs Laboratory Inc.) (DuRand et al., 2001; Bec et al., 2008; Chen et al., 2011). The best fit curve yielded from the relationship between the bead diameter and mean FSC was \(d = 0.1048 \times V_{FSC}^{0.6901} \) \((r^2=0.98, p < 0.05)\). Where, \(d\) and \(V_{FSC}\) denote mean diameter (\(\mu\)m) and the bead normalized mean FSC value, respectively. The FSC and chlorophyll signals of *Synechococcus* cell, as well as reference size beads (2 \(\mu\)m) were collected in blue and red
fluorescence channels, respectively. We applied the derived power-law relationship to estimate mean cellular diameter from the mean FSC value of *Synechococcus* cluster in the bivariate contour plot.

*Exopolysaccharide quantification*

**TEP measurement**

TEP fraction was measured by following the centrifugation method of Passow and Alldredge, (1995) modified by Claquin et al., (2008). Briefly, centrifugation of 10 mL of culture was performed at 3200×g for 30 min at 12 °C to separate the cells from the supernatant. To stain the TEP, two mL of aqueous solution of 0.02% alcian blue (8GX) in 0.06% acetic acid (pH 2.5) was added to the cell pellet. Excess dye was removed by centrifugation (3200×g for 20 minutes at 4 °C) immediately after staining. Pellet was rinsed with 2 mL of distilled water and centrifuged several times (3-5) until supernatant became clear. The cell pellet was digested with 4 mL of 80% sulfuric acid, and absorbance was measured after 2 h at 787 nm by spectrophotometer (UV-VIS-2550, SHIMADZU). A calibration was performed using xanthan gum by following the above protocol. TEP fraction was measured in xanthan equivalent weight and converted to carbon using the factor of 0.75 as mentioned by Engel and Passow, (2001).

**S-EPS measurement**

The S-EPS fraction was quantified from the supernatant that remained unused during TEP measurement. 0.5 mL of supernatant was added to 1 mL of 5% phenol solution and 5 mL of 80% sulfuric acid in a glass tube. After 30 min, the absorption of the solution was measured at 485 nm in a spectrophotometer (UV-VIS-2550, SHIMADZU) (Dubois et al., 1956). The absorption value was converted to equivalent glucose using a standard calibration curve of different glucose concentration. Glucose was also converted into carbon, using the factor of 0.40 as for hexoses (Pannard et al., 2016).

The S-EPS consists of all types of polysaccharides and can be estimated as total carbohydrates by phenol-sulfuric acid method by using glucose as standard (Dubois et al., 1956). Concentrated sulfuric acid hydrolysates glycosidic linkages, and these hydrolysed neutral sugars are dehydrated to form furfural or furfural derivatives. The coloured compounds developed by condensation of furfural or furfural derivatives with phenol are measured at 485 nm. On the other hand, TEP consists largely of acidic polysaccharides and stainable with alcian blue (Passow and Alldredge, 1995; Passow, 2002a). TEP is enriched in fucose, rhamnose and arabinose, but relatively depleted in glucose and galactose (Mopper et al., 1995; Zhou et al., 1998). An aqueous alcian blue dye solution at a pH of 2.5 can stain
carboxyl and sulfated polysaccharides, but not the neutral sugars. Hence, we have not used alcian blue staining method for quantifying the S-EPS which is enriched in neutral sugars.

