

## **Synergistic effect of elevated temperature, pCO<sub>2</sub> and nutrients on marine biofilm**

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### **Abstract**

Natural marine biofilms provide signatures of the events that occur over a period of time and can be used as bioindicators of environmental changes. Hence, the effects of temperature (30 and 34°C), pCO<sub>2</sub> (400 and 1500 µatm) and nutrients (unenriched and enriched f/2 media) on the marine biofilm were evaluated using a 2×2×2 factorial design. In unenriched condition, acidification significantly increased the abundance of phytoperiphytes whereas reduced that of bacteria and it was vice versa in the enriched condition. Warming had significant negative effect on the abundance of both phytoperiphytes and bacteria, except in unenriched condition wherein it favoured bacterial growth. Synergistically, acidification and warming had deleterious effects resulting in further reduction in the abundance of both phytoperiphytes and bacteria, except in enriched condition wherein bacterial abundance increased. Such changes in biofilm communities in response to warming and acidification can have cascading effect on the subsequent build-up of macrofouling community.

**Keywords:** Biofilm, warming, acidification, nutrient enrichment, phytoperiphyton, bacteria

## Introduction

Diatoms, cyanobacteria (autotrophic) and heterotrophic bacteria are the major periphytic communities in a biofilm (Costerton et al., 1995), whose complex biological systems play a major role in benthic trophodynamics (Sommer, 1999) and also provide cues for the settlement of higher organisms (Qian et al., 2007). Biofilm formation, growth and development are dependent on temperature, pCO<sub>2</sub> and nutrient availability in the surrounding waters (Melo and Bott, 1997; Qian et al., 2007; Russell et al., 2013). Biofilm communities reorganize and respond rapidly to the environmental changes (Tolker-Nielsen and Molin, 2000), thus providing signatures of the events that occur over a period of time. As such biofilms can be used as bioindicators of environmental changes (Burns and Ryder, 2001 Sabater and Admiraal, 2005).

Warming and acidification are considered as major threats to the marine biota (Stocker et al., 2013). These processes are the consequences of the CO<sub>2</sub> emission by anthropogenic activities. While the impact of warming on marine biota is well known, the acidification might bring more complicated irreparable changes in the ecosystem. Climatologists have predicted a further three times increase in pCO<sub>2</sub> concentration, thus resulting in pH drop by 0.3 – 0.4 units and temperature increase by 1-4 °C by the end of this century (Stocker et al., 2013). Studies conducted so far reported varying effects of these stressors on microalgae and bacteria, however the results are either conflicting or scarce. Therefore, it is very difficult to draw a conclusion or to predict the patterns of future ecological impacts. Moreover, the potential effects of acidification on biofilms are still completely unknown (Cartaxana et al., 2015; Johnson et al., 2013; Lidbury et al., 2012; Russell et al., 2013).

So far, studies done on the effects of warming and acidification on biofilms are skewed towards unialgal diatoms (Torstensson et al., 2012; Torstensson et al., 2013); but, very few snapshots are available on multispecies natural biofilms (Cartaxana et al., 2015; Lidbury et al., 2012; Russell et al., 2013). In an earlier investigation using a unialgal fouling diatom (*Navicula distans*) it was observed that warming and acidification synergistically had a significant negative impact on its growth (Baragi et al., 2015). In the case of natural biofilms, complex interaction exists between different types of periphytic organisms and thus, it is hypothesised that the discrete response of each component to these environmental changes could modify the behavioural response of the whole biofilm.

Nutrients and CO<sub>2</sub> play vital role in the growth of photoautotrophs in a biofilm matrix. It has been reported that their influence on the photoautotrophic community can be better understood by using a nutrient-load hypothesis (Brauer et al., 2012), modified by Verspagen et al. (2014). The modified nutrient-load hypothesis describes the resource limitation patterns as a function of two limiting factors: nutrients (nitrate, phosphate and silicate) and carbon. It predicts that at low nutrient concentration and high pCO<sub>2</sub>, nutrient will be the limiting resource for microalgae; whereas, at high nutrient concentration and low pCO<sub>2</sub>, carbon will become limiting. However, both high nutrients and high pCO<sub>2</sub> will produce dense microalgal population that will increase the turbidity of water column thus creating a light limited condition.

The present study was designed to assess the response of natural biofilm community to temperature, pCO<sub>2</sub> and nutrients at community level. Biofilms were grown for 2 days under natural conditions (in the field) and their subsequent development in the laboratory under different combinations of

temperature, pCO<sub>2</sub> and nutrients to address the following questions (a) How does acidification and warming affect the natural biofilm communities (diatom, picophytoplankton and bacteria)? and (b) How does the nutrient concentration affect the response of biofilm community to acidification and warming?

## **2. Materials and Methods**

### *2.1. Natural biofilm development*

Natural biofilms were developed (April, 2013) by submerging polystyrene slides (7.6 × 2.5 cm) for 2 days at Dona Paula (15° 31'N, 73° 59'E), west coast of India. For this purpose, a total of 45 slides were fitted in a slide holder and suspended in the subtidal environment (depth ~ 1 m). After 2 days, the slide holders were retrieved and slides were used for the laboratory experiment. Out of 45 slides, 40 slides were used for the experiment [5 slides (5 replicates) X 8 treatments] and 5 slides for day 0 analysis.

### *2.2. Manipulation of pCO<sub>2</sub> and temperature levels*

The two pCO<sub>2</sub> levels used in the present study were ~400 µatm (present day pCO<sub>2</sub>) and ~1500 µatm (pCO<sub>2</sub> level projected for the year 2100) (Stocker et al., 2013). The projected 2100 year pCO<sub>2</sub> concentration was manipulated by adding NaHCO<sub>3</sub> and HCl (0.1 N) following the method described by Riebesell et al. (2010) in which total alkalinity (TA) was kept constant, but dissolved inorganic carbon (DIC) was increased. This is one of the methods recommended for manipulating the seawater CO<sub>2</sub> for small-volume experiments.

TA and pH values of the seawater were used to determine the pCO<sub>2</sub> concentration. TA was quantified by Gran titration (Gran, 1952) and the seawater pH was measured using a pH electrode calibrated using buffers (National Bureau of Standards). The TA, pH, temperature and salinity measurements were used to calculate the carbonate chemistry parameters of the treatments using CO<sub>2</sub> Sys program developed by Lewis and Wallace (1998), using the dissociation constant of Mehrbach et al. (1973) and then modified by Dickson and Millero (1987).

The two temperature levels used in the present study were ~30°C (ambient - present day temperature) and ~34°C (elevated - projected for the year 2100). Since the average annual temperature of Dona Paula Bay (Goa, India), where the biofilm was developed, is 30°C, it was used as present day temperature. Incubators (REMI, Microprocessor cooling incubator) were used to maintain the experimental temperatures, with ± 0.05°C accuracy.

