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## **Influence of elevated temperature and pCO<sub>2</sub> on the marine periphytic diatom *Navicula distans* and its associated organisms in culture**

Lalita V. Baragi, Lidita Khandeparker, Arga C. Anil\*

*Academy of Scientific and Innovative Research (AcSIR)*

*CSIR – National Institute of Oceanography, Dona Paula, Goa – 403 004, INDIA*

**Running title:** Influence of temperature and pCO<sub>2</sub> on diatom

### **Abstract**

Influence of temperature and pCO<sub>2</sub>, reflecting the future climatic scenario, on the marine periphytic diatom (*Navicula distans*) and its associated organisms was evaluated. *Navicula distans* along with its associated picoperiphyte and heterotrophic bacteria were exposed to two temperatures (30 °C - present day and 34 °C - projected for year 2100) and pCO<sub>2</sub> levels (~500 µatm - present day and ~1500 µatm - projected for year 2100) in a 2 × 2 factorial design. It was observed that rising temperature reduced the abundance of *N. distans* and picoperiphyte; but increased that of heterotrophic bacteria. On the other hand, rising pCO<sub>2</sub> favored the growth of *N. distans* and picoperiphyte and had no significant effect on the bacterial growth. Synergistically, rising temperature and pCO<sub>2</sub> had a negative effect on *N. distans*, and a positive effect on picoperiphyte and heterotrophic bacteria. Additionally, this also resulted in the reduction of diatom cell size. This study suggests that in the future climatic scenario, increased abundance of picoperiphyte and heterotrophic bacteria along with smaller *N. distans* cells might influence the carbon budget and may have a cascading effect on higher trophic levels.

**Keywords:** Ocean acidification, biofilm, *Navicula distans*, picoperiphyte, heterotrophic bacteria

\*Corresponding author at: CSIR-National Institute of Oceanography, Dona Paula 403004, Goa, India. Tel: +918322450404; fax: +918322450615.

Email address: [acanil@nio.org](mailto:acanil@nio.org) (A.C.Anil)

## **Introduction**

Rapid industrialization across the globe has led to an increase in the concentration of carbon dioxide (CO<sub>2</sub>), a potent greenhouse gas, in the atmosphere. A quarter of this is absorbed by the ocean as a major sink (Canadell et al., 2007), resulting in a fall in pH (Ocean acidification). Since the pre-industrial era the concentration of CO<sub>2</sub> has increased from 280 μatm to 390 μatm resulting in a fall in pH by 0.1 unit and rise in the global average surface temperature by 0.8 °C. However, this will continue further and is expected to reach 750 μatm resulting in a change in pH (0.3- 0.4 units) and temperature (1-4 °C) by the end of this century (Stocker et al., 2013).

Temperature and pCO<sub>2</sub> are the two crucial factors that influence the ecological interactions in the marine ecosystem. Temperature stimulates growth and metabolism of all marine biota including phytoplankton at different rates (Brown et al., 2004; Eppley, 1972); whereas, elevated pCO<sub>2</sub> influences the photosynthetic carbon fixation and calcification of phytoplankton (Sett et al., 2014; Traving et al., 2014). Several investigators have studied the effect of either ocean acidification or warming separately. However, in nature these stressors influence the organism synergistically. The combined effect of these stressors have been addressed in few studies, however only snapshots are available for diatoms (Sett et al., 2014; Torstensson et al., 2013).

Diatoms are the major primary producers and dominate the fouling community in the shallow water photic system. Fouling diatoms are: (a) a source of primary production for higher herbivores (Hawkins et al., 1992; Sommer, 1999); (b) a source of settlement cues for invertebrate larvae (Patil & Anil 2005a). Diatoms showed positive, negative or no response to elevated pCO<sub>2</sub> (Johnson et al., 2013; Torstensson et al., 2012; Boelen et al., 2011). In this study, we evaluated the effect of elevated pCO<sub>2</sub> and temperature on *N. distans*, a dominant fouling diatom. This diatom was found to be associated with picoperiphyte and heterotrophic bacteria. Picoperiphytes can also include cyanobacteria as observed in earlier studies (Buck & Bentham 1998; Janson et al., 1995). Keeping this in mind, experiments were designed to elucidate the independent and synergistic effects of pCO<sub>2</sub> and temperature on diatom and associated organisms (picoperiphyte and heterotrophic bacteria).

## **Materials and methods**

### **Culture conditions**

*Navicula distans*, a pennate periphytic diatom, was isolated from a biofilm developed on a glass slide submerged in the waters of Dona Paula Bay located along the west coast of India (15° .31'N, 73° .59'E). This species was identified through Scanning Electron Microscopy (SEM) and was found

to be associated with the picoperiphyte and heterotrophic bacteria (Fig. 1). The stock culture of *N. distans* is maintained at the CSIR-National Institute of Oceanography, Goa, India at room temperature ( $28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ) in f/2 medium (Guillard & Ryther, 1962), prepared with autoclaved seawater (salinity 35).

### Temperature and pCO<sub>2</sub> manipulation

Experiments were carried out at  $30\text{ }^{\circ}\text{C}$  (ambient temperature - present day temperature) and  $34\text{ }^{\circ}\text{C}$  (elevated temperature - the projected year 2100 temperature). Temperature  $30\text{ }^{\circ}\text{C}$  was used as present day temperature and it corresponds to the average annual seawater temperature ( $29.3 \pm 1.8\text{ }^{\circ}\text{C}$ ) of the source locality of the strain. Cultures were maintained in incubators (REMI, Microprocessor cooling incubator) with an accuracy of  $\pm 0.05^{\circ}\text{C}$ . The two pCO<sub>2</sub> levels were chosen based on the present day (pCO<sub>2</sub> =  $\sim 500\text{ }\mu\text{atm}$ ; pH =  $\sim 8$ ; Non-acidified condition) and predicted concentration for the year 2100 (pCO<sub>2</sub> =  $\sim 1500\text{ }\mu\text{atm}$ ; pH =  $\sim 7.6$ ; Acidified condition) (Stocker et al., 2013). To achieve the projected 2100 year pCO<sub>2</sub> we adopted a method described by Riebesell et al. (2010), wherein NaHCO<sub>3</sub> and HCl (0.1 N) are added to seawater to increase the dissolved inorganic carbon (DIC) by keeping the total alkalinity constant. The seawater pH, total alkalinity (TA) temperature and salinity were used to determine the carbonate chemistry of different treatments using CO<sub>2</sub> sys program (Lewis & Wallace, 1998) by using the dissociation constant of Mehrbach et al. (1973), modified by Dickson & Millero (1987). A pH electrode calibrated using buffers (National Bureau of Standards) was used to measure the seawater pH and Gran titration method (Gran, 1952) was used to quantify TA.

### Experimental design

*Navicula distans* along with its associated organisms were exposed to two temperature ( $30\text{ }^{\circ}\text{C}$  (ambient temperature- present day) and  $34\text{ }^{\circ}\text{C}$  (elevated temperature - projected for year 2100)) and pCO<sub>2</sub> levels ( $\sim 500\text{ }\mu\text{atm}$  (ambient pCO<sub>2</sub>- present day) and  $\sim 1500\text{ }\mu\text{atm}$  (elevated pCO<sub>2</sub> - projected for year 2100)) in a  $2 \times 2$  factorial design. Four treatments were used in this study (1) AT-AC: Ambient Temperature ( $30\text{ }^{\circ}\text{C}$ ) and Ambient pCO<sub>2</sub> ( $\sim 500\text{ }\mu\text{atm}$ ); (2) AT-EC: Ambient Temperature ( $30\text{ }^{\circ}\text{C}$ ) and Elevated pCO<sub>2</sub> ( $\sim 1500\text{ }\mu\text{atm}$ ); (3) ET-AC: Elevated Temperature ( $34\text{ }^{\circ}\text{C}$ ) and Ambient pCO<sub>2</sub> ( $\sim 500\text{ }\mu\text{atm}$ ); and (4) ET-EC: Elevated Temperature ( $34\text{ }^{\circ}\text{C}$ ) and Elevated pCO<sub>2</sub> ( $\sim 1500\text{ }\mu\text{atm}$ ). *Navicula distans* along with its associated picoperiphyte and heterotrophic bacteria were initially adapted to the respective pCO<sub>2</sub> and temperature conditions for at least 7 days (i.e. more than 10 generations) to avoid measurement of any potential adaptation effects during the experiments. The

pre-adapted cells were grown in two different culturing techniques: batch and semi-continuous, in order to understand how these two different approaches would affect the experimental outcome.

