

Dark survival in biofilm-forming microalgae: potential for colonizing benthic ecosystems

Jagadish S. Patil* and Samantha D'souza

CSIR – National Institute of Oceanography, Dona Paula – 403 004, Goa, India.

Tel: 91(0)832-2450513; Fax: 91(0)832-2450615;

*Correspondence to patilj@nio.org

Abstract

The biofilm-forming microalgae are known to experience periods of continuous darkness (upto several days), in addition to the natural day-night cycle, especially in the intertidal sediment regions (when transported to deeper sediments) and the ships' ballast water tanks (during voyages). However, the information on community and physiological responses to different periods of darkness is limited. Here, the survival capability of biofilm-forming microalgae to varying periods of darkness (7-35 days) and the growth patterns upon resumption of normal 12h light:dark photocycle has been addressed through simulation experiments. Diatoms dominated the seawater biofilms, but the dark survival period varied and was species-specific. Of the 25 diatoms, only *Amphora*, potential toxin producer, followed by *Navicula* remained viable and photosynthetically healthy under darkness without undergoing asexual reproduction. Both diatoms are essential contributors to fouling and microphytobenthic community. Upon re-exposure of dark-adapted biofilms to 12h light:dark photocycle, the improvement in photosynthetic efficiency and resumption of growth via asexual-reproduction was observed. However, the lag-phase duration increased with a long dark history. Nevertheless, eurytolerant nature and high dark survival capability (with its quick response to light) of *Amphora* and *Navicula* indicated that they have the potential to colonize benthic-ecosystems thus impacting the benthic and fouling community.

Keywords: microalgae, biofilms, darkness, photosynthetic efficiency, growth, *Amphora*, *Navicula*

1. Introduction

In the marine environment, microalgal biofilms are ubiquitous in the intertidal regions. They are found at the surface interface (both artificial and natural) and water in an illuminated zone. However, the microalgal community in biofilms is distinctly different from those present in the water (Mitbavkar and Anil, Patil and Anil 2005, 2015). Therefore these biofilms are the subject of a large number of studies focusing on the regulations of the organic carbon content in shallow environments (Battin et al. 1999), forming a crucial link at the base of the trophic web (Steinman 1996), purifying water (Percival et al. 2000) or, conversely, causing a nuisance by biofouling (Gawne et al. 1998). Literature suggests that marine microalgal biofilms are dominated mainly by diatoms (Mitbavkar and Anil 2002, 2007, and 2008). To date, a considerable amount of information is available on the influence of environmental factors such as light, temperature, salinity, nutrients, etc., on the life cycle, physiology, and the behavior of the diatoms (Mcquoid and Hobson 1996). For example, nitrogen and phosphorus depletion lead to increased exopolysaccharide production (Staats et al. 2000) or may induce resting stage formation (Round 1971). Several biofilm-forming microalgae experience a period of dark stress when they get transported from the (i) surface to intertidal sediments through water movements (eg., waves, tides, and currents) and (ii) to distant places through ships ballast tanks (Drake et al., 2007). Further, within a ships ballast tank, microalgae can be found in contiguous compartments, that is, in water, sediment, and biofilm, and its discharges across their bioregions is widely considered as the most important vector for marine bioinvasion (Drake et al., 2007), which causes detrimental impacts to coastal communities and ecosystems (Ruiz et al., 1997, 2015; Gollasch et al., 2000, 2015; Carlton and Ruiz, 2003; Muirhead et al., 2014). Even the ships' biofouling seems to be a powerful vector for microalgae dispersal at a global scale due to the capacity of microalgae to attach to the biofouling film and cope by adaptation mechanisms with antifouling compounds (Costa et al. 2013). As a result, biofilm-forming microalgae also experience extended periods of darkness in ships' ballast water tanks during voyages. However, the periods of darkness ranged from hours to days (McMinn and Martin 2013) in the intertidal regions, and it depends on the ship's voyage period (days to months) from the ballast tank.