**Cell Cycle analysis**

Samples from each treatment (triplicates) were collected at 2 h interval for 50 h during exponential growth phase and used for analyzing the DNA frequency distribution. One milliliter of culture (∼10^5–10^7 cells mL⁻¹) was preserved in glutaraldehyde solution (0.2% final concentration) followed by dipping into liquid nitrogen. Samples were stored at −80°C for the cell cycle analysis. Before cell cycle analysis, the samples were thawed at 37°C; later a 0.1 g L⁻¹ mixture of RNase A and B (1:1) (Sigma) was added and incubated for 30 min to improve the precision of the DNA histogram. SYBR Green I (30 μL; 10^4× concentration of commercial stock solution; Invitrogen) was used to stain nucleic acid material and incubated in the dark for 20 min (Marie et al., 1997). DNA fluorescence was detected at 530/30 nm band pass filter against chlorophyll fluorescence (695/45 nm). Flow cytometric data was further analyzed by the Mod Fit LT v. 3.2 (Verity Software House) software package to compute the fraction of cells in G1, S and G2 phases. The software deconvolutes the flow cytometer DNA fluorescence data into the cell fraction of the three cell cycle phases. The cellular DNA content differs during these phases. The histograms of the DNA fluorescence were analyzed with the software. The percentage of cells in each phase of the cell cycle (G1, S and G2) was obtained from the histogram analysis. The different phases of bimodal DNA distribution were quantified by a model that included two Gaussian peaks (G1 and G2) and a broadened rectangle (S) population. Duration (h) of each cell cycle phase was calculated by using equation provided by Slater et al., (1977).

\[
T(G1) = - \left( \frac{T_d}{\ln 2} \right) \ln \left\{ 1 - \frac{P(G1)}{2} \right\}
\]

\[
T(G2) = \left( \frac{T_d}{\ln 2} \right) \ln \left\{ 1 + P(G2) \right\}
\]

\[
T(S) = \left( \frac{T_d}{\ln 2} \right) \ln \left[ \frac{1 + P(S)}{1 + P(G2)} \right].
\]

The doubling time (T_d) was measured during the exponential growth phase of the experiment. P(G1), P(G2) and P(S) are the fraction of the cell population in G1, G2 and S phases, respectively.

Specific growth rate (μ_{cc}; day⁻¹) was also estimated from cell cycle phase duration over a diel cycle using the formula of Carpenter and Chang, (1988).

\[
\mu_{cc} = \frac{\sum_{i=1}^{n} \ln \left( 1 + \frac{f_i(t_i) + f_{G2}(t_i)}{n \times (T_G + T_{G2})} \right)}{24}
\]
Where $\mu_{cc}$ is an estimate the division rate; $n$ is the number of samples collected at fixed intervals during one subjective day. $T_S$ and $T_{G2}$ (h) is the sum of the duration of S and G2 phases, computed by using the formula, $[2(t_{G2max} - t_{Smax})]$, twice the delay between the peaks of cells in these phases. $f_S(t)$ and $f_{G2}(t)$ are the fractions of cells in S and G2 phases at time $t$. The doubling time ($T_d^*$) was calculated by using formula $24\times (0.6931/\mu_{cc})$.

Results

Growth rate, photosynthetic efficiency and cell size variation under different salinity conditions

The growth rate ($\mu$) differed significantly ($F_{29, 3}; p < 0.001$) among the four experimental conditions indicating salinity influence on cellular growth during exponential phase. The growth rate was maximum (0.36±0.04 day$^{-1}$) in salinity 10, while the minimum growth rate (0.19±0.02 day$^{-1}$) was recorded in salinity 30. The *Synechococcus* cultures of 0, 10 and 20 salinity entered into stationary phase after 27 days, 25 days and 31 days, respectively. The culture grown in the 30 salinity took the longest time (43 days) to reach the early stationary phase with lower cell density (four to six times) than the other salinity conditions (Fig. 1A).

The maximum photochemical efficiency (Fv/Fm) values varied between 0.25 and 0.66 in all four salinity conditions (Fig. 1B). The Fv/Fm values showed a steep increment during the exponential growth phase and maintained a maximum plateau during stationary phase. A significant ($F_{40, 3}; p < 0.001$; *post hoc* Tukey test) difference in Fv/Fm values indicated that the photochemical yield of PS II in CCAP1405 varied among the four salinity conditions.