### *2.3. Experimental design in the laboratory*

The experiment was conducted to test the effects of temperature (30°C and 34°C), pCO<sub>2</sub> (400 µatm and 1500 µatm) and nutrients (unenriched and enriched f/2 media) on the response of marine biofilms, using a 2 × 2 × 2 factorial design (total 8 combinations) on the marine biofilm. f/2 media was used for enriched conditions. The condition with ambient temperature (30°C) and pCO<sub>2</sub> (400 µatm) with unenriched nutrients was considered as control condition. The average initial concentrations of NO<sub>3</sub>+NO<sub>2</sub>, PO<sub>4</sub> and Si(OH)<sub>4</sub> in unenriched seawater were 3.0±0.4, 2.0±0.5, and 13.1±1.3 µmol l<sup>-1</sup> respectively; whereas, in enriched seawater were 238±43, 45±11 and 101.3±32

$\mu\text{mol l}^{-1}$ , respectively. In the laboratory, the 2 days old natural biofilms were incubated in the 500 ml polycarbonate bottles, at different treatment condition. The polycarbonate bottles were incubated under  $70 \text{ mmol photons m}^{-2} \text{ s}^{-1}$  illumination with a 12Light: 12Dark photoperiod, for 10 days. A total of 45 slides were used in the experiment (5 slides X 8 treatments = 40 slides and 5 slides for day 0 analysis). Every day the slides were transferred to new 500 ml polycarbonate bottles containing seawater of appropriate condition, this was done to maintain the  $\text{pCO}_2$  and nutrient levels stable. Five slides from each treatment were sampled, on day 10 of the experimental period, and used for analysis.

#### 2.4. Biofilm abundance and community analysis

In this paper the terms ‘periphytes’ refer to both autotrophic and heterotrophic organisms in the biofilm, ‘picoperiphytes’ to both autotrophic and heterotrophic organisms with cell size  $\leq 2\mu\text{m}$ , ‘phytoperiphytes’ to autotrophic organisms in the biofilm, and ‘picophytoperiphytes’ to autotrophic organisms with cell size  $\leq 2\mu\text{m}$ .

Diatoms were enumerated, by direct light microscopy of 400X magnification, in 10 randomly selected fields (area =  $0.38 \text{ dm}^2$ ) on each slide used for natural biofilm development. Diatoms were identified to genus level based on standard identification keys (Tomas, 1996). Final counts are expressed as diatoms  $\text{dm}^{-2}$ . This data are used to determine the Margalef’s generic richness (d), Pielou’s evenness (J’) and Shannon-Wiener’s diversity ( $H'$ ) of diatoms.

After diatom analysis each slide was scraped, with 10 ml of  $0.22 \mu\text{m}$  autoclaved filtered sea water, using a nylon brush as this method is identified to be efficient in removing the adhered cells (Patil and Anil, 2005); subsequently, the scrapped material was sonicated. For picophytoperiphyton and heterotrophic bacterial analysis, the sonicated sample (1 ml for each) was preserved with paraformaldehyde (0.2% final concentration) and stored at  $-80 \text{ }^\circ\text{C}$  until further analysis. The frozen samples were thawed and analysed through a BD FacsAria II flowcytometer equipped with a laser emitting at 488 nm following standard methods (Marie et al., 1997; Mitbavkar et al., 2012). For heterotrophic bacteria, samples were analysed after staining with nucleic acid stain, SYBR Green I (final concentration  $10^{-4}$ ) (Marie et al., 1997).

#### 2.5. Chlorophyll *a* (Chl *a*) and photosynthetic quantum yield analysis

Chl *a* estimation was done by filtering a known volume of sample (5 ml) onto the GF/F filter paper and extracting the pigments in 90% acetone for 24 hrs at  $4 \text{ }^\circ\text{C}$ . Absorbance of the extract was measured in a spectrophotometer. These values were then used to calculate the chl *a* concentration using the method described by Parsons et al. (1984).

The status of chlorophyll in the biofilms under different treatments was assessed using Fluorescence Induction and Relaxation (FIRe) technique. The FIRe fluorometer measures the initial ( $F_0$ ), maximum ( $F_m$ ) and variable ( $F_v = F_m - F_0$ ) chlorophyll fluorescence, which is later used to measure photosynthetic quantum yield ( $F_v/F_m$ ).  $F_v/F_m$  can be used to assess the physiological status of the photosynthesizing organisms in the biofilm. Lower values of  $F_v/F_m$  indicates unhealthy cells and vice versa (Patil and Jagadeesan, 2011).

## 2.6. Carbon contribution of different biofilm communities

Autotrophic carbon biomass was derived from chl *a* using a carbon to chlorophyll ratio of 40 (Gallegos, 2001). This value was used as the biofilm was dominated by diatoms. Picophytoperiphyton carbon biomass was calculated by measuring the carbon biomass from each of the 3 picophytoperiphyton groups. For measuring the carbon biomass from each group, initially right angle light scatter (RALS) data obtained from flowcytometry was converted to cell diameter (*d*) as explained by Worden et al. (2004). Later, the cell diameter was used to estimate the biovolume using the equation,  $V = \pi/6 (d^3)$ , assuming that picophytoperiphyton cells are spherical. The factor to convert cell abundance to carbon biomass for different picophytoperiphyton groups was derived from the biovolume to carbon conversion factor of 254 fg C mm<sup>3</sup> (Baudoux et al., 2007). From these estimations, the percentage contribution of picophytoperiphyton (< 2 μm) and larger phytoperiphyton (> 2 μm) to total carbon biomass was calculated. Heterotrophic bacterial carbon biomass was calculated using a carbon conversion factor of 20 fg C cell<sup>-1</sup> (Lee and Fuhrman, 1987).

## 2.7. Data analyses

Margalef's species richness (*d*), Pielou's evenness (*J'*) and Shannon-Wiener's diversity (*H'*) of the fouling diatom community were calculated, using PRIMER software version 6. Prior to statistical analysis, data was checked for normality by Shapiro-Wilk test and homogeneity of variances by Levens test to meet the assumptions of parametric tests. Data for each taxonomic group (diatom, picophytoperiphyton and bacteria) were log-transformed to meet assumptions of parametric tests. The variation in the descriptors of biofilm (abundance of diatom, picophytoperiphyton, and bacteria, chl *a* concentration and Fv/Fm ratio) was analysed by three-way MANOVA, with temperature (30 and 34°C), pCO<sub>2</sub> (400 vs 1500 μatm) and nutrients (Enriched and unenriched) as factors. Due to a significant 3-way interaction, we performed 2-way ANOVAs on each nutrient level (unenriched and enriched) independently, to investigate the effect of pCO<sub>2</sub> (400 vs 1500 μatm) and temperature (30 and 34°C) on the responses of biofilm. All statistical analysis was performed using STATISTICA software.