Available reports suggested that the organisms use different strategies (for example production of resting spores and cysts, obtaining nutrition from alternative sources, using stored energy reserves and reducing their metabolic rates, alteration in pigment concentrations) to survive long periods of darkness (see review paper McMinn and Martin 2013, Naik and Anil 2018). However, the duration of dark survival or tolerance varies and is species-specific (Peters 1996, Peters

and Thomas 1996). Diatoms are found to survive under dark stress conditions by various mechanisms such as carrying out dissimilatory nitrate reduction (Kamp et al. 2011, 2013) and entering into the resting phase to prevent cellular degradation and death (McMinn and Martin 2013 and references therein). Simulation experiments indicated that the phytoplankton maintains their photosynthetic efficiency under continuous darkness (to several days), but regain their growth after exposure for normal day-night photocycle (Patil and Anil 2018). Hence, it is hypothesized that the diatoms' photosynthetic activity can be regained only if the resurfacing from the sediments occurs before or up to the 'critical period' for survival under the dark. Short exposure of diatoms in the euphotic zones also influences their viability (Anil et al., 2007). Most of the findings have come from the planktonic (e.g., ocean mixing), benthic (e.g., burial), and polar (e.g., winter) systems (e.g., Carney et al. 2011, McMinn and Martin 2013 and references therein, Kvernvik et al. 2018). However, to our knowledge, there is not much information available on the response of diatom biofilms, i.e., growth, photosynthetic efficiency, and survival to prolong darkness despite knowing their (i) rich diversity, (ii) importance as major primary producer in both planktonic and benthic systems, (iii) as ecosystem engineers in intertidal sediment stabilization (de brouwer et al. 2005) (iv) mediators of subsequent macro fouling recruitment (eg. Wieczorek and Todd 1998, Qian et al. 2007) and (v) model organisms in biofouling and stress (including antifouling, pollution etc) studies (Patil and Jagadeesan 2011). The biofilm-forming microalgae, like planktonic forms, do experience periods of continuous darkness (up to several days), in addition to the natural day-night cycle, especially in the intertidal sediment regions (when transported to deeper sediments) and the ships' ballast water tanks (during voyages). Given this, here an attempt was made to evaluate the effect of length of darkness on the growth and photosynthetic response of microalgae within the biofilms using seawater biofilms and dominant fouling diatoms. In this study, the biofilms and fouling diatoms selected (i) contribute significantly to phytoplankton composition, biomass, and production to adjacent water column and benthic systems (eg. Mitbavkar and Anil 2002), (ii) might facilitate secondary invasion as they are known for their role as mediators in macrofouling (eg. Patil and Anil 2005), particularly barnacles (which is well-known ship fouling organisms and thus many species no doubt owe parts of their modern distribution to human-mediated translocations over the past several centuries; Carlton et al. 2011), and (iii) the fouling diatoms, *Amphora coffeaeformis* and *Navicula distans*, selected are generally use as the model organisms in the biofouling and stress (including antifouling, pollution, toxicity) related studies (eg. Patil and Jagadeesan 2011, Patil and Anil 2018). Therefore, the information obtained will have implications in the studies relevant to the ecology of micro-phytobenthos, ships ballast water management, and antifouling strategies.

2. Materials and Method

Effect of dark conditions on the development of seawater biofilms

All the experiments were conducted in the laboratory and the schematic with details of the experimental setup using seawater and fouling diatom cultures are presented in Fig.1. For carrying out experiments with marine biofilms, the surface seawater was collected from Dona Paula Bay, located on the west coast of India (15° 27' N, 73° 48' E) in a clean, 5L carboy. The collected seawater was transported immediately to the laboratory within an hour. The collected seawater (~1.5L) was added to two pre-cleaned and dried acrylic tanks, followed by enrichment with f/2 media nutrients (i.e macronutrients, trace metals and vitamins at a concentration similar to that use for preparing f/2 media; Gullard and Ryther, 1962) and mixing before immersion of glass slides. For the experiment, a total of 42 new, grease-free glass slides admeasuring (25.4mm x 76.2mm x 1.2mm) were labeled and immersed in each of these tanks. The slides were placed in the tanks with one side sticking to container walls using forceps so that only one surface was exposed to develop biofilms. Of the two tanks, one tank was tank set in 12h light: dark photocycle and total darkness, wherein former and latter are referred to as Tank L and Tank D (Fig. 1a). The illumination was provided using cool white fluorescent light at a light intensity of 70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. The tanks L and D was covered with cling film having holes for aeration i.e. assess to oxygen while the tank D was covered with a black cardboard box with a provision for air exchange. Both tanks were kept for incubation under at room temperature $25\pm 2^\circ\text{C}$.