### Batch culture

Stock suspensions of pre-adapted cells from the exponential growth phase, were cultured in polycarbonate bottles (500 ml capacity) containing 0.22  $\mu\text{m}$  filtered f/2 medium under 100-120  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  illumination with a 12L: 12D photoperiod, until the late exponential phase or early stationary phase. A total of 120 bottles were used in the experiment (4 treatments x 3 bottles (triplicates) x 10 sampling days). These bottles were sealed with parafilm in order to avoid gas exchange and is considered as a closed system for  $\text{CO}_2$  manipulation (Riebesell et al., 2010). Every alternate day, three bottles from each treatment were used for the analyses. To avoid any possible influence of diurnal rhythm in the cell division, the cultures were sampled at noon on each occasion. A sterile soft nylon brush was used to scrape the biofilm from the flask as this method is known to be efficient in removing the adhered cells (Patil & Anil 2005b). The scraped material was sonicated for 4 min to get a homogenous cell suspension and the sample from each of the bottles was used for the following analysis: (1) total alkalinity by Gran titration method (100 ml); (2) dissolved inorganic nutrients (50 ml); (3) enumeration of *N. distans* cell abundance and cell size (10 ml); (4) enumeration of picoperiphytic cell abundance (2 ml); and (5) enumeration of heterotrophic bacterial abundance (2 ml). On each sampling day, total alkalinity and pH was measured in each treatment and these values were used to calculate the amount of base and acid required to adjust the  $\text{pCO}_2$  to the initial levels as described by Riebesell et al. (2010). Briefly, due to the utilization of  $\text{CO}_2$  during diatom growth the pH of culture media increased. To adjust the pH and TA to initial values, 0.1N HCl was added to decrease the pH which also decreased the TA (0.1 ml HCl was added to 100 ml of seawater to decrease the pH and TA by 0.02 units and 103  $\mu\text{mol kg}^{-1}$ , respectively). The TA was then adjusted to initial value by adding  $\text{NaHCO}_3$  (addition of 0.58 mg of  $\text{NaHCO}_3$  to 100 ml increased the TA by 100  $\mu\text{mol kg}^{-1}$ ) (Supplementary table 1).

### Semi-continuous culture

The semi-continuous culture is based on the principle in which only a portion of the culture is harvested at a time and the remaining culture is adjusted to initial volume with seawater and nutrients (Bourne et al., 1989). This culturing technique allows us to understand the effect of  $\text{pCO}_2$  and temperature on the organism, at constant carbonate chemistry and nutrient concentration. In this culturing method, pre-adapted cells from the exponential growth phase, were cultured in polycarbonate bottles (500 ml capacity) containing 0.22  $\mu\text{m}$  filtered f/2 medium, under 100-120

$\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  illumination with a 12L: 12D photoperiod. A total of 120 flasks were used in the experiment (4 treatments x 3 bottles (triplicates) x 10 sampling days). Every alternate day, three bottles from each of the treatments were used for the analyses as it was done for batch cultures. From day 8, i.e on day 8, 12 and 16, the remaining bottles were sonicated (without damaging the cells) and a portion (40%) of culture media was removed and replaced by the fresh media with the calculated amount of nutrients in order to maintain the culture in the exponential growth phase.

## Analytical methods

### Analysis of *N. distans* cell abundance and cell size

Ten milliliters of the sample was fixed with filtered glutaraldehyde to a final concentration of 2.5%. Cell abundance was determined by counting three aliquots of cultures using a haemocytometer at a magnification of 400X. The maximum growth rate ( $\mu$ ,  $\text{day}^{-1}$ ) refers to the highest growth rate attained by the diatom over the experimental period and was calculated from the equation:

$$\mu = (\ln N_{t_2} - \ln N_{t_1}) / t$$

Where  $\mu$  = growth rate;  $N_{t_1}$  and  $N_{t_2}$  = cell count at initial and final time, respectively; and  $t$  = time (days).

*Navicula distans* cell size was measured as surface area/volume ratio. For this, 25 cells were selected at random to measure the two linear dimensions in order to calculate cell volume (V) and surface area (A) with a standard geometric shape (Sun & Liu, 2003). The measurements were made using an image analysis system (Image PRO) attached to a light microscope (OLYMPUS) and confocal microscope (OLYMPUS) at a magnification of 1000X.

### Analysis of picoperiphyte and heterotrophic bacterial abundance

The scraped homogenized samples for picoperiphyte abundance (in triplicate) were preserved using paraformaldehyde (0.2% final concentration) and stored at  $-80^{\circ}\text{C}$  until further analysis. For picoperiphyte analysis, a BD FACS Aria II flowcytometer equipped with 15-mW Argon laser emitting at 488 nm was used. Forward light scatter (FLS), side light scatter (SSC), green fluorescence (515–545 nm), orange fluorescence from phycoerythrin (564–606 nm), and red fluorescence from chlorophyll ( $>650$  nm) were measured for each particle after excitation by laser. Fluorescent beads (2  $\mu\text{m}$  “Fluoresbrite”, Polysciences) were used as internal standard and for the calibration of the above parameters (Marie et al., 1997). The samples for heterotrophic bacterial abundance (in triplicate) were preserved with 1% formaldehyde and stored at  $-20^{\circ}\text{C}$  until further

analysis. The sonication level (without disrupting the diatom cells) could segregate the loosely associated bacteria from diatom, therefore only free living and loosely associated bacteria were quantified from the samples. For analysis the samples were thawed and after addition of SYBR – Green I (nucleic acid stain, final concentration  $10^{-4}$ ; Molecular probes) were incubated at 37 °C for 30 mins and were analysed with a BD FACS Aria II flowcytometer (Marie et al., 1997).

### Nutrient analysis

The sample was filtered through GF/F filter paper (~0.7 µm pore size) and the filtrate was used for nutrients analysis [nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ) and silicate ( $\text{SiO}_3$ )] by SKALAR SANplus ANALYSER. Because of the possible interference of silicate from GF/F filter paper in silicate estimation a bench experiment was also carried out to derive a correction factor for silicate ( $6.93 \mu\text{mol L}^{-1}$ ).

### Data analyses

The examined parameters from different treatments (triplicate values), batch and semi-continuous cultures, were tested to ascertain if the detected variation was statistically significant. The level of significance considered was  $p \leq 0.05$ . Before analyses, the data was checked for normality and homogeneity of variances. A two-way ANOVA was performed with temperature and  $\text{pCO}_2$  as the factors. To test if the abundance of diatom, picoperiphyte and heterotrophic bacteria differed significantly over time with respect to changes in  $\text{pCO}_2$  and temperature, repeated measures of ANOVA were applied. A Pearson correlation was performed to assess the correlation between the diatom, picoperiphyte and heterotrophic bacterial abundance. All the statistical analyses were performed using STATISTICA software (Stat Soft Inc., Tulsa, OK, USA). Factor analysis was carried out for batch and semi-continuous culture datasets (abundance of diatom and its associated organisms) by using SPSS statistical package (Windows Ver. 16). This analysis was performed to understand the factors controlling the observed variations in the measured parameters.