## 3. Results

At ambient temperature (30 °C), the initial ambient and elevated pCO<sub>2</sub> was 514±19 μatm and 1665±18 μatm, respectively. At elevated temperature (34 °C), the initial ambient and elevated pCO<sub>2</sub> was 493±29 μatm and 1560±36 μatm, respectively. The temperature and pCO<sub>2</sub> at ambient and elevated levels were significantly different, throughout the experiment (*p* < 0.05). The seawater pCO<sub>2</sub> concentration and nutrients were kept stable by every day transferring the slides to new polycarbonate bottles containing seawater of appropriate conditions. The three-way MANOVA revealed significant main effects of temperature, pCO<sub>2</sub> and nutrients; and interactions between these factors on the biofilm (Wilks' Lambda: *P* < 0.001).

### 3.1. Chlorophyll *a* and photosynthetic quantum yield (Fv/Fm)

The Chl *a* concentration on day 0 was 0.53 ± 0.12 μg dm<sup>-2</sup> (Fig. 1). On day 10, the maximum Chl *a* concentration (99.74 ± 4.36 μg dm<sup>-2</sup>) was observed at 514 μatm and 30°C condition with enriched nutrients (ANOVA, Post Hoc Tukey's HSD *p* < 0.001). The rise in pCO<sub>2</sub> to 1665 μatm at 30°C

increased the chl *a* concentration in both unenriched ( $3.03 \pm 0.47 \mu\text{g dm}^{-2}$ ) and enriched conditions ( $16.92 \pm 0.62 \mu\text{g dm}^{-2}$ ); however the pCO<sub>2</sub> effect was significant only in enriched condition ( $p < 0.001$ ). Temperature had significant negative effect on chl *a* concentration ( $p < 0.001$ ) with lower concentration at elevated temperature (34°C) ( $p < 0.001$ ); however, compared to unenriched condition the concentration was higher in enriched condition (ANOVA, Tukey's HSD  $p < 0.001$ ).

On day 0, the mean Fv/Fm was 0.75 (Fig. 2). On day 10, Fv/Fm values of biofilms at 514  $\mu\text{atm}$  and 30°C were not significantly different in both enriched and unenriched conditions ( $p = 0.872$ ). Irrespective of nutrient levels, the value was lower at 1665  $\mu\text{atm}$  than 514  $\mu\text{atm}$  pCO<sub>2</sub> ( $p < 0.01$ ). Significant negative effect of temperature was observed only in unenriched condition, with lower values in treatments with 34°C ( $p < 0.001$ ).

### 3.2. Periphytic community

#### 3.2.1. Autotrophs

##### 3.2.1.1. Diatoms

The mean diatom abundance on day 0 was  $1.9 \times 10^4$  cells  $\text{dm}^{-2}$  (Fig. 3A). On day 10, the abundance trend was similar to chl *a* concentration with highest abundance at 514  $\mu\text{atm}$  pCO<sub>2</sub> and 30°C in enriched condition ( $10 \times 10^5$  cells  $\text{dm}^{-2}$ ) (ANOVA, Post Hoc Tukey's HSD  $p < 0.001$ ). Irrespective of nutrient levels, the higher pCO<sub>2</sub> (1665  $\mu\text{atm}$ ) at 30°C enhanced the diatom abundance: unenriched ( $1.7 \times 10^5$  cells  $\text{dm}^{-2}$ ) and enriched ( $3.4 \times 10^5$  cells  $\text{dm}^{-2}$ ). However, in enriched condition the abundance was lower (~77%) compared to 514  $\mu\text{atm}$  pCO<sub>2</sub> and 30°C ( $p < 0.001$ ). Temperature significantly reduced the abundance of diatoms in conditions with 34°C and with a combination of 1560  $\mu\text{atm}$  and 34°C compared to 514  $\mu\text{atm}$  pCO<sub>2</sub> and 30°C ( $p < 0.01$ ). In the synergistic condition of elevated pCO<sub>2</sub> (1560  $\mu\text{atm}$ ) and temperature (34°C), the diatom abundance was 2-3 fold higher in enriched than unenriched conditions.

The diatom community composition was altered by temperature and pCO<sub>2</sub> conditions (Fig. 4A). Diatom belonging to 12 genera were recorded with *Navicula* as the dominant genus in all the treatments, throughout the experiment. At 30°C, either enrichment of nutrients or pCO<sub>2</sub> increased the diatom abundance whereas nutrients and pCO<sub>2</sub> synergistically decreased the diatom abundance except *Thalassionema* spp and *Achnanthes* spp. Irrespective of nutrient concentrations, increased temperature either reduced the diatom abundance (*Navicula* spp., *Nitzschia* spp., *Amphora* spp. and *Pleurosigma* spp.) or eliminated the diatoms (*Striatella* spp., *Thalassionema* spp., *Licmophora* spp. *Melosira* spp.) in the biofilm.

Changes in the diatom community were recorded in terms of Shannon-Wiener diversity ( $H'$ ), Margalef's species richness ( $d$ ), and Pielou's evenness ( $J'$ ). In both the nutrient levels, the mean generic diversity ( $H'$ ) of fouling diatom significantly varied with temperature with lower diversity at 34°C ( $p < 0.001$ ). The diversity was similar in both the pCO<sub>2</sub> levels ( $p > 0.05$ ). Biofilm grown at 514  $\mu\text{atm}$  and 30°C had highest diversity ( $0.62 \pm 0.01$ ) compared to other treatments (ANOVA, Tukey's HSD,  $p < 0.05$ ). The influence of temperature and pCO<sub>2</sub> on diatom generic evenness ( $J'$ ) was significant only in enriched condition with highest ( $0.79 \pm 0.1$ ) in condition with 1560  $\mu\text{atm}$  and 34°C ( $p < 0.05$ ). Diatom generic richness ( $J'$ ) was significantly influenced by temperature and pCO<sub>2</sub> in both

the nutrient conditions with a lower richness in conditions with 34°C and combination of 1560  $\mu\text{atm}$   $\text{pCO}_2$  and 34°C ( $p < 0.001$ ).