The duration of the experiment was 35 days, and for the analysis, six slides (3 each for growth measurements and light: dark incubation experiment) were retrieved from both the tanks using forceps, at regular intervals, i.e., days 3, 7, 14, 21, 28, and 35. The slides retrieved on each occasion were dip-rinsed in a beaker containing autoclaved seawater to wash off loosely attached algae. Further to evaluate the growth of the seawater biofilms exposed to different periods of continuous darkness (days 3, 7, 14, 21, 28, and 35), three slides from Tank D were retrieved and placed in a Petri dish containing f/2 media. These slides were then incubated for ten days under normal 12h:12h light: dark photocycle similar to the control but in a Petri dish containing f/2 media in a temperature-controlled room to obtain differently aged biofilms. This was done to evaluate the effect of prolonging darkness on the growth of diatom biofilms. The exposure of dark-treated water samples to 10 days of natural light: dark cycle was referred to as dark to light treatment (dark-light). Considering the the heterogenous distribution of diatoms in biofilms the above experiment was performed on two occasions, i.e., from 11th November to 26th December 2013 & from 20th January to

6th March 2014, for repeatability. The recorded temperature (i.e. 25 and 27 °C) and salinity (33.8 and 33.5 PSU) of the seawater collected on both the occasions (i.e. 11th November 2013 and 20th January 2014) for the experiments were almost same. The growth of these microalgal biofilms was assessed based on the estimation of pigments (chlorophyll and phaeopigments), variable fluorescence, exopolysaccharides (EPS) production, and cell counts. In each experiment biofilms in triplicates were used for the measurements..

The chlorophyll (chl *a*) and phaeopigment concentration of the biofilms (mono-species and mixed community) in triplicates were estimated using 90% acetone. The biofilm from each glass slide was removed with a nylon brush into a sterile Petri dish with 50 ml of filtered seawater followed by filtration through GF/F Whatman filter paper (Patil and Anil 2005a, 2005b; Patil and Jagadeesan 2011). The filters were immediately subjected to acetone extraction overnight in the dark at 4°C. The acetone extracts were later used for fluorescence measurement (with and without acidification) for the estimation of chl *a* and phaeopigment (with acidification) concentrations using a Turner designs (Trilogy) fluorometer equipped with a dedicated chlorophyll *a* extracted – acidification module (ex ≤ 500 nm , em - ≥ 665 nm; P/N 7200-040). Chl *a* and phaeopigment are considered as an indicator for algal biomass and degradation.

The monitoring of biofilm development non-destructively was done using the FIRE technique (Patil and Jagadeesan 2011, Patil and Anil 2018). The FIRE fluorometer measures a suite of photosynthetic parameters such as the initial (F_0), maximum (F_m), and variable ($F_v = F_m - F_0$) components of photosystem II (PSII) fluorescence, quantum yield (F_v/F_m), and functional absorption cross-section (σ_{PSII}), which can be used to assess the physiological status of the photosynthesizing organisms such as diatoms. The fluorescence transients were measured using a FIRE fluorometer (Satlantic Inc.) equipped with a fiber-optic probe. Before measurements, the biofilms were adapted to darkness for 30 min. The gain was adjusted depending upon the chl *a* concentration of the sample taken for measurement. Curves were fitted using the FIREPro software.

The petri dish along with the biofilms was routinely observed microscopically (10x objective as this will provide larger field area compared with higher magnification) and photographed (10 fields of view based on the heterogeneous distribution in multispecies biofilms) for monitoring growth (cell counts) and community changes using an inverted microscope (Olympus IX2-ILL-100) equipped with a digital camera operated using the ImagePro software. The biofilm microalgae were identified initially (up to 40x magnification) and thereafter counted using identification keys.

To confirm EPS production in the biofilms developed under the light: dark cycle and continuous darkness, the biofilm slides were stained by flooding with 1mL of 0.02% aqueous Alcian blue. The slides were then incubated for 30 min and 1 hour. The excess stain was drained off, and the slide was dipped in seawater until the stain was washed off. The slides were then placed in Petri dishes with autoclaved filtered seawater and observed under an Olympus microscope at 10x magnification for stained EPS regions.