## Results

### Carbonate chemistry

In both batch and semi-continuous cultures, at ambient temperature (30 °C), the initial ambient and elevated  $\text{pCO}_2$  was  $594 \pm 9$  and  $1725 \pm 28$ , respectively. At elevated temperature (34 °C), the initial ambient and elevated  $\text{pCO}_2$  was  $513 \pm 39$  and  $1480 \pm 46$ , respectively. Throughout the experiment the  $\text{pCO}_2$  and temperature at ambient and elevated levels were significantly different in batch ( $p \leq 0.05$ ) as well as semi-continuous cultures ( $p \leq 0.05$ ).

## Batch culture

Experiments under batch culture lasted for 18 days and the nutrient concentration changed with time. Nitrate concentration decreased from day 14, whereas silicate and phosphate concentrations started depleting from day 10 (Fig. 2a, b, c). Taking this into consideration the experimental duration was divided into 2 phases: phase I (days 0-8) with high nutrient concentration (nutrient-replete condition) and phase II (days 10-18) with low nutrient concentration (nutrient-limited condition). The changes in the growth of *N. distans*, picoperiphyte and heterotrophic bacteria have been discussed in the context of phase I and phase II.

## Growth of *N. distans* in different experimental treatments

The abundance of *N. distans* increased exponentially as a function of time over the cultivation period (Fig. 3a). At elevated temperature, *N. distans* attained maximum abundance and growth rate in phase I whereas at ambient temperature it did so in phase II. Temperature and pCO<sub>2</sub> either in isolation or in combination significantly influenced the maximum *N. distans* abundance and growth rate with higher values at ambient temperature (Table 1). Elevated temperature and pCO<sub>2</sub> synergistically resulted in the lowest cell abundance ( $p \leq 0.05$ ,  $4.9 \times 10^3$  cells mL<sup>-1</sup>). pCO<sub>2</sub> significantly affected the diatom abundance in phase II ( $p \leq 0.01$ ) compared to phase I ( $p \geq 0.05$ ). Temperature significantly influenced the diatom growth rate, with lower growth rate at elevated temperature ( $p \leq 0.05$ ). The diatom growth rate decreased at AT-EC (~23 % reduction) relative to AT-AC (however the decrease was not significant) (Fig. 4a). The lowest growth rate was observed in synergistic condition of elevated temperature and pCO<sub>2</sub> (ET-EC, ~57 % significant reduction relative to AT-AC) ( $p \leq 0.05$ ).

The diatom surface area/volume ratio (representing cell size) did not vary in phase I (Fig. 5a). In phase II, the ratio increased (cell size decreased) at elevated temperature (34 °C) with the highest ratio at elevated pCO<sub>2</sub> (ET-EC) ( $p \leq 0.001$ ). Temperature had a significant effect on the surface area/volume ratio ( $p \leq 0.05$ ). A reduction in the diatom cell size in the ET-EC treatment can also be seen in the confocal image (Fig. 6).

## Growth of picoperiphyte and heterotrophic bacteria in different experimental treatments

The abundance of picoperiphyte (Fig. 3b) and heterotrophic bacteria (Fig. 3c) were higher in phase I. In both the phases, temperature and pCO<sub>2</sub> in isolation and in combination had significant effect on picoperiphyte and heterotrophic bacterial abundance ( $p \leq 0.05$ ) except in phase II where temperature and pCO<sub>2</sub> in isolation had no significant effect on picoperiphytic abundance ( $p \geq 0.05$ ). Regardless of the temperature condition, picoperiphyte attained maximum abundance at elevated pCO<sub>2</sub>, with the

highest abundance in the AT-EC treatment ( $4.6 \times 10^3$  cells mL<sup>-1</sup>) followed by ET-EC treatment ( $2.6 \times 10^3$  cells mL<sup>-1</sup>) (Fig. 3b). Temperature alone and synergistically with pCO<sub>2</sub> had significant effect on the growth rate of picoperiphyte (Table 1) with higher growth rate at elevated temperature and pCO<sub>2</sub> (Fig. 4b). Higher bacterial abundance was observed at elevated temperature and the maximum was observed at elevated pCO<sub>2</sub> ( $7.2 \times 10^6$  cells mL<sup>-1</sup>), followed by ambient pCO<sub>2</sub> ( $3.9 \times 10^6$  cells mL<sup>-1</sup>).

Coupling between *N. distans* and its associated organisms (picoperiphyte and heterotrophic bacteria)

In phase I, Pearson's correlation analysis showed significant positive correlation between diatom and its associated organisms in all the treatments ( $p \leq 0.05$ ). In phase II, no significant correlation was observed between diatom and picoperiphyte ( $p \geq 0.05$ ). A significant positive correlation was observed between diatom and heterotrophic bacteria at AT-EC and significant negative correlation was observed at ET-EC. Factor analyses (FA) was done for two phases separately (Fig. 7). Two factors of FA together accounted for 69.61 % and 62.49 % variation in datasets in phase I and phase II, respectively. In phase I, this analysis indicated strong association between temperature and pCO<sub>2</sub> and picoperiphyte; and anti-association between nutrients and diatom and its associated organisms. In phase II, a strong association between temperature and pCO<sub>2</sub> and heterotrophic bacteria was observed.

Semi-continuous culture

In semi-continuous culture, nutrient concentrations were not limiting and the variations in the concentration of each nutrient over the course of experiment is presented in Fig. 2d, e, f.

Growth of *N. distans* in different experimental treatments

Temperature alone and in interaction with time (days) had significant influence on the diatom abundance ( $p \leq 0.05$ ) as observed in phase I (nutrient-replete condition) of batch culture. Temperature and pCO<sub>2</sub> either independently or synergistically had significant influence on the maximum diatom abundance and growth rate (Table 1). Maximum diatom abundance was observed at ambient temperature with the highest at ambient pCO<sub>2</sub> ( $8.8 \times 10^5$  cells mL<sup>-1</sup>) followed by elevated pCO<sub>2</sub> ( $6.6 \times 10^5$  cells mL<sup>-1</sup>) (Fig. 3d). Lowest diatom abundance ( $4.9 \times 10^3$  cells mL<sup>-1</sup>) was observed in synergistic condition of elevated temperature and pCO<sub>2</sub> (ET-EC;  $p \leq 0.05$ ). In all the treatments, maximum diatom abundance observed in batch cultures was higher compared to semi-continuous



cultures. Cells grown at ambient temperature (30 °C) exhibited higher growth rate in both the pCO<sub>2</sub> levels (Fig. 4c). The diatom surface area/volume ratio did not vary among the treatments (Fig. 5b).

### Growth of picoperiphyte and heterotrophic bacteria in different experimental treatments

The growth of picoperiphyte and heterotrophic bacteria is presented in Fig. 3e and f. Irrespective of temperature, higher picoperiphyte abundance was observed at elevated pCO<sub>2</sub> and the maximum abundance was observed at ambient temperature (AT-EC;  $4.9 \times 10^3$  cells mL<sup>-1</sup>), followed by elevated temperature (ET-EC;  $2.5 \times 10^3$  cells mL<sup>-1</sup>) (Fig. 3e). Temperature and pCO<sub>2</sub>, either in isolation or in combination, had significant influence on the picoperiphytic abundance and growth rate; and heterotrophic bacterial abundance (Table 1). Picoperiphytic growth rate increased with increasing pCO<sub>2</sub> and temperature (Fig. 4d). Higher bacterial abundance was observed at elevated temperature and the maximum abundance was observed at elevated pCO<sub>2</sub> (ET-EC;  $7.7 \times 10^6$  cells mL<sup>-1</sup>) followed by ambient pCO<sub>2</sub> (ET-AC;  $3.9 \times 10^6$  cells mL<sup>-1</sup>) (Fig. 3f).