Flowcytometric bivariate contour plots of cell size showed that FSC value (a proxy of cell diameter) shifted towards the higher side of the axis with increasing salinity conditions (Supplement Fig. 1). A significant difference ($F_{387, 3}; p < 0.001$) in the mean cellular diameter was observed among the four salinity conditions (Fig. 1C). The cell diameter varied least in 0 salinity with a mean value of 1.53 (±0.07) µm throughout growth period, while maximum mean cellular diameter 2.40 (±0.85) µm was measured in 30 salinity. A *post hoc* analysis revealed that the cellular diameter in 20 and 30 salinity varied significantly ($p < 0.05$) throughout the exponential growth phase, however, cellular diameter did not vary significantly ($p > 0.05$) in the cases of 0 and 10 salinity.

Exopolysaccharide production under different salinity conditions

Exopolysaccharide production was not detectable before the 11th day in 0, 10 and 20 salinity conditions due to lower cell concentration, whereas this period extended up to 27th day in the case of 30 salinity. The exopolysaccharide production varied with cellular growth phases; the S-EPS production was two (0, 10 and 20 salinity) to five (30 salinity) times higher during the stationary
The specific growth rate and doubling time were measured higher in 30 salinity than 20, 10 and 0 salinity conditions during exponential ($F_{186}, 3; p < 0.001$; post hoc Tukey test) and stationary ($F_{646}, 3; p < 0.001$; post hoc Tukey test) phases. However, observations showed that TEP production was comparable among the salinity 0, 10 and 20 during exponential and stationary phases ($p > 0.05$; post hoc Tukey test). The cellular S-EPS production increased significantly ($F_{657}, 3; p < 0.001$) in higher salinity conditions; with a maximum production of $1205.27\pm39.08$ fg C cell$^{-1}$ observed in 30 salinity while the least production was recorded in 0 salinity condition ($62.64\pm9.91$ fg C cell$^{-1}$) during the stationary phase. However, a post hoc analysis revealed that S-EPS production did not vary significantly ($p = 0.56$) between 0 and 10 salinity conditions (Fig. 2A and B).

**Cell cycle analysis under different salinity conditions**

The bimodal DNA frequency distribution and diel pattern of cell cycle phases of CCAP1405 grown in different salinity conditions are presented in Fig. 3. The DNA fluorescence histogram is comprised of three peaks; the larger peak (65 — 98% of cell population) corresponds to G1 (1 copy of DNA) and the smallest peak (1— 3% of cell population) at the far right represents as G2 peak (G2/ G1=1.98). The peak in between G1 and G2 is identified as S phase (1— 27% of cell population). The G1 peak CV (< 15%) and lower reduced chi-square values (< 5) indicated that flow cytometric datasets in all treatments were a good fit for the selected model (Fig. 3A—D). The cell cycle pattern of *Synechococcus* in all the four salinity conditions was strongly synchronized with 12:12 h LD cycle. The maximum cell fractions in G1 (pre-replication) phase was recorded during the dark phase (03:00- 05:00 h) and did not change until the middle of subsequent light phase (13:00- 17:00 h). The maximum cell fraction in S (DNA synthesis) phase and minimum cell fractions of G1 phase co-occurred during the same time; light to dark transition (21:00 h) or early dark phase (23:00- 03:00 h). The cell fraction in G2 (post-replication) phase peaked during the mid-dark phase, 4 – 8 h after the S phase maxima (Fig. 3E—H). The G1 and S phase duration significantly differed among the four salinity conditions ($F_{215}, 3$ and $F_{106}, 3; p < 0.05$), however, this was not the case with G2 phase duration ($p = 0.69$) (Fig. 4A—C). The duration of G1 phase varied between 73 and 93% of the generation time in all four salinity conditions. The S phase duration, represented maximum (~24%) and minimum (~5%) of the generation time, was observed in salinity 20 and 30, respectively.