Responses of fouling diatoms grown under the different nutrient regimes to continuous darkness

In order to strengthen the findings from the above experiments the following experiments using cultures of dominant fouling diatoms were conducted. For the experiments, the monocultures of two pennate diatoms, *Amphora coffeaeformis*, and *Navicula transitans* var. *derasa* f. *delicatula*, were selected. The cultures are being maintained in the Biofouling and Bioinvasion Division, CSIR-National Institute of Oceanography, Goa, India (Patil and Anil 2005b). *A. coffeaeformis* and *N. transitans* are the dominant in fouling and in the microphytobenthic community in Dona Paula Bay, west coast of India). Both the diatoms were isolated from the intertidal sediments of Dona Paula Bay (15° 27' N, 73° 48' E) and the cultures of these diatoms were maintained in the exponential growth phase by periodic re-inoculation into the fresh culture medium, prepared in autoclaved seawater (~35 PSU) enriched with nutrients (f/2 media; Guillard & Ryther 1962). Cells were grown in Erlenmeyer flasks (100 ml) containing 50 ml of culture medium and were maintained at 25 ± 1°C under a similar light condition as stated above i.e. light photoperiod of 12:12 h light: dark at a light intensity of 70 μmol photons m⁻²s⁻¹.

The experiments were carried out using eight sets (6 and 2 sets for dark and light: dark treatments) of 6-well multiwells (Corning-25820; Fig. 1b). The multiwells containing f/2 media with and without adding micronutrients (Nitrate -NO₃; Phosphate – PO₄; Silicate – SiO₃) were inoculated aseptically under a laminar flow with exponentially growing *Amphora* and *Navicula* cultures at densities of 10⁴ cells ml⁻¹. After inoculation, the multiwells were kept overnight for the formation of films. This experiment was done to evaluate the interactive effect of prolonging darkness and micronutrients on the growth of diatom biofilms. All the treatments were conducted in triplicates, and the growth was monitored daily for 12 days. The nutrient concentration was determined at the start (day 0) and last day of (day 12) of the experiment using Skalar Autoanalyzer. Similar to previous experiments, the growth of *Amphora* and *Navicula*, exposed to different periods of continuous darkness (days 3, 5, and 7) upon re-exposed to normal light: dark conditions as that of control were also evaluated in triplicates. To achieve this, of the 6 sets (used for dark treatments), two sets were re-exposed to light: dark

treatments, and this was repeated for 3, 5, and 7 days of dark-adapted culture plates. The diatom growth was assessed through microscopic analysis (counts and morphology) and variable fluorescence measurements using FIRE fluorometer as detailed above. Additionally cell morphologies were measured microscopically using an ocular micrometer fitted to microscope. Further the all the data (such as chlorophyll, pheopigments, F_v , F_v/F_m and cell counts) were subjected to analysis of the variance (ANOVA) to assess the significance of the differences if any between treatments and days of the incubation.

Results

Effect of dark conditions on the development of seawater biofilms

Chlorophyll and phaeopigments

The results showed that the concentration of the chlorophyll a, an indicator of micro-algal biomass, in the biofilm showed an increasing trend with the incubation period under light conditions whereas, under dark conditions, biofilm chlorophyll remained constant irrespective of the incubation period (Fig.2). The above results are consistent in both the experiments (1 and 2) conducted on different occasions (Suppl. Fig. 1). The biofilm chlorophyll under light: dark conditions ranged between 0.04 to 2.5 $\mu\text{g cm}^{-2}$ in experiment 1 and 0.1 to 9.7 $\mu\text{g cm}^{-2}$ whereas, under the continuous dark condition, the ranges are 0.01 to 0.3 $\mu\text{g cm}^{-2}$ and 0.03 to 0.1 $\mu\text{g cm}^{-2}$ respectively in experiments 1 and 2. The phaeopigments also showed a similar trend to chlorophyll under light: dark and continuous dark conditions (Fig. 2) in both the experiments (1 and 2; Suppl. Fig. 2). The phaeopigments under light:dark conditions ranged between 0.03 to 1.4 $\mu\text{g cm}^{-2}$ in experiment 1 and 0.07 to 5.6 $\mu\text{g cm}^{-2}$ whereas under continuous dark condition the ranges are 0.01 to 0.2 $\mu\text{g cm}^{-2}$ and 0.01 to 0.08 $\mu\text{g cm}^{-2}$ respectively in experiments 1 and 2. The increase in biofilm chlorophyll under light: dark conditions are mainly due to the growth of the micro-algae, and the no increase in continuous dark indicated no growth of the micro-algae. However, the slides from continuous dark when exposed to normal light: dark conditions, a significant increase in the chlorophyll ($p < 0.05$), and phaeopigment ($p < 0.05$) were observed. The increase in chlorophyll and phaeopigment was observed only up to 2 to 3 weeks of dark incubation. Beyond the three weeks of dark incubation, the biofilm algae did not respond when re-exposed to light conditions.