### Coupling between *N. distans* and its associated organisms (picoperiphyte and heterotrophic bacteria)

A strong positive correlation was observed between diatom and its associated organisms ( $p \leq 0.05$ ). In Factor analysis, the two factors together accounted for 73.69 % variation in the dataset (Fig. 8). It revealed an anti-association between nutrients and diatom and its associated organisms; and a strong association between temperature and pCO<sub>2</sub> and picoperiphyte.

### Discussion

This study demonstrates that the periphytic diatom, *N. distans* is sensitive to elevated pCO<sub>2</sub> and temperature. Among the two stressors, temperature seems to be the overriding factor determining its abundance and growth. In the present study we employed two culturing techniques, batch and semi-continuous, in order to understand how these two different approaches would affect the experimental outcome. In general, the results from the semi-continuous culturing technique were similar to those obtained from Phase I of batch culture (nutrient-replete condition).

It has been reported that warming is the causative factor behind the rapid decline (~1% annually) of phytoplankton abundance globally (Boyce et al., 2010); among the phytoplankton the most affected group is diatom (Toseland et al., 2013). In both batch and semi-continuous cultures, lower growth rate and abundance of *N. distans* at elevated temperature (34 °C) indicate the impact of warming, within an average predicted range, on the growth of this species.

In batch culture, at elevated temperature the diatom culture collapsed on day 10 because of nutrient depletion, whereas at ambient temperature even after day 10 the diatom abundance increased. This is possibly because of the nutrient accumulation by the diatoms from nutrient-replete phase (phase I) which was utilized for growth under nutrient-limiting phase (phase II) and this also supports the findings of Dortch et al. (1984). In contrast, at elevated temperature the inability of diatoms to grow after nutrient depletion could be because of increase in the cellular metabolism (Brown et al., 2004). Warming, along with nutrient-limiting condition (phase II, batch culture), also resulted in the reduction of *N. distans* cell size. It has been reported earlier that nutrients can modify the effect of temperature on the diatom cell size (Peter & Sommer, 2012). The reduction in diatom cell size may have a cascading effect on the higher trophic levels and influence the food web dynamics. The possible reason for this could be the cell size, which drives various ecological and physiological functions of diatoms including nutrient diffusion, intake and requirements and metabolic rate (Aksnes & Egge, 1991; Hein et al., 1995; Pasciak & Gavis, 1974; Shuter, 1978). This is also one of the important factors that determine the export of primary production into the deep ocean and higher trophic levels (Finkel et al., 2009).

Elevated pCO<sub>2</sub> increased the abundance of *N. distans* which might be due to downregulation of Carbon Concentrating Mechanisms (CCMs) (Hopkinson et al., 2011), that actively transports HCO<sub>3</sub><sup>-</sup> into the cell and catalyses the build-up of CO<sub>2</sub> around the RuBisCO under CO<sub>2</sub> limited condition at the expense of high metabolic energy (Raven et al., 2008). Several studies have reported varying response of diatoms to elevated pCO<sub>2</sub>: positive (Low-Décarie et al., 2011; Li & Campbell, 2013); neutral (Riebesell et al., 1993; Boelen et al. 2011); and negative (Low-Décarie et al., 2011; Torstensson et al., 2012). Studies also showed positive effect of elevated pCO<sub>2</sub> on benthic microalgae (Johnson et al., 2013, Lidbury et al., 2012). Such diverse response in diatoms may result from diverse CCMs in diatoms (Reinfelder, 2011). However, elevated pCO<sub>2</sub> combined with elevated temperature had significant negative effect on the growth of *N. distans*; the exact mechanism for such negative response is currently unknown and needs further attention.

Picoperiphytes were found on the diatom surface however, the exact cause of association is still unknown. Irrespective of temperature, elevated pCO<sub>2</sub> increased the abundance and growth rate of picoperiphytes in both batch and semi-continuous cultures. Since picophytoplankton (include cyanobacteria) are undersaturated with respect to carbon at present CO<sub>2</sub> concentration, therefore future climatic condition (with increased pCO<sub>2</sub>) might favour their growth (Fu et al., 2007; Schulz et al., 2013). The faster growth rate of picoperiphytes compared to that of *N. distans* at elevated pCO<sub>2</sub>

is suggested by Newbold et al. (2012), because of superiority in the rapid utilization of CO<sub>2</sub> by picoplankton compared to other photosynthetic organisms. The association between diatom and picoperiphytes was also influenced by nutrient concentration. In nutrient-replete conditions (batch (phase I) and semi-continuous cultures), the growth of picoperiphytes was tightly coupled with diatom. However, when the nutrients were exhausted (batch cultures (phase II) the abundance of both the groups reduced and no significant correlation was observed. This is probably the outcome of competition between diatom and picoperiphytes for nutrients under nutrient limited condition.

Till date, very little or no effect of the elevated pCO<sub>2</sub> has been found on the bacterial abundance. In the present study, heterotrophic bacterial abundance was strongly influenced by the temperature instead of pCO<sub>2</sub>. An increase in bacterial abundance in response to increasing temperature is a general phenomenon, which is also evident in the present study. Previous studies reported increased bacterial activities in response to increasing temperature (Hoppe et al., 2008; Piontek et al., 2009). The abundance of bacteria did not exhibit any difference either in the present or elevated pCO<sub>2</sub>. Even in previous studies on the community response to elevated pCO<sub>2</sub>, the total bacterial abundance did not significantly differ between pCO<sub>2</sub> treatments. However, the changes in free-living community composition were observed, and were linked to elevated pCO<sub>2</sub> (Allgaier et al., 2008; Kerfahi et al., 2014; Witt et al., 2011). Lidbury et al. (2012) while investigating the community-level response of biofilms to ocean acidification in a natural vent ecosystem reported an increased bacterial diversity in response to elevated pCO<sub>2</sub>.

The important source of food for bacteria is the organic matter derived from phytoplankton, which results in the tight coupling between phytoplankton and bacterial production and abundance (Cole et al., 1988). Based on the review from freshwater, estuarine and coastal waters, it is concluded that dissolved organic carbon (DOC) released from phytoplankton meets <50% of the bacterial carbon requirements (Baines & Pace, 1991). The phytoplankton and bacterial interaction may be both “down up” and “top down” at the same time. Bacteria affect the amount and composition of DOM released by phytoplankton (Bruckner et al., 2011; Gärdes et al., 2012); and the composition of DOM released by the phytoplankton will affect the growth and structure of the bacterial community (Pete et al., 2010). Marine bacteria are highly efficient in utilizing carbon and nutrients and compete with phytoplankton for the same. In the present study, the abundance of heterotrophic bacteria was tightly coupled with the *N. distans* development in phase I of batch culture and semi-continuous cultures, when nutrients were high. However, when nutrients were exhausted (in phase II) there was no significant correlation between the two groups except at elevated pCO<sub>2</sub>. Under elevated pCO<sub>2</sub>,

diatom and bacteria exhibited positive correlation at ambient temperature whereas at elevated temperature they showed significant negative correlation. Collapse of diatom did not result in an increase in the bacterial abundance, and a separate heterotrophic phase was not observed in phase II except for synergistic condition of elevated pCO<sub>2</sub> and temperature, where increased bacterial abundance was observed. A previous study (Grossart et al., 2006) has indicated that with the changing pCO<sub>2</sub>, the response of heterotrophic bacteria is primarily linked to phytoplankton rather than a direct effect of pCO<sub>2</sub> or pH. Decoupling between phytoplankton and bacteria was mainly observed in phase II. Previous studies on the biomass standing stocks of phytoplankton and bacteria during phytoplankton blooms showed some decoupling in response of bacteria to phytoplankton (Kirchman et al., 1994; Lochte et al., 1997). There may be several possible reasons for this decoupling. The first possibility is the existence of a dormant state in most marine bacteria under severe carbon, phosphate and/or energy starvation. A second possibility is the presence of non-dividing bacterial subpopulations, which are in turn overgrown by the growing populations (De Kluijver et al., 2010; Ducklow et al., 1999). A third possibility is viral lyses of bacteria, which controls bacterial production in aquatic environments, thus influencing ecological and biogeochemical processes (Bratbak et al., 1992; Fuhrman 1999); this cannot be ruled out and needs to be considered in future studies. A fourth possibility for the decoupling is the quality and quantity of Dissolved Organic Matter (DOM) produced by the diatom, as bacteria utilizes DOM for its growth. The quality and quantity of DOM may affect the bacterial growth and might have resulted in decoupling between diatom and bacteria. The last possible reason could be the effect of low nutrient concentration on bacteria and diatom. In low nutrient environment bacteria directly compete with diatom for dissolved inorganic nutrients; and bacteria also grow slowly to survive in these conditions (Abreu et al., 2003).