The specific growth rate and doubling time in the four salinity conditions were estimated separately by using two different datasets: cell abundances and cell cycle phase duration. The comparable doubling time measurements indicated no significant difference between the approaches. Among
four salinity conditions, the shortest doubling time ($T_d^*$), $44.1\pm4.6$ h was measured in salinity 10. The *post hoc* analysis revealed no significant difference ($p = 0.49$) in doubling time between salinity 0 and 10. However, significantly longer doubling time $76.6\pm10.5$ and $92.6\pm5.1$ h was measured between 20 and 30 salinity conditions, respectively. A strong positive correlation ($r = 0.84$, $p < 0.05$) was observed between doubling time and G1 phase duration. However, a negative correlation ($r = -0.30$) and lower correlation coefficient ($r = 0.18$) was found for the G2 and S phase duration, respectively (Table 1).

**Discussion**

The maximum photosynthetic efficiency and highest growth rate of freshwater *Synechococcus* CCAP1405 are observed in salinity 10, suggesting an optimum condition among all four salinity levels. A comparable growth rate between 0 and 10 salinity indicates that the freshwater *Synechococcus* strain is well adapted to both the salinity conditions. However, significantly lower photosynthetic efficiency and slower growth rate in salinity 20 and 30 suggest that freshwater *Synechococcus* is stressed above salinity 10. The optimal preference of salinity 10 and restricted growth of freshwater strain in higher salinity can be explained by two cellular adaptive mechanisms. 1) The ion exchange between Na$^+$ and H$^+$ across the cell wall of freshwater *Synechococcus* continues efficiently at low salinity conditions. Under high salinity condition, the low Na$^+$ exclusion activity interrupts the ion exchange pathway, and cells are unable to cope with increased Na$^+$ influx resulting in cessation of growth (Ladas and Papageorgiou, 2000; Waditee et al., 2002). 2) Under elevated salt concentration, the freshwater cyanobacteria strains can accumulate low-molecular-weight organic solutes inside the cell to maintain cellular turgor pressure without changing the primary metabolism (Brown, 1976). However, increased stress at higher salinity disturbs this osmotic balance, resulting in cell death or restricted cellular growth. A breakdown of photosynthetic oxygen evolution and a decrease in the photosynthetic efficiency have been attributed to cyanobacterial slow growth rate under salt stress conditions (Reed et al., 1985; Allakhverdiev et al., 2000). A *Synechococcus* strain, isolated from salt-field, maintained an optimum growth between 105 to 135 g L$^{-1}$ NaCl concentrations. The maximum growth was obtained in 120 g L$^{-1}$ NaCl concentration, whereas growth ceased above 175 g L$^{-1}$ NaCl concentration (Roux, 1996). A balanced growth of *Synechococcus elongatus* in ASW medium was maintained between 2.1 and 52.5 practical salinity units, whereas no growth was observed above 72.5 practical salinity units (Qiu et al., 2012). Fry et al., (1986) showed increased respiration (~10 folds) of freshwater *Synechococcus* 6311 under elevated salt concentration resulting in inhibition of growth and photosynthesis. Rosales et al., (2005) observed that growth rate of a *Synechococcus* strain isolated from hypersaline pond followed the
order of 35‰ > 70‰ > 0‰ > 100‰ in different salinity conditions, while the longest duration of stationary phase was witnessed at 100‰. They also pointed out that exopolysaccharide production may be a favored option for maintaining optimum osmoregulation at higher salinity condition.