### 3.2.1.2. *Picophytoperiphytes*

The mean picophytoperiphytic abundance on day 0, was  $2 \times 10^4$  cells  $\text{dm}^{-2}$  (Fig. 3B). Picophytoperiphytic community comprised of *Synechococcus* spp., *Prochlorococcus*-like cells and picoeukaryotes. Irrespective of treatments, *Synechococcus* spp. was the dominant group (Fig. 4B). On day 10, the total picophytoperiphytic abundance was highest in enriched condition at 514  $\mu\text{atm}$  and 30°C ( $11 \times 10^6$  cells  $\text{dm}^{-2}$ ) ( $p < 0.001$ ) with *Prochlorococcus*-like cells as dominant group followed by picoeukaryotes. Regardless of nutrient levels at 30°C, elevated  $\text{pCO}_2$  (1665  $\mu\text{atm}$ ) enhanced the picophytoperiphytic abundance: unenriched ( $5.5 \times 10^5$  cells  $\text{dm}^{-2}$ ) and enriched ( $3 \times 10^6$  cells  $\text{dm}^{-2}$ ). However, in enriched condition the abundance was lower (~73%) compared to 514  $\mu\text{atm}$   $\text{pCO}_2$  ( $p < 0.001$ ). In unenriched condition, all the groups showed increasing trend at elevated  $\text{pCO}_2$  (1665  $\mu\text{atm}$ ) compared to 514  $\mu\text{atm}$ ; *Synechococcus* spp. (~58%), *Prochlorococcus*-like cells (~35%) and picoeukaryotes (~145%); however in enriched condition only *Synechococcus* spp. increased (10 fold). The total picophytoperiphytic abundance also increased in conditions with 34°C and with a combination of 1560  $\mu\text{atm}$  and 34°C compared to 514  $\mu\text{atm}$   $\text{pCO}_2$  and 30°C ( $p < 0.01$ ). The total picophytoperiphytic abundance in enriched conditions was 30-60% higher than unenriched conditions, however was 94% lower than condition with ambient  $\text{pCO}_2$  and temperature (514  $\mu\text{atm}$   $\text{pCO}_2$  and 30°C). The abundance of only *Synechococcus* spp. increased in these conditions irrespective of nutrient conditions.

### 3.2.2. *Heterotroph*

#### 3.2.2.1. *Bacteria*

The mean initial bacterial abundance was  $1.7 \times 10^7$  cells  $\text{dm}^{-2}$  (Fig. 3C). On day 10, the enrichment of nutrients at 514  $\mu\text{atm}$   $\text{pCO}_2$  and 30°C increased the abundance by ~23% compared to unenriched condition ( $p < 0.001$ ). Irrespective of temperature treatments, elevated  $\text{pCO}_2$  decreased the bacterial abundance in unenriched condition whereas increased the abundance in enriched condition ( $p < 0.01$ ). Temperature had significant effect on bacterial abundance with increasing and decreasing bacterial abundance with temperature in unenriched and enriched conditions, respectively (Fig. 3C,  $p < 0.001$ ).

### 3.3. *Diatom, picophytoperiphytic and bacterial carbon contribution*

The variation in phytoperiphytic (autotrophic) and bacterial carbon biomass is presented in Fig. 5A and B. Temperature and nutrients significantly influenced the pico ( $< 2 \mu\text{m}$ ) and larger phytoperiphytic ( $> 2 \mu\text{m}$ ) carbon contribution to total autotrophic carbon biomass (Fig. 6). Irrespective of  $\text{pCO}_2$  levels, the picophytoperiphytic carbon contribution was lower in conditions with 30°C: unenriched (0.07-0.1 %) and enriched (0.06-0.1%); whereas the contribution increased at elevated temperature (34°C): unenriched (1.07 %) and enriched (0.27 %) and with combination of 1560  $\mu\text{atm}$   $\text{pCO}_2$  and 34°C: unenriched (2.03 %) and enriched (0.26 %).

Bacterial biomass (BB) to autotrophic carbon biomass (AB) ratio (BB:AB) was significantly influenced by temperature and nutrients ( $p < 0.05$ ), with a higher ratio at elevated temperature (34°C): unenriched (0.078-0.102) and enriched (0.004-0.011) (Fig. 5C).

#### 4. Discussion

The present study highlights that acidification, warming and elevated nutrient significantly affect the biofilm community. The altered community structure in response to these factors is indeed a function of either the direct effect of these factors on the physiology of individual organisms or the effect of species interaction, such as competition, among the different groups within the biofilm under different experimental treatments.

Nutrient enrichment increased the abundance of phytoperiphytes (diatom and picophytoperiphyte) and bacteria in the biofilm, which corroborates the earlier studies (Cermeño et al., 2008; Chiu et al., 2008; Falkowski et al., 1998; Kirchman, 1994). According to nutrient-load hypothesis (Verspagen et al., 2014), at lower pCO<sub>2</sub> and nutrient concentration, both carbon and nutrients act as limiting factors for microalgal growth, resulting in lower abundance as observed in the present study. However, at high nutrient and low pCO<sub>2</sub> condition, carbon behaves as the sole limiting factor for microalgae (Verspagen et al., 2014). In contrast to this, recent studies have observed that carbon is not a limiting factor for microalgal growth at ambient atmospheric CO<sub>2</sub> level (400 µatm) because of the presence of the Carbon Concentrating Mechanisms (CCMs) which actively utilize bicarbonate and converts it to CO<sub>2</sub> (Giordano et al., 2005). This is also supported by the present study wherein, under ambient pCO<sub>2</sub> and enriched nutrients phytoperiphytes attained highest abundance.

Picophytoperiphytes dominated the phytoperiphytic community irrespective of nutrient levels, the reason of which could be attributed to their competitive strategies (Donald et al., 1997; Fogg, 1986). Because of the smaller size, picophytoperiphytes acquire and use nutrients more efficiently than larger phytoperiphytes (Donald et al., 1997; Fogg, 1986). Nutrient enrichment increased the abundance of picoperiphytes, however, its contribution to total carbon biomass was similar to unenriched condition. This could be due to the dominance of larger sized cells, *Synechococcus* spp. in unenriched condition whereas smaller sized *Prochlorococcus*-like cells in enriched condition. However, further studies should advocate this contrasting pattern of these two picophytoperiphytes in response to nutrient requirements.

In the present study, the abundance of phytoperiphytes increased in response to elevated pCO<sub>2</sub> which is in accordance with previous studies (Fu et al., 2007; Hicks et al., 2011; Johnson et al., 2013; Newbold et al., 2012). This is because, some microalgae take advantage of rising CO<sub>2</sub> level through downregulation of CCMs that cause reduction in the energetic cost and also by improving resource allocation (Hopkinson et al., 2011; Rost et al., 2008; Trimborn et al., 2009). At elevated pCO<sub>2</sub> and enriched condition phytoperiphytic (diatom and picophytoperiphyte) abundance increased supporting the nutrient-load hypothesis (Verspagen et al., 2014). However, the percentage increase was significantly lower than that at ambient pCO<sub>2</sub>, the reason of which could be attributed to 3 possibilities: (1) the phytoperiphytes are light-limited as evidenced from the presence of stalked preiphytic diatom *Achnanthes* spp., and larger sized picophytoperiphyton such as *Synechococcus* spp. compared to smaller sized such as *Prochlorococcus*-like cells at ambient pCO<sub>2</sub>; which might have resulted in overcrowding in the biofilm thus resulting in light-limiting conditions. (2) The lower pH, as a result of increased pCO<sub>2</sub>, affects the acid base balance (Beardall et al., 2009) and enhance mitochondrial respiratory (Wu et al., 2010) or photorespiratory (Gao et al., 2012) processes in

microalgae hence demanding high cellular energy which might have reduced the phytoperiphytic abundance at this condition. (3) The dominance of *Synechococcus* spp., which attains high growth rate at elevated pCO<sub>2</sub> compared to ambient pCO<sub>2</sub> (Fu et al., 2007) and is also known to produce substances with allelopathic effect on prokaryotic and eukaryotic cells (Martins et al., 2008) that may have inhibited the other periphytic organisms in the biofilm.