Although interesting results with wider implications were obtained from this experiment, the experimental setup used does not fully mimic the environmental conditions in coastal waters where there are wide fluctuations in pH, CO<sub>2</sub> and temperature due to biological activity and physical forcing.

A recent study has shown that the organisms in the tropical environment are at their upper limit of their temperature tolerance therefore, slight increase can have deleterious effects on the organisms (Thomas et al., 2012). For example, if the results obtained from this study are mimicked in the Dona Paula Bay, it would influence the carbon budget and have a cascading effect on the higher trophic levels.

## Conclusion

In batch culture, two phases were observed: phase I (nutrient-replete condition) and phase II (nutrient-limited condition). The results from the semi-continuous culturing conditions were similar to the results obtained from Phase I of batch culture (nutrient-replete condition). Elevated pCO<sub>2</sub> had positive influence on the abundance of *N. distans* and picoperiphyte whereas, temperature had positive influence on the abundance of heterotrophic bacteria.

Through the experiments carried out in this study, it is also evident that elevated pCO<sub>2</sub> and temperature synergistically had a negative influence on the diatom growth rate and under nutrient-limiting conditions (Phase II) this resulted in the reduction of diatom size. Under this condition, the abundance of picoperiphytes and heterotrophic bacteria also increased. Such a change might influence the carbon budget and may have a cascading effect on the higher organisms. Further studies are required to elucidate the changes in the trophic level and food web dynamics.

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## Legends to figures

**Fig. 1 The association of *Navicula distans* with picoperiphyte and heterotrophic bacteria.** (a) Epifluorescence image of (a) *N. distans* and picoperiphyte with Chlorophyll autofluorescence and (b) *N. distans* and heterotrophic bacteria stained with SYBR Green stain. D- diatom, P- picoperiphyte and B- heterotrophic bacteria

**Fig. 2 Mean concentration ( $\mu\text{mol L}^{-1}$ ) of nutrients with respect to time (Days), in different treatments.** Batch culture (a) nitrate, (b) phosphate and (c) silicate; and semi-continuous culture (d) nitrate (e) phosphate and (f) silicate. Vertical lines indicate standard deviation from the mean.

**Fig. 3 Mean cell abundance (cells  $\text{ml}^{-1}$ ) with respect to time (Days), in different treatments.** Batch culture (a) *N. distans*, (b) picoperiphyte and (c) heterotrophic bacteria; and semi-continuous culture (d) *N. distans*, (e) picoperiphyte and (f) heterotrophic bacteria. Vertical lines indicate standard deviation from the mean

**Fig. 4 Maximum growth rate ( $\text{Day}^{-1}$ ) in different treatments.** Batch culture (a) *N. distans*, (b) picoperiphyte; and semi-continuous culture (c) *N. distans*, (d) picoperiphyte. Vertical lines indicate standard deviation from the mean.

**Fig. 5 Mean diatom surface area/volume ratio with respect to time (Days), in different treatments.** (a) Batch culture and (b) semi- continuous culture. Vertical lines indicate standard deviation from the mean

**Fig. 6. The cell size of *N. distans* at AT-AC and ET-EC in batch culture.**

**Fig. 7 Factor Analysis biplot for batch culture.** It shows the relationship between the abundance of different groups (diatom, picoperiphyte and heterotrophic bacteria) and the abiotic factors: carbonate concentration ( $\text{CO}_3^{2-}$ ),  $\text{pCO}_2$  concentration ( $\text{pCO}_2$ ), temperature, silicate, phosphate and nitrate. (a) Phase I and (b) Phase II

**Fig. 8 Factor Analysis biplot for semi-continuous culture.** It shows the relationship between the abundance of different groups (diatom, picoperiphyte and heterotrophic bacteria) and the abiotic factors: carbonate concentration ( $\text{CO}_3^{2-}$ ),  $\text{pCO}_2$  concentration ( $\text{pCO}_2$ ), temperature, silicate, phosphate and nitrate