Variable fluorescence (F_v) and photosynthetic efficiency(F_v/F_m)

Variable fluorescence, a proxy for live cells, also followed a similar pattern as chlorophyll, i.e., F_v values increased with the incubation period under the light: dark condition and no increase were

observed under continuous dark conditions (Fig. 3). F_v values ranged from 200 to 650 under light: dark conditions, whereas F_v values were around 200 under continuous dark conditions (Fig. 3). However, when the dark-adapted biofilms are exposed to normal light: dark conditions, a significant increase in F_v values (up to 400; $p < 0.05$) was observed similarly to chlorophyll, and this response was observed in both the experiments (Fig. 3; Suppl. Fig. 3).

In both the experiments, F_v/F_m was around 0.5, indicating that the biofilms used for the experiments were photosynthetically active. The biofilms which were exposed to light: dark conditions showed a slight increase in the F_v/F_m values with the incubation period, whereas the biofilms exposed to continuous dark conditions did not show any change in the F_v/F_m values irrespective of the incubation period (Fig. 3). This indicated that the micro-algae were photosynthetically active even though there was no growth under prolonged darkness. These results were consistent in both experiments (Suppl. Fig. 4). However, when the dark-adapted biofilms were exposed to normal light: dark conditions for ten days, an increase in F_v/F_m values were observed, and this response was observed in both the experiments (Fig. 3 and Suppl. Fig. 4). The increase in F_v/F_m values can be attributable to the growth of the micro-algae in biofilms.

Micro-algal abundance and composition

The cell abundance in seawater biofilms under light: dark conditions showed a steady state of growth with the incubation period (Fig.4). The cell abundance ranged between 10^4 - 10^6 cells/cm². In experiment 2, an increase in cell number was seen up to day 14. Thereafter, an overgrowth of filamentous algae dominated the biofilm, and the fouling diatoms could not be visible. Hence, the cell numbers are not recorded from day 21 onwards. In continuous dark conditions, the cell abundance remained constant for up to 1-2 weeks, and after that, the population decreased with a further increase in dark exposure. In experiment 2, no diatom cells were recorded after four and five weeks of exposure. The cell abundance was found to be less than 10^4 cells/cm² till the last day of the experiment. The dark-adapted biofilms, irrespective of the duration of dark adaptation, when exposed to light: dark conditions for ten days, a significant increase in the cell abundance ($p < 0.001$) was observed in both experiments (Fig.4).

Micro-algal composition revealed that the diatoms were the most dominant group concerning cell abundance and many species recorded during the experiment. Altogether a total of 25 species belong to centrics (6 species), and pennates (19 species) were recorded (Table 1a). Pennate diatoms dominated the micro-algal community concerning the number of species and cell abundance. It was also observed that the maximum and minimum numbers of species were recorded during the start

and end of the experiments, respectively. *Coscinodiscus*, *Amphora coffeaformis*, *Cylindrotheca closterium*, *Cymbella gasteroides*, *Navicula spp*, *Nitzschia spp*, *Pleurosigma*, and unidentified pennate diatom were found to be present in the biofilms up to day 35. In the dark-adapted biofilms, *Navicula* was the dominant species.

Under 12h light: dark conditions, the growth of *Amphora* increased with the increase in the length of the incubation, whereas the population of *Navicula* and other diatoms decreased with the increased incubation period (Fig. 4). Similar population growth and species exclusion pattern were evident when the dark-adapted biofilms were exposed to 12h light: dark cycle for ten days. However, such an increase in population growth and species exclusion was not observed under dark conditions (Fig. 4). Even though chlorophyll was detected in the dark-adapted biofilms diatoms were not detected. This is because the chlorophyll was analyzed from the total surface area of the glass slide, whereas the counts were made in fields under the microscope.