The addition of pCO<sub>2</sub> had no significant effect on the total bacterial abundance in enriched condition, which supports the earlier reports (Allgaier et al., 2008; Grossart et al., 2006; Oliver et al., 2014). However, under unenriched condition the lower abundance may be possibly due to the effect of lower pH which increases the respiration and in turn metabolic and energy cost of bacteria (Del Giorgio et al., 1999; Siu et al., 2014). This highlights the significant influence of nutrients on the response of bacteria to ocean acidification.

Elevated temperature, irrespective of pCO<sub>2</sub>, had detrimental effect on biomass (Chl *a*), photosynthetic efficiency (Fv/Fm) and diatom abundance whereas the picophytoperiphytic abundance remained unaltered. Studies have reported negative effect of elevated temperature on microphytobenthic biomass (Cartaxana et al., 2015; Hicks et al., 2011). In the present study under warming condition, the magnitude of biomass reduction was higher in unenriched than enriched condition. Moreover, the Fv/Fm ratio, a proxy for phytoperiphytic health (Patil and Jagadeesan, 2011), also significantly decreased under unenriched condition. Thus, indicating that the magnitude of temperature impact on the phytoperiphytes depends on the nutrient availability.

It has been observed that the microalgae in the tropical environment are at their upper limit of temperature tolerance therefore, a slight increase can have detrimental effects on them (Thomas et al., 2012). Among diatoms, some were temperature tolerant with reduced abundance (*Navicula* spp., *Nitzschia* spp., *Amphora* spp. and *Pleurosigma* spp.) and others were temperature sensitive and were eliminated from the biofilm (*Striatella* spp., *Thalassionema* spp., *Licmophora* spp. *Melosira* spp). These differences in the response of diatoms to temperature may be attributed to species specific response to temperature (Eppley, 1972; Suzuki and Takahashi, 1995). It is also known that microalgae with a narrow range of temperature for growth are expected to be affected by the future rise in temperature (Beardall and Raven, 2004). Boyce et al. (2010) reported the rapid decline (~1% annually) of global phytoplankton abundance with warming; with diatoms being the most affected group (Toseland et al., 2013).

Warming, under unenriched condition, favoured the growth of bacteria and this could be due to two reasons: (1) the positive effect of temperature on bacteria with higher bacterial production at elevated temperature (Apple et al., 2006), and (2) the availability of dissolved organic matter (DOM), source of nutrition for bacteria, from periphytic microalgae as microalgae are known to release high amounts of DOM under stressful conditions such as nutrient limitation. Under this condition, bacterial abundance was negatively correlated with phytoperiphytic abundance and had higher BB:AB ratio indicating less energy will be available to higher trophic organisms as the energy transfer from bacteria to higher trophic organisms is less efficient than phytoplankton to higher trophic organisms, because of the higher number of trophic levels in the food chain between bacteria and higher organisms.

In future, both factors, pCO<sub>2</sub> and temperature, will rise and make the ocean acidic as well as warmer. Under this condition with low nutrient concentration, diatoms attained lower abundance that may have resulted from combined stressful effects of warming and acidification. This could be due to higher energy requirement to cope up with an elevated temperature. Along with this, the additional energy is also required to meet the energy demand in maintaining the acid-base balance at lower pH. However, the addition of nutrients to this condition slightly increased the total phytoplankton abundance, thus, indicating that availability of nutrients compensated the negative effect of temperature and pCO<sub>2</sub>.

Warming either alone and in synergy with acidification increased the percentage contribution of picophytoplankton carbon to total autotrophic carbon biomass under unenriched condition. This is in accordance with an earlier study (Agawin et al., 2000) wherein they reported that picophytoplankton dominate the biomass in the warm and nutrient poor waters. To sustain maximum growth rates, picophytoplankton require 10 fold less nutrient concentration compared to larger phytoplankton. Hence, under low nutrient concentration picophytoplankton are able to maintain high growth rate where larger phytoplankton are nutrient limited (Agawin et al., 2000). Additionally, the increase in pCO<sub>2</sub> had additive effect as it enhanced the growth of picophytoplankton, especially *Synechococcus* spp. (Fu et al., 2007). However, the lower contribution in enriched condition could be due to the increased numbers of larger phytoplankton.

This study utilizes a multispecies approach, natural periphytic organisms that naturally co-occur in the nature, which has advantages over single species to predict the response of the biofilm to anthropogenic drivers. This experiment however, does not fully reflect the natural environment as we have eliminated the impact of grazers. Nevertheless, the results allow us to understand the response of biofilm including the interaction among the components (autotrophs vs heterotrophs) occurring in the biofilm.

## **5. Conclusion**

Our study points out a significant effect of warming on the biofilm growth and community composition than acidification, however the enrichment of nutrients decreased the magnitude of warming effect. Acidification and warming, individually and synergistically, resulted in altered biofilm community structure, which can have cascading effect on the subsequent build-up of macrofouling community. The response of microbial biofilms to the shifts in pCO<sub>2</sub> and temperature could be used as a biological indicator of future climatic scenario.

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## Legends to figures

**Fig. 1.** Chlorophyll *a* concentration ( $\mu\text{g dm}^{-2}$ ) in the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

**Fig. 2.** Photosynthetic quantum yield ( $F_v/F_m$ ) of the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

**Fig. 3.** Abundance ( $\text{cells dm}^{-2}$ ) of (A) diatom, (B) picophytoperiphyton, and (C) bacteria in the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

**Fig. 4.** Relative abundance of different species of (A) diatom and (B) picophytoperiphyton in the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

**Fig. 5.** Carbon biomass ( $\mu\text{g dm}^{-2}$ ) of (A) autotrophic periphytes, (B) bacteria, and (C) the ratio between bacterial carbon biomass and autotrophic carbon biomass (BB:AB ratio) in the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

**Fig. 6.** Carbon biomass ( $\mu\text{g dm}^{-2}$ ) and percentage carbon contribution to total autotrophic carbon biomass from (A) larger ( $> 2\mu\text{m}$ ) phytoperiphytes and (B) picophytoperiphytes ( $< 2\mu\text{m}$ ) in the biofilm grown under different  $\text{pCO}_2$  ( $\sim 400 \mu\text{atm}$  and  $\sim 1500 \mu\text{atm}$ ), temperature ( $\sim 30^\circ\text{C}$  and  $\sim 34^\circ\text{C}$ ) and nutrient (unenriched and enriched) combinations. Values are mean  $\pm$  SE ( $n=3$ ). T = temperature, C =  $\text{pCO}_2$ , N = nutrients, + = elevated levels. TCN refers to control condition.