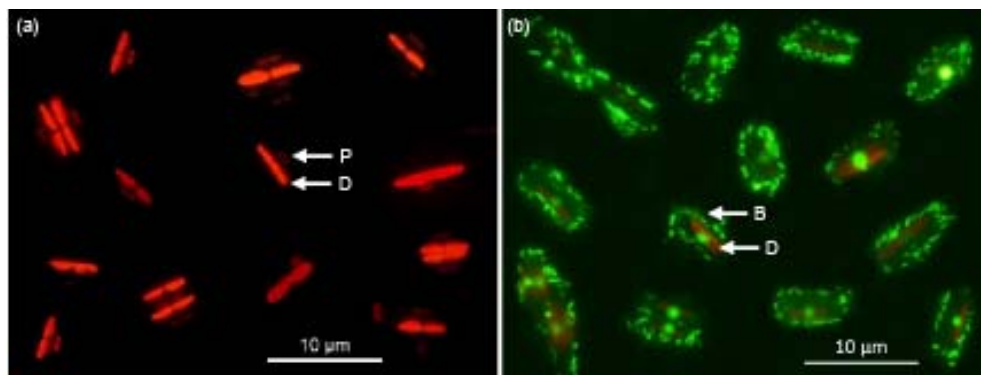


Fig. 1

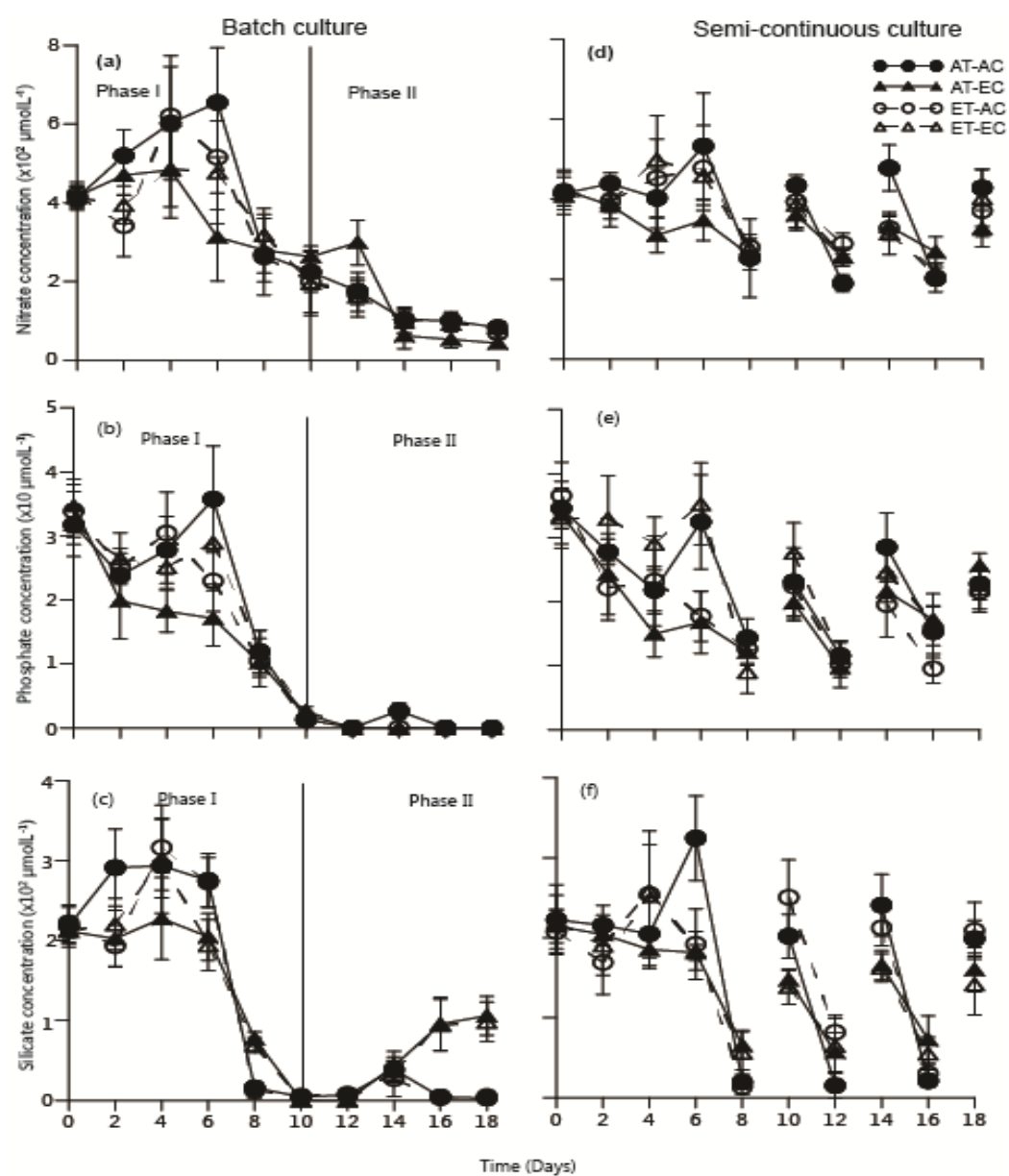


Fig. 2

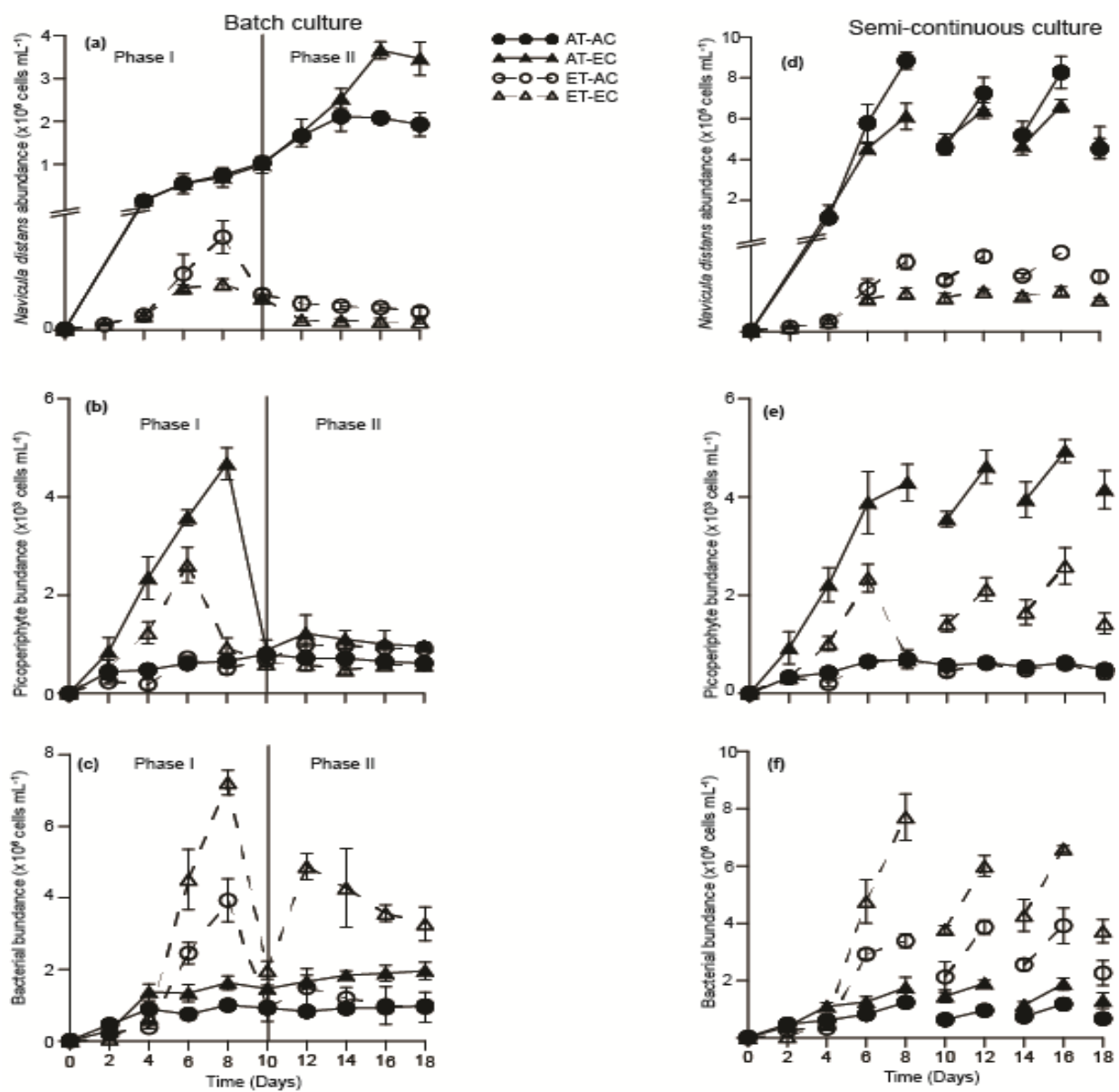


Fig. 3