The response of dominant fouling diatoms to continuous darkness

The cultures were grown under different media, i.e., with and without nitrate, phosphate, and silicate in 12h light: dark cycle showed a characteristic growth curve up to day 12. In this experiment, nutrient variations did not play a major role in the growth pattern as the cell counts did not show a significant difference between different media due to elevated nutrient concentration in the seawater used for preparing the media. Even though there was no significant difference, the growth of *Amphora* and *Navicula* was relatively better in f/2 media than f/2 -P-Si, f/2 -N-Si, and seawater media (Figs. 5). Irrespective of the growth media, the cultures incubated in the continuous dark showed no increase in the abundance even up to seven days, indicating the arrest of the growth (Figs. 5). The dark-adapted cultures started to resume their growth, usually upon exposure to the 12h light: dark photocycle. However, the time required for the cultures to attain the stationary phase was longer because of the more extended lag phase.

The variable chlorophyll fluorescence also followed a similar trend to that of the cell counts in light and dark treatments. In 12h light: dark conditions F_v values increased logarithmically during the lag phase and fluctuated during the stationary phase. Compared to cell counts, variable fluorescence values showed clear variations among the different media used for the growth. Variable fluorescence values were high in f/2 media, followed by f/2-P-Si, f/2-N-Si, and seawater. Irrespective of the growth media the variable fluorescence values remained constant/ decreased when the cultures were incubated in the continuous dark due to the arrest of the growth (Figs. 6). The increase in

variable fluorescence values was observed when the dark-adapted cultures of *Amphora* and *Navicula* started to resume their growth normally upon exposure to the 12h light: dark cycle.

F_v/F_m values of both *Amphora* and *Navicula* were around 0.55, indicating that the cultures used for the experiments were photosynthetically active. The cultures incubated under the 12h light: dark cycle showed a slight increase in the F_v/F_m values with the incubation period, whereas the cultures exposed to continuous dark conditions did not show any change in the F_v/F_m values irrespective of the incubation period (Figs.7). This indicated that the micro-algae were photosynthetically active even though there was no growth under prolong darkness. These results were consistent with both cultures. However, when the dark-adapted cultures were exposed to normal 12h light: dark cycle, an increase in F_v/F_m values was observed, and this response was observed for both *Amphora* and *Navicula* (Figs. 7). The increase in F_v/F_m values can be attributable to the growth of the micro-algae.

Discussion

Biofilm forming microalgae are mostly dominated by diatoms (in particular, pennates), which are also known to contribute significantly to benthic microalgal biomass. Reports are indicating that microalgae can survive for an extended period of darkness (even exceeding 12 months, but the proportion of cells is low; Veuger and Oevelen 2011). Some studies suggested that the microalgae, known for forming biofilms, have developed mechanisms such as resting stage formation, dissimilarity use of nitrate, heterotrophy to survive extended periods of dark conditions (Anderson 1975, Veuger and Oevelen 2011, Cullen JJ. 1982, Kamp et al. 2011, Schaub et al. 2017). Nevertheless, to our knowledge, to date, the explicit information on the growth and photosynthetic response of biofilm-forming microalgae on continuous darkness and subsequent response upon re-exposure to normal light: dark photocycle is not available. Earlier findings from the studies conducted on water (phytoplankton) and sediments (benthic microalgae) suggested that the shrinkage of chloroplasts and a decrease in photosynthetic pigment content and efficiency of photosynthetic electron transport are the long-term response of the microalgae for continuous darkness (Veuger and Oevelen 2011, Kvernvik et al. 2018). Though there is some literature on the dark survival of benthic microalgae using sediment samples, a direct assessment of the growth and photosynthetic efficiency of biofilm-forming microalgae is warranted. Generally, in addition to biofilm-forming microalgae, sediment samples contain different stages of microalgae (both planktonic and benthic) and other organic matter (including fresh and degraded). As a result, viable, dormant, and non-viable cells will have dominant chlorophyll pigment, and quantifying the same will not provide a better physiological assessment. Therefore a direct study was undertaken using variable fluorescence fluorometry