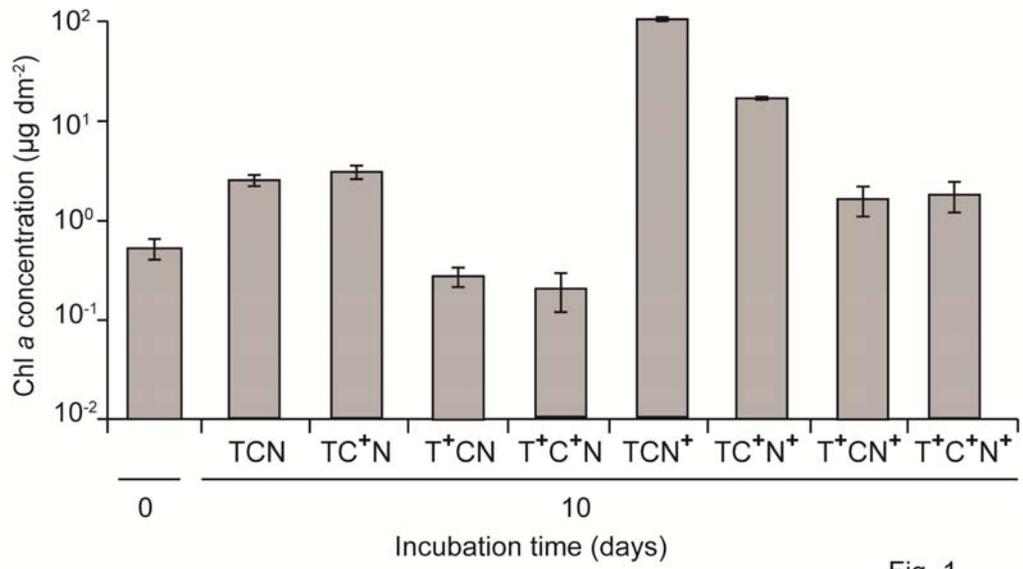


Fig. 1

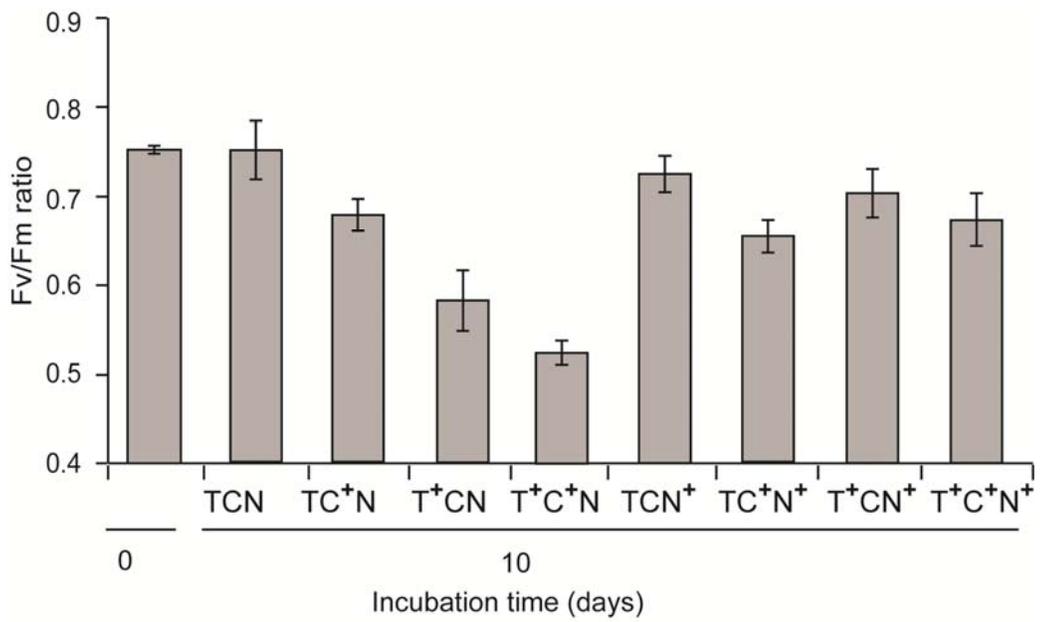


Fig. 2