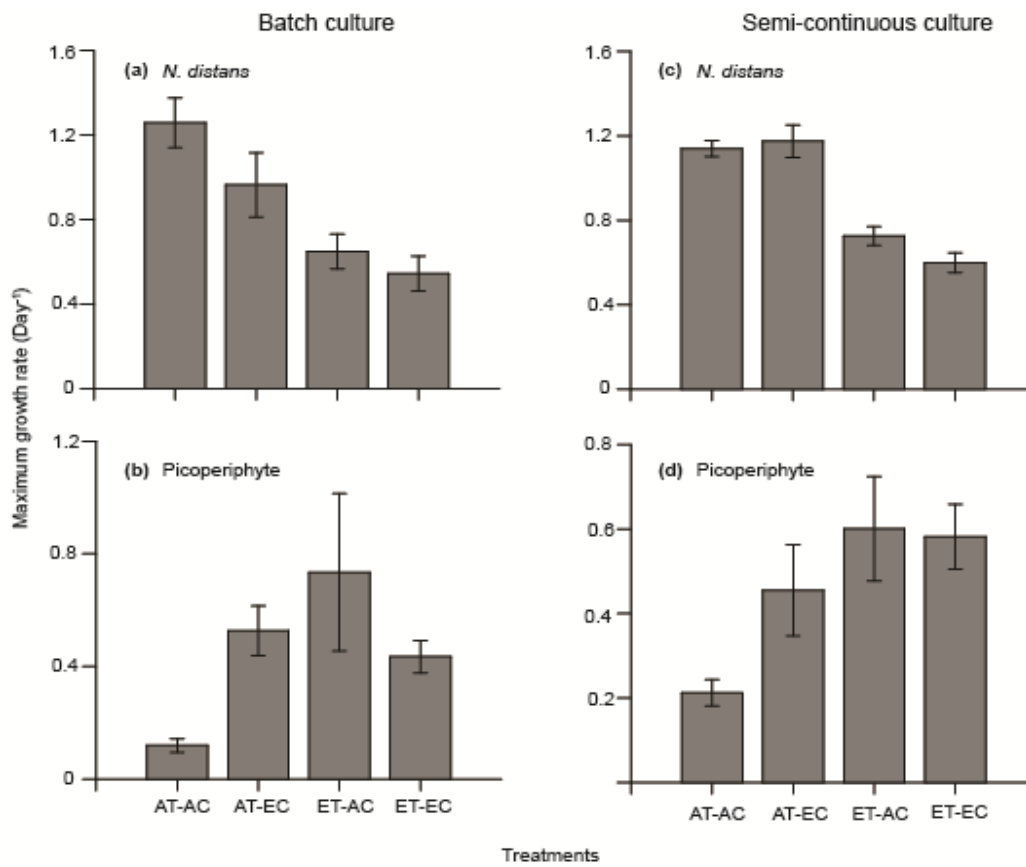


Fig. 4

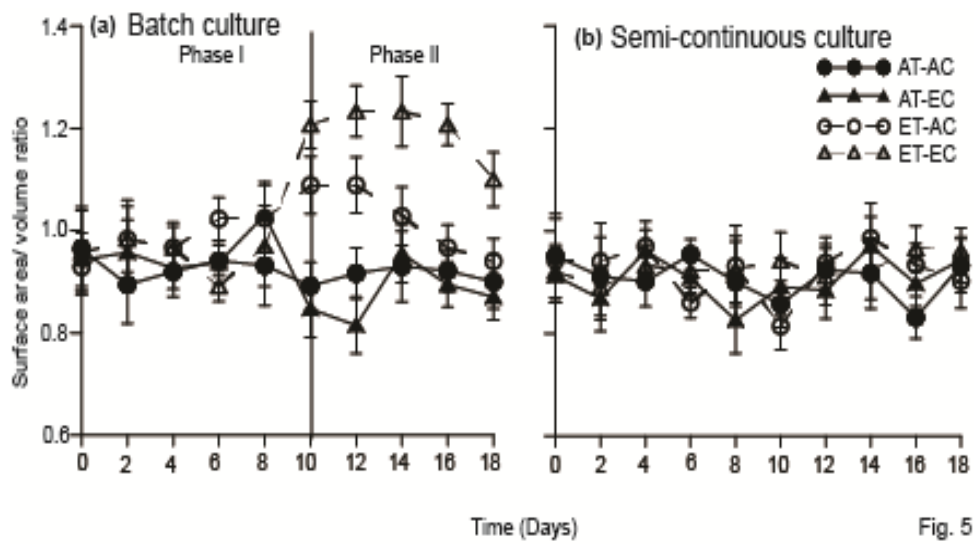
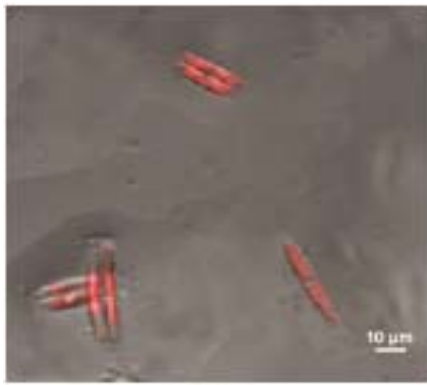
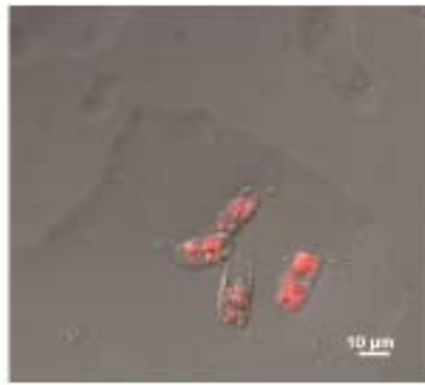