The different physicochemical factors (suboptimal temperature, limited nutrients, and osmotic stress) that restrict the cellular activities may enhance the microbial exopolysaccharide production (Sutherland, 1982; De Philippis et al., 1993; De Philippis and Vincenzini, 1998; Moreno et al., 1998; Poli et al., 2010). Our observations confirm freshwater *Synechococcus* strain with slower growth rate can survive in high salinity conditions by producing exopolysaccharide in the vicinity of the outer cell boundary. Under salinity stress, cells investment in increased exopolysaccharide production by slowing down the growth rate can be related to investing energy for survival rather than replication. An enhanced exopolysaccharide layer can protect the cells from dehydration by creating a microenvironment surrounding the cell wall that buffers the osmotic disequilibrium across the cell membrane and provides a repository for water and slows down the ion influx under hyper saline condition (De Philippis and Vincenzini, 1998; Sheng et al., 2006). Our results show that the S-EPS and TEP production increases with the age of the culture and salinity. Similar findings in an earlier investigation suggested a strong positive linear correlation ($r^2 = 0.99$) between EPS production by *Synechococcus elongatus* and salinity (Qiu et al., 2012). Our observations point out that TEP is closely attached to the outer boundary of *Synechococcus* cells resulting in increased formation of cell aggregates in higher salinity conditions (Supplement Fig. 2). Deng et al., (2016) noted the existence of TEP as cell coating on a marine *Synechococcus* strain WH8102, indicating a major component of the large cell-aggregate matrix. Authors further suggested that increasing of TEP production and formation of *Synechococcus* cell aggregation were associated with slower growth rate.

The cyanobacteria cell synthesizes compatible solutes to support osmotic equilibrium and extrusion of Na$^+$ under the salt stress condition, and these activities consume energy which is not available for cell growth (Jeanjean et al., 1993). The cell cycle analysis describes how cellular growth characteristics are regulated by different physicochemical factors and how does *Synechococcus* strain interact with the surrounding environments in cellular level (Carpenter and Chang., 1988). Two specific cell cycle modes, fast and slow growth models of cell cycle have been identified in different *Synechococcus* strains (freshwater, coastal and marine) in situ as well as under laboratory conditions (Binder and Chisholm, 1995). In fast growth mode, generation time is shorter than the total time required together for chromosome replication (C) and the time between termination of chromosome replication and cell division (D) (Nordström and Austin, 1993; Binder and Chisholm, 1995). On the contrary, generation time is longer than the sum of the time required for C and D phases in the slow
growth mode. Both the cell cycle modes of *Synechococcus* are regulated by different environmental factors such as nutrient concentration and ratios (nitrate and phosphate) (Vaulot et al., 1996; Bemal and Anil, 2016b), available spectral intensity and quality (Mann and Carr, 1974; Olson et al., 1986; Binder and Chisholm 1990; Bemal and Anil, 2016b). Various studies showed that both in culture (Binder and Chisholm, 1995; Bemal and Anil, 2016a) as well as in natural environment (Jacquet et al., 1998; Vaulot and Marie, 1999; Jacquet et al., 2002) cell cycle of *Synechococcus* is coupled to daily light-dark cycle. Such information on cell cycle analysis provide insights into cellular responses of different *Synechococcus* strains to varied environmental conditions that control the picophytoplankton community structure in riverine and estuarine waters. The understanding of cell cycle phases provides insights into varied growth characteristics (generation time) of the freshwater *Synechococcus* strain CCAP1405 under different salinity conditions. The bimodal DNA distribution of CCAP1405 is the first evidence for a freshwater *Synechococcus* strain, suggesting the slow growth mode of cell cycle can exist in all four salinity conditions. The freshwater *Synechococcus* strain PCC6301 exhibited unimodal to multimodal DNA distribution pattern indicating asynchronous fast growth mode of cell division (Binder and Chisholm, 1990; Binder and Chisholm, 1995). On the contrary, the synchronous cell division of the same strain (PCC6301) was reported in several earlier observations (Asato, 1979; 1983; 1984). The cell division of PCC6301 ceased during the onset of dark phase and the broad unimodal DNA distribution pattern was resolved into several peaks, resulting in multimodal DNA distribution. The cell division resumed during the light period and the DNA distribution returned to the unimodal pattern (Binder and Chisholm, 1990). Authors further showed that the multimodal DNA distribution was associated with faster growth rate (T_d = 3 h) condition and it shifted to the bimodal pattern when growth rate (T_d = 35 h) slowed down. The DNA distribution pattern of the four *Synechococcus* strains (CSIRNIO1, CSIRNIO2, CSIRNIO3 and CSIRNIO4), isolated from coastal and open ocean of Eastern Arabian Sea, was found to be bimodal in optimal cellular physiology conditions with cell division restricted to the light period and synchronized with the light-dark cycle (Bemal and Anil, 2016a). The open-ocean strain WH7803 has exhibited the fast growth multimodal DNA distribution pattern, whereas the other two open-ocean strains, WH7805 and WH8103 are characterized with bimodal slow growth model (Binder and Chisholm, 1995). However, Liu et al., (1999) found bimodal DNA distribution pattern for the WH7803 under a wide range of growth rate (0.1 – 0.9 day\(^{-1}\)) in chemostat continuous culture. The contrasting cell cycle data of specific strain indicate that the growth conditions of a culture can significantly influence the DNA distribution pattern (Scanlan, 2003). Our observations show that the DNA synthesis (S phase) of the freshwater CCAP1405 in all four salinity conditions reach to a maximum during the light-dark transition and replication (G2 phase) is completed in the dark period.
The synchronized DNA replication and doubling of cells in freshwater *Synechococcus leopoliensis* were completed during dark period (Döhler and Datz, 1989). The G1 and S phase duration of CCAP1405 significantly varied among the all four salinity conditions, while the G2 phase duration did not change with salinity. This consistency in G2 phase indicates that the post-replication duration is not dependent on salinity. The slower growth rate of freshwater strain CCAP1405 can be attributed to a significant lengthening of G1 and S phase duration and indicate cells spend a longer time in pre-replication and DNA synthesis phase under the higher salinity conditions. The increased exopolysaccharide (TEP) deposition surrounding the cell helps to maintain the cellular osmotic balance and is associated with slower growth rate in higher salinity condition. Exopolysaccharide secretion requires a significant share of energy that can cost up to 70% of total reserved energy (Poli et al., 2011). Claquin et al., (2008) showed that notable fraction of photosynthetically assimilated carbon is utilized in the production and release of TEP. The *Synechococcus* cells invest a larger proportion of energy in exopolysaccharide production than on cell division under salinity stress conditions, leading to reduced growth rate. The metabolic cost that goes into salt adaptation may reduce growth rates to the point of complete growth inhibition (Moisander et al., 2002). Consistent with the observations in this study, several previous studies related to different phytoplankton groups have indicated that increased TEP production is associated with a reduction of growth rate. The growth rate reduction was attributed to nutrient depletion in the growth medium (Corzo et al., 2000; Mari et al., 2005; Deng et al., 2016), natural ageing of organisms, self-shading (Kiørboe et al. 1990; Passow, 2002a), different environmental stressors and oxidative states (Berman-Frank et al., 2007).