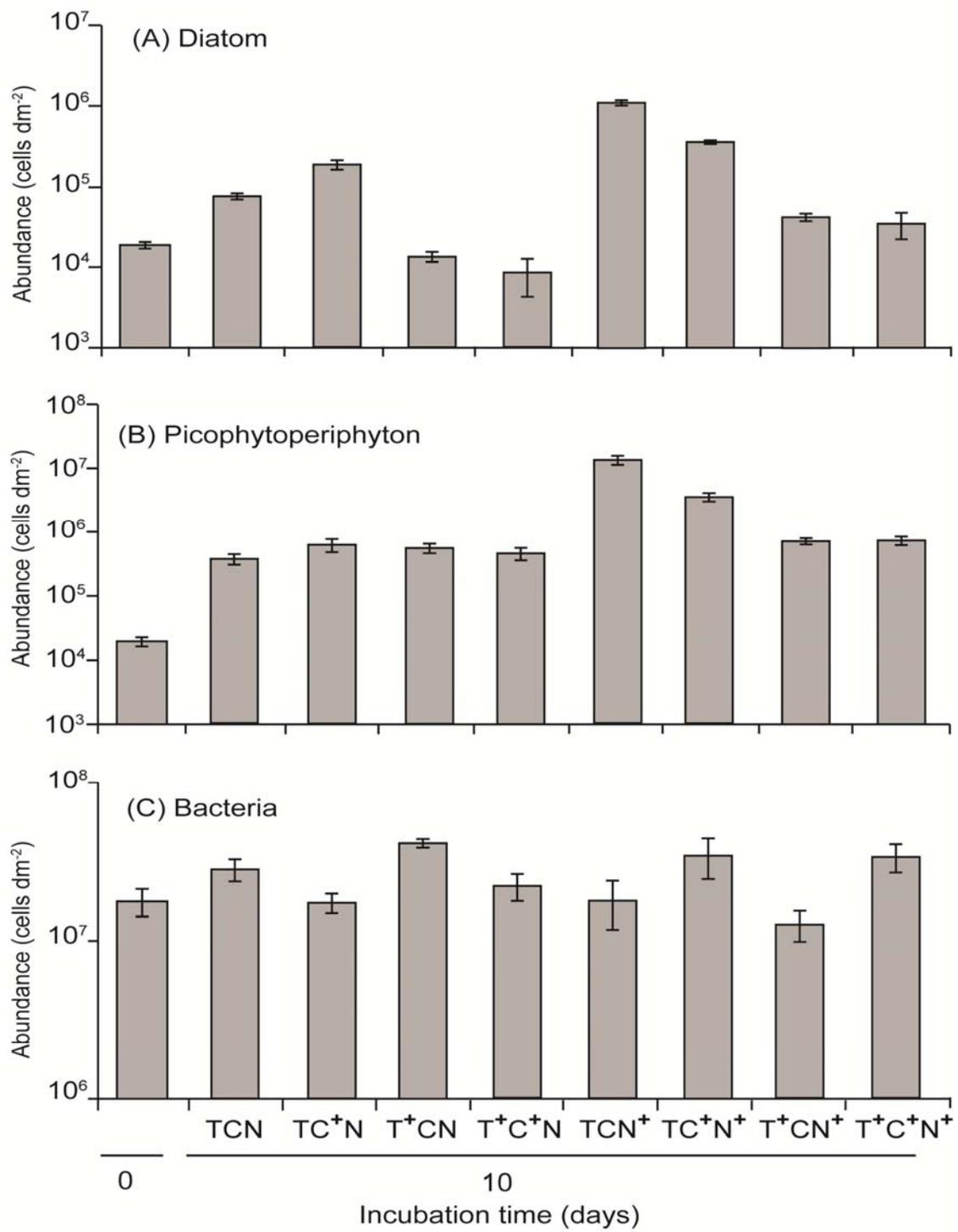


Fig. 3

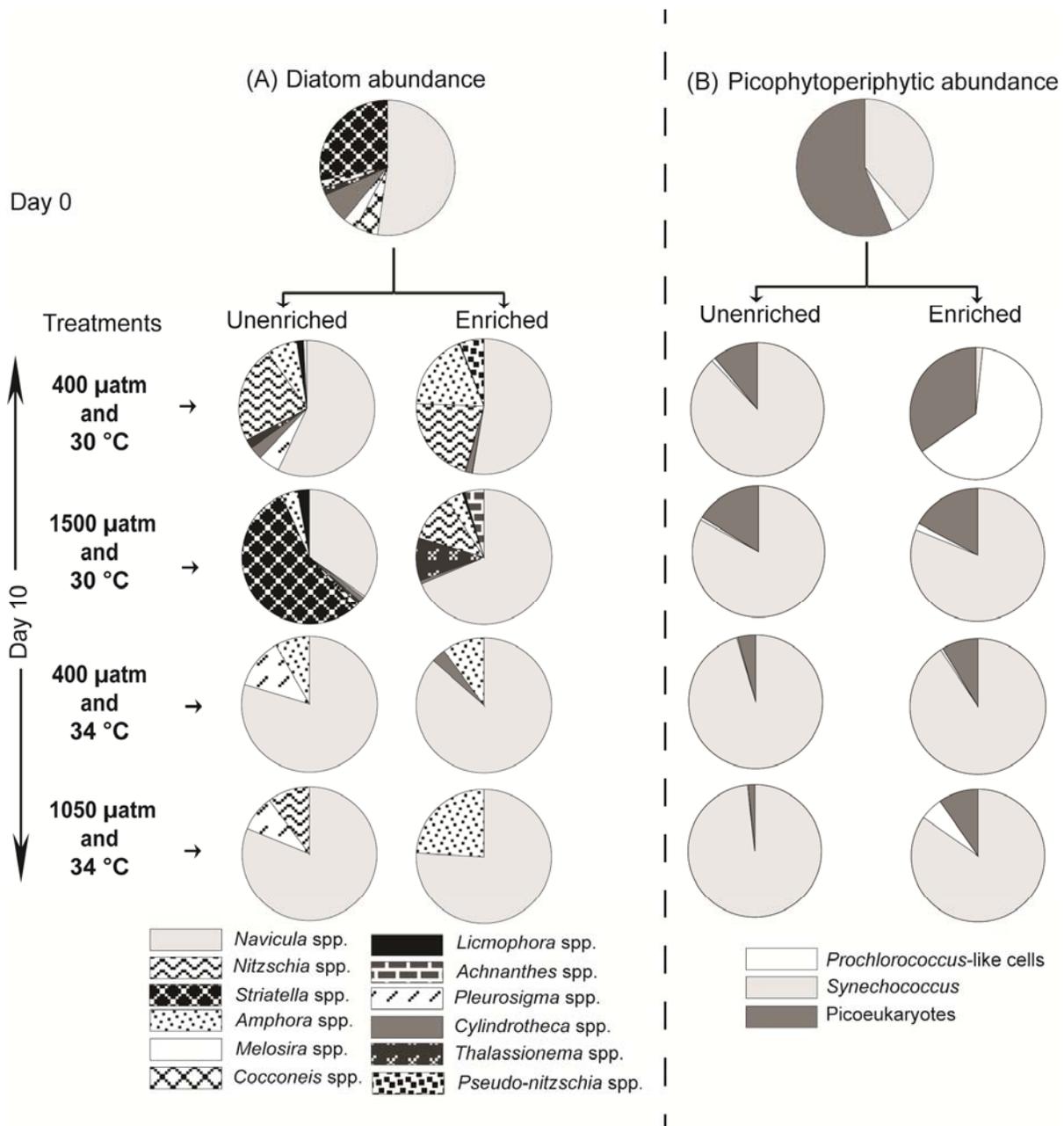


Fig. 4