Fig. 5



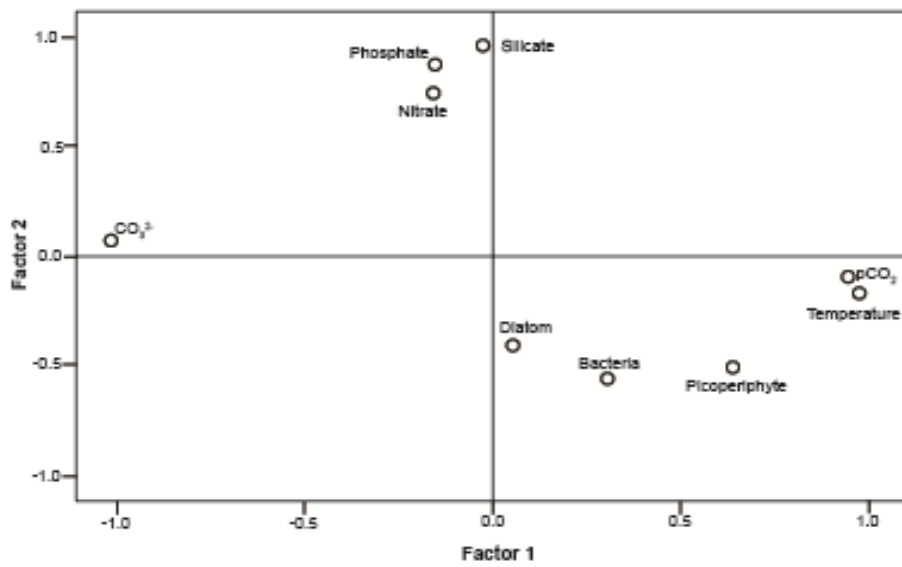
AT-AC



ET-EC

Fig. 6

(a) Batch culture - Phase I



(b) Batch culture - Phase II

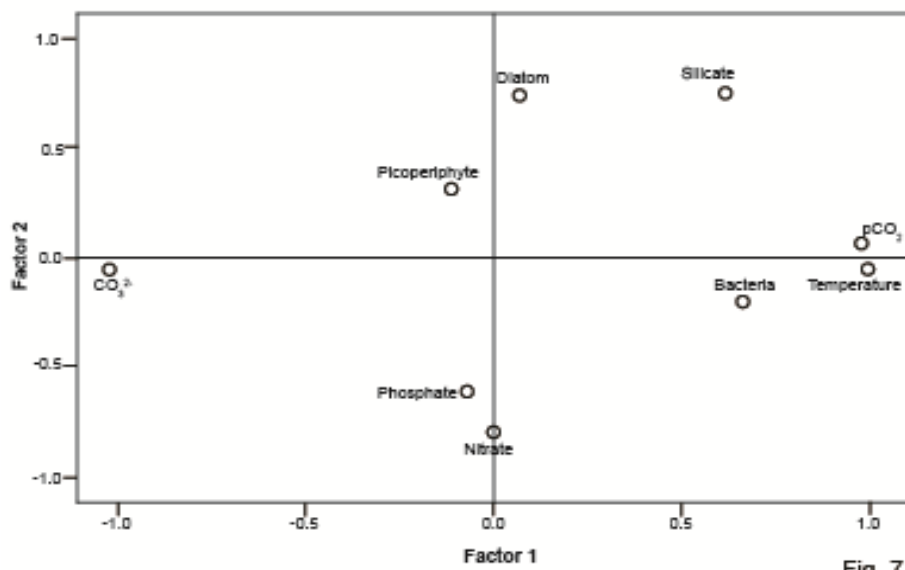


Fig. 7

Semi-continuous culture

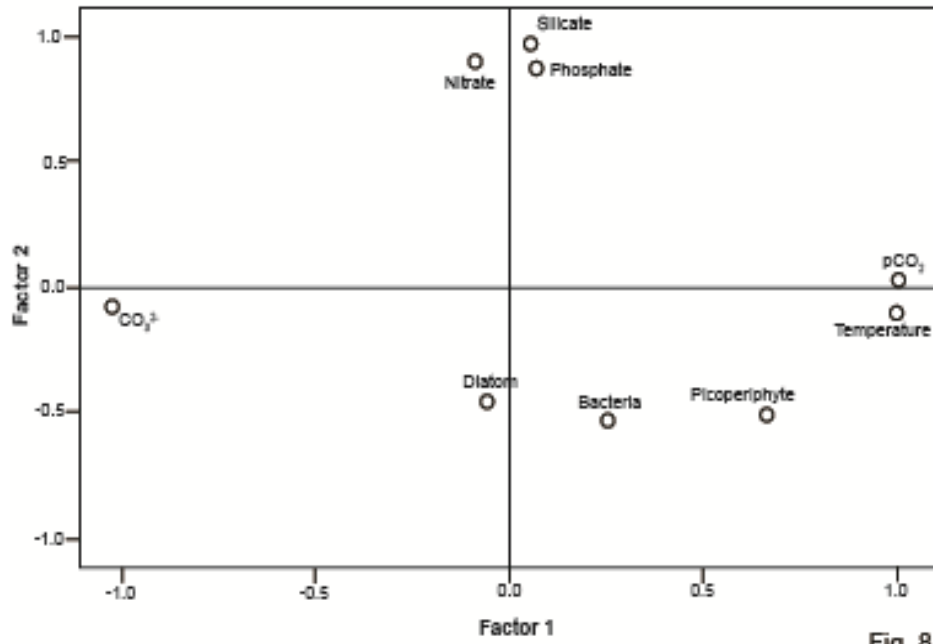


Fig. 8



Table 1. Two-way analysis of variance (ANOVA) for interaction and main effects of temperature and pCO<sub>2</sub> treatments. Significant results are in bold

Variable		df	Batch culture		Semi-continuous culture		
			F-value	p-value	df	F-value	p-value
Maximum diatom abundance (Cells mL <sup>-1</sup> )	Temp	1	635.38	<b>≤0.001</b>	1	2554.60	<b>≤0.001</b>
	pCO <sub>2</sub>	1	45.49	<b>≤0.001</b>	1	55.26	<b>≤0.001</b>
	Temp x pCO <sub>2</sub>	1	46.09	<b>≤0.001</b>	1	50.75	<b>≤0.001</b>
	Error	8			8		
Maximum picoperiphyte abundance (Cells mL <sup>-1</sup> )	Temp	1	9.32	<b>0.016</b>	1	35.62	<b>≤0.001</b>
	pCO <sub>2</sub>	1	537.14	<b>≤0.001</b>	1	901.70	<b>≤0.001</b>
	Temp x pCO <sub>2</sub>	1	47.27	<b>≤0.001</b>	1	34.86	<b>≤0.001</b>
	Error	8			8		
Maximum heterotrophic bacterial abundance (Cells mL <sup>-1</sup> )	Temp	1	369.22	<b>≤0.001</b>	1	331.22	<b>≤0.001</b>
	pCO <sub>2</sub>	1	99.53	<b>≤0.001</b>	1	63.45	<b>≤0.001</b>
	Temp x pCO <sub>2</sub>	1	30.02	<b>≤0.001</b>	1	3.24	0.1095
	Error	8			8		
Maximum diatom growth rate (day <sup>-1</sup> )	Temp	1	9.95	<b>0.014</b>	1	254.83	<b>≤0.001</b>
	pCO <sub>2</sub>	1	1.49	0.258	1	2.17	0.179
	Temp x pCO <sub>2</sub>	1	0.34	0.577	1	6.75	<b>0.032</b>
	Error	8			8		
Maximum picoperiphyte growth rate (day <sup>-1</sup> )	Temp	1	9.11	<b>0.017</b>	1	23.66	<b>0.001</b>
	pCO <sub>2</sub>	1	0.39	0.551	1	4.46	0.068
	Temp x pCO <sub>2</sub>	1	16.74	<b>0.003</b>	1	6.08	<b>0.039</b>
	Error	8			8		

**Supplementary table 1.** Measurement of the pH and Total alkalinity (TA) and the amount of HCl (0.1N) and NaHCO<sub>3</sub> added to maintain the pH and TA to initial levels, under different treatments in both batch and semi-continuous cultures

Batch culture

Days	AT-AC				AT-EC				ET-AC				ET-EC			
	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)
0	8.04	2256			7.63	2254			8.05	2243			7.65	2240		
2	8.23	2383	0.89	0.52	7.85	2406	0.97	0.23	8.15	2331	0.68	0.35	7.75	2333	0.68	0.2
4	8.37	2463	1.23	0.26	7.99	2466	1.29	0.56	8.21	2354	0.83	0.54	7.91	2402	1.06	0.53
6	8.43	2492	1.37	0.28	8.02	2491	1.37	0.28	8.29	2401	1.01	0.35	8.07	2493	1.45	0.28
8	8.69	2621	1.98	0.34	8.14	2548	1.65	0.36	8.34	2410	1.13	0.83	8.06	2478	1.42	0.57
10	8.73	2641	2.08	0.35	8.32	2649	2.08	0.06	8.28	2400	0.99	0.24	7.96	2429	1.19	0.55
12	8.79	2671	2.22	0.37	8.37	2664	2.19	0.36	8.26	2390	0.94	0.23	7.87	2394	0.98	0.23
14	8.83	2694	2.31	0.28	8.46	2706	2.41	0.47	8.25	2385	0.92	0.23	7.82	2362	0.86	0.45
16	8.81	2687	2.26	0.19	8.53	2733	2.57	0.69	8.25	2374	0.92	0.55	7.79	2352	0.79	0.3
18	8.77	2668	2.17	0.16	8.49	2733	2.48	0.1	8.23	2365	0.87	0.51	7.74	2330	0.67	0.2

Semi-continuous culture

Days	AT-AC				AT-EC				ET-AC				ET-EC			
	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)	pH (NBS)	TA ( $\mu\text{mol kg}^{-1}$ )	0.1N HCl (mL)	NaHCO <sub>3</sub> (mg)
0	8.03	2286			7.62	2268			8.06	2286			7.64	2255		
2	8.2	2411	0.85	0.28	7.79	2390	0.85	0.39	8.12	2356	0.59	0.28	7.72	2337	0.64	0.23
4	8.29	2448	1.06	0.53	7.88	2440	1.06	0.24	8.19	2390	0.76	0.33	7.87	2409	0.99	0.32
6	8.46	2545	1.46	0.19	7.97	2480	1.27	0.41	8.25	2414	0.89	0.49	7.92	2435	1.11	0.31
8	8.71	2659	2.05	0.55	8.06	2523	1.49	0.49	8.31	2447	1.04	0.41	8.04	2492	1.39	0.39
10	8.37	2499	1.25	0.21	8.01	2501	1.37	0.39	8.27	2435	0.94	0.17	7.95	2445	1.18	0.46
12	8.62	2620	1.84	0.38	8.12	2562	1.63	0.22	8.34	2469	1.11	0.22	8.07	2503	1.46	0.52
14	8.43	2525	1.39	0.34	7.99	2481	1.32	0.68	8.26	2427	0.92	0.26	7.99	2472	1.27	0.27
16	8.73	2673	2.1	0.44	8.15	2570	1.69	0.45	8.36	2474	1.16	0.37	8.08	2520	1.49	0.17
18	8.39	2504	1.3	0.39	8.02	2511	1.39	0.25	8.24	2415	0.87	0.31	7.9	2426	1.06	0.27