The TEP cell-aggregation increases the functional size of *Synechococcus* cells, making them better at avoiding microzooplankton grazing but readily available for larger zooplankton (Pernthaler, 2005; Jezberová and Komárková, 2007). Deng et al., (2016) found that the higher *Synechococcus* cell-normalized TEP production was associated with lower growth rate under nutrient-limited conditions. The increased TEP production results in higher cellular stickiness which in turn facilitates the *Synechococcus* cell aggregation. The grazing on TEP-microaggregate of *Synechococcus* cells by mesozooplankton links the microbial loop with the classical food web (Passow, 2002b). A recent series of studies have shown significantly higher picophytoplankton contribution (~73 % of total carbon export) to deep water carbon transport pathways in the Pacific Ocean and Arabian Sea (Richardson et al., 2004; 2006; Richardson and Jackson, 2007). Deng et al., (2016) showed that the *Synechococcus* could contribute significantly to deep water carbon export via gravitational sinking of TEP-cell-aggregates in oligotrophic oceanic condition.
Conclusion

The present study suggests that salinity has marked effect on cell cycle phase duration as well as the growth rate of freshwater *Synechococcus* strain. The lengthening of G1 and S phase duration results in slower growth rate under higher salinity condition. The increased exopolysaccharide production and cell aggregate formation under salinity stress condition may alter the picophytoplankton contribution to the food web as well as deep water carbon export. Further studies elucidating the influence of rapid salinity change on the cellular physiology of *Synechococcus* will enable quantification of the changes and its implication in food web dynamics and biogeochemistry.