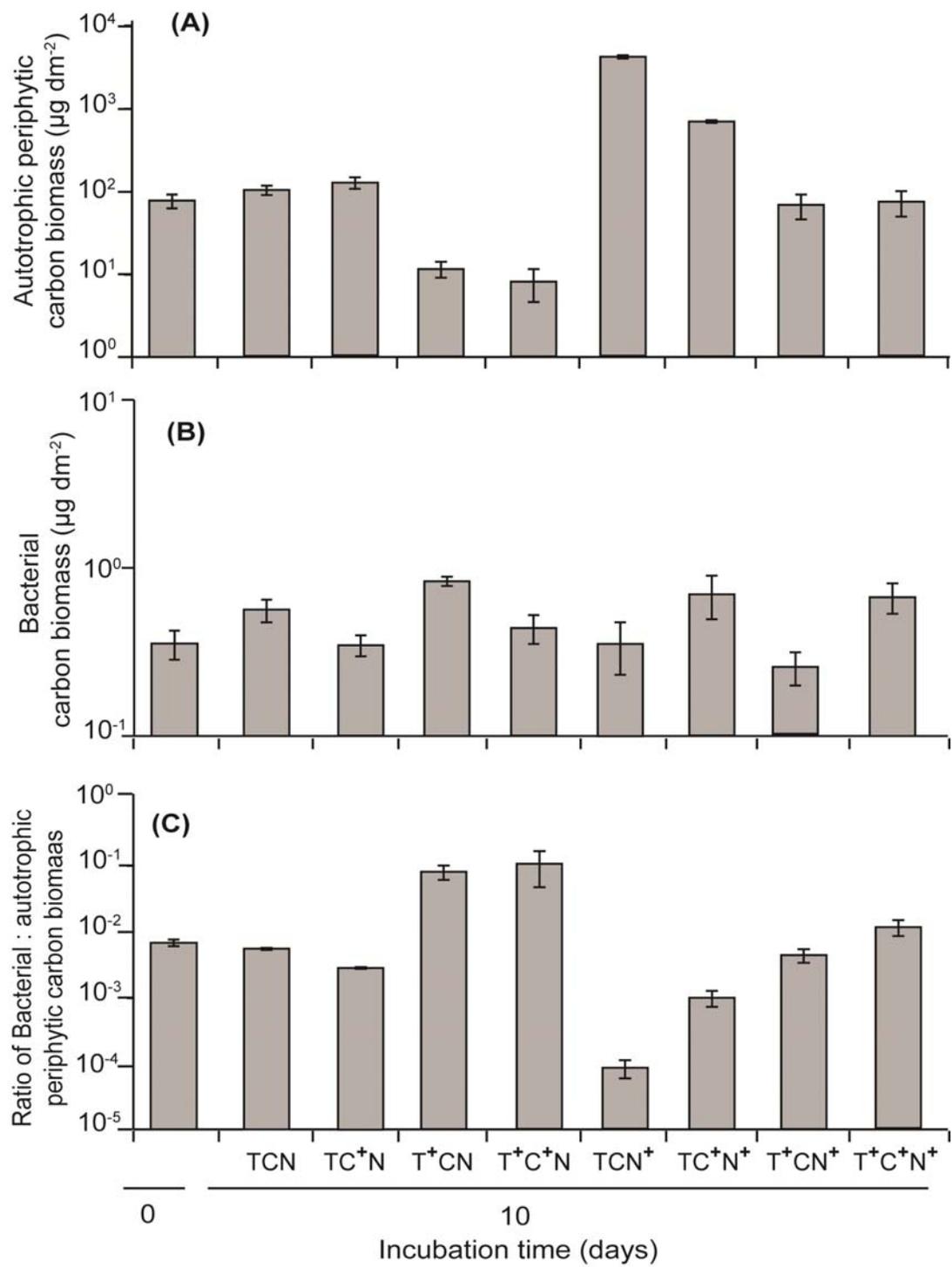


Fig. 5

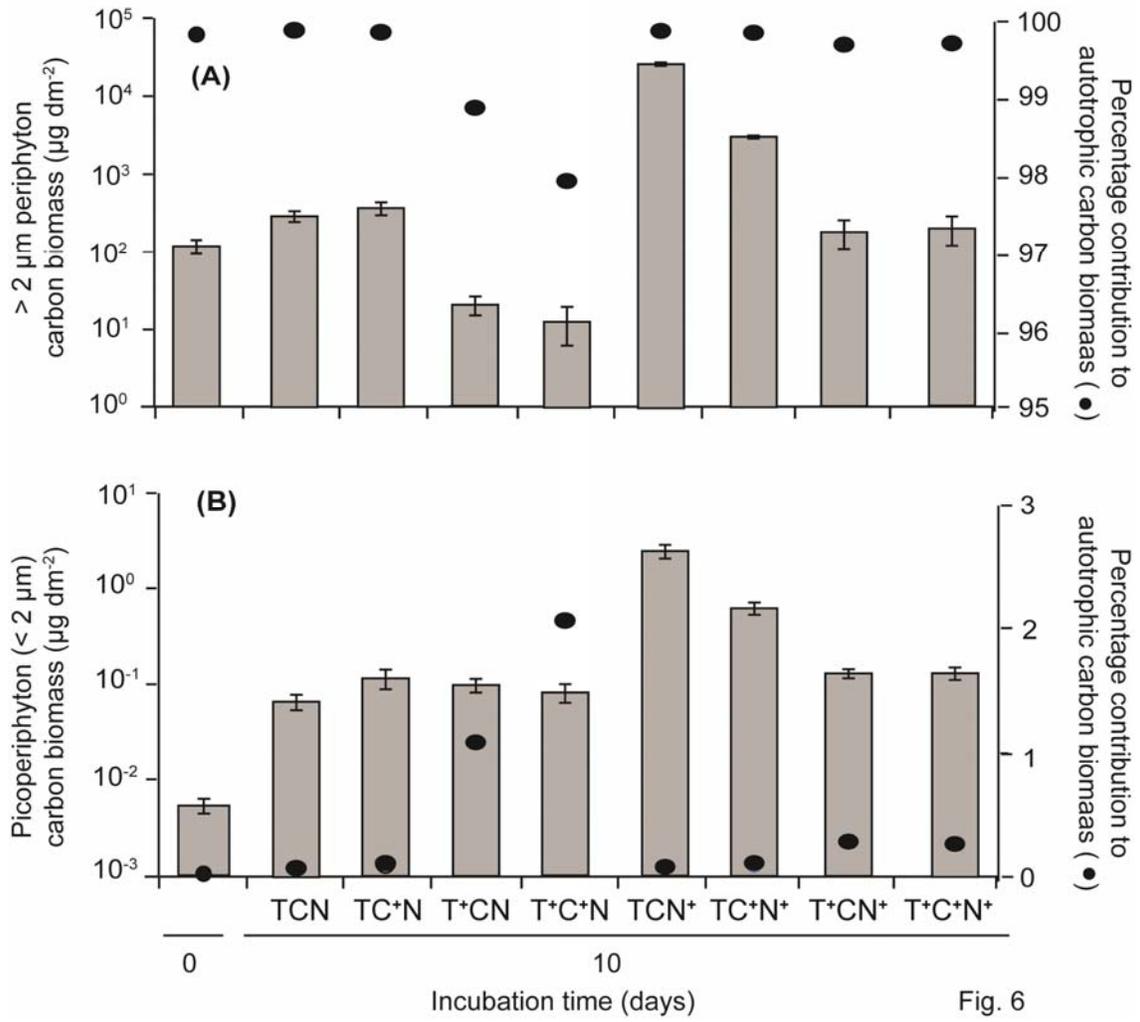


Fig. 6