Acknowledgement

The authors thank the Director, CSIR–National Institute of Oceanography (NIO). S. Bemal acknowledges CSIR, New Delhi for providing the Research Fellowship. Authors greatly appreciate the discussions with Dr. Dattesh V Desai, Dr. Lidita Khandeparker and Dr. Shankar Doraiswamy. This is NIO contribution no..

References


Legends

Table 1: Cell cycle parameter estimates with the bimodal DNA frequency distributions for *Synechococcus* strain CCAP1405 in four different salinity treatments. The cultures were growing exponentially with the doubling times indicated. Population fractions obtained from the ModFit LT ver. 3.2 software and duration of the cell cycle phases were determined from the equations mentioned in Material and Methods. The specific growth rate calculated from cell number variation ($\mu$) and cell cycle ($\mu_{cc}$) over a diel cycle.

Figure 1: The growth curve (A), photosynthetic efficiency (B) and cell diameter (C) variation of *Synechococcus* CCAP1405 grown under different salinity treatments.

Figure 2: TEP (A) and S-EPS (B) produced by *Synechococcus* CCAP1405 during exponential growth (white area) and stationary growth (grey area) phase under different salinity treatments. *, ▲ and ■ depict significantly different ($p < 0.05$) from the first, second and third bar, respectively in each treatment condition. Error bar represents the standard error of the mean.

Figure 3: The bimodal DNA frequency distribution of *Synechococcus* CCAP1405 during exponential growth phase under different salinity conditions (A–D). Coefficient of variation of G1 peak was below 15% and RCS value of selected model was below 5 as obtained from ModFit LT v. 3.2 software. The diel pattern of cell cycle phase of the isolates is presented in (E–H). Light phase was maintained from 09:00 h to 21:00 h and the dark phase was continued from 21:00 h to 09:00 h. The light-dark transition time was fixed at 21:00 h. Open and filled horizontal bars indicate the light–dark phase of the growth period.

Figure 4: The cell cycle phase, G1 phase (A), S phase (B) and G2 phase (C) duration of *Synechococcus* CCAP1405 compared in different salinity treatments. *, ▲ and ■ depict significantly different ($p < 0.05$) from the first, second and third bar, respectively in each treatment condition. Error bar represents the standard error of the mean.

Supplement Fig. 1: The bivariate contour plot of *Synechococcus* strain CCAP1405 grown under different salinity treatments. The X-axis denotes cell size (FSC) and Y-axis denotes chlorophyll fluorescence (PerCP-Cy5-5) of *Synechococcus* CCAP1405. The pink contour represents the relative frequency of *Synechococcus* population. The cell size fluorescence values have followed the order 30> 20> 10> 0 salinity. Green cluster indicates the relative frequency of 2 micron size bead population.

Supplement Fig. 2: The bright field image of Alcian blue stained *Synechococcus* cell-aggregate.
Table 1

Growth rate and doubling time are given in day$^{-1}$ and hour, respectively.

<table>
<thead>
<tr>
<th>Salinity</th>
<th>% of population</th>
<th>Cell cycle parameters (h)</th>
<th>Mean ± SE (n=3)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>G1</td>
<td>S</td>
<td>G2</td>
</tr>
<tr>
<td>0</td>
<td>82.6±3.9</td>
<td>14.5±6.3</td>
<td>2.8±0.1</td>
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<tr>
<td>10</td>
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<td>8.6±5.1</td>
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<tr>
<td>20</td>
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<td>20.1±5.4</td>
<td>2.4±0.2</td>
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<tr>
<td>30</td>
<td>93.2±5.5</td>
<td>4.9±3.6</td>
<td>1.3±0.2</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Supplement Fig. 1
Supplement Fig. 2