(measured by single turnover protocol), which has been suggested as an important tool during the evaluation of ballast water, testing/assays (e.g., toxicity, water treatments, and antifouling), and ecological studies involving microalgae (Eg. Drake et al. 2015, First et al 2018, Patil and Anil 2018). This is because the interpretation of the variable fluorescence parameters provides direct insights into the biomass (F_0), viability (F_v), photosynthetic efficiency (F_v/F_m), and electron transport rate. For instance, F_v/F_m is known to range from 0.2 to 0.65, and low F_v/F_m indicates that a fraction of the PSII reaction center is functionally damaged/photoinhibited (Kraus and Weis 1991; Greene et al. 1992) or the presence of more dead/dying cells (Franklin et al. 2009; Perkins et al. 2010). Further, F_v/F_m is also an excellent index to follow photoacclimation during a transition from continuous dark to light-dark cycle (Katayam et al., 2015). Stress experiments confirmed that, typically in the live or healthy microalgae, F_0 is always less than F_m , and $F_v (=F_m - F_0)$ will always be positive. The magnitude is directly proportional to the density of the healthy biomass. On the other hand, in dead or non-viable chlorophyll F_0 and F_m are very close to each other, and F_v will be near zero or very small (Patil and Anil 2018). The subsequent discussion is based on the above criteria.

The experimental results revealed that the F_v (which is several times higher than F_0) and F_v/F_m (relatively higher) of the dark biofilms didn't change significantly with the incubation days suggesting that photosynthetic viability of the cell is maintained without growth (no increase in cell abundance/biomass) for up to 35 days. This is because F_v of the non-viable and viable cells will be negligible and higher than the F_0 , respectively (Patil and Anil 2018). On the contrary, in light: dark biofilms, a distinct increase in F_v values, chlorophyll, cell abundance, and F_v/F_m were observed with the incubation days suggesting enhanced growth with improvement in photosynthetic efficiency. However, the dark-adapted biofilms, when returned to the normal 2h light: dark regime was able to reproduce and photosynthesize quickly, indicating that they remained viable and photosynthetically active. Observation of pronounced chlorophyll induction curves in dark biofilms further suggests that the functionality of the photosystem is unaffected. Similar findings were also reported by Kvernvik et al. (2018) on microalgae experiencing polar night wherein pronounced chlorophyll induction curves were recorded and suggested that fully functional photosystem II and downstream electron acceptors seem to be present. Since photodamage is absent, maintenance of basic photosynthetic machinery may not impose a high metabolic cost on microalgae and become active immediately upon illumination and recover full photosynthetic activity within a short duration (Kvernvik et al. 2018). However, the present study revealed that the recovery of full photosynthetic activity varied (Figs. 3), and probably the length of the dark history and the taxonomic composition (including the stages) could be the reason. For instance, prolonged darkness exposure either triggers resting stage

formation (no variable fluorescence signals; Patil and Anil 2018) or develops alternative mechanisms to remain viable without undergoing resting stages. Upon reillumination, the photosynthetic activity is regained only after germination in the former (unpublished data) and quickly later, as seen during polar night (Kvernvik et al. 2018). The acclimation of dark-adapted diatoms to growth irradiance is associated with the enhancement of photosynthetic activity with changes in xanthophyll pigments to dark duration and silicate availability (Katayama et al. 2015). Here, the nutrient limitation is not an issue as the experiments with seawater biofilms were performed under high nutrient conditions, and dark treatments mediate the observed changes.

Here, the analysis of the community composition of the seawater biofilms shows that all fouling diatoms cannot survive under continuous darkness. As the length of the dark incubation increases, the weaker diatoms are wiped out, whereas the sturdy organisms survive and multiply. This study found that *Amphora* followed by *Navicula* dominated the biofilms (Table 1, Fig. 4). Their growth and photosynthetic parameters (variable fluorescence and photosynthetic activity) improved quickly upon incubation under 12h light: dark cycle (Fig. 3). This was evident in both the monoculture experiment as well in the experiments conducted with the seawater biofilms. The ability of the fouling microalgae, mainly *Amphora* and *Navicula*, to survive extended periods of darkness may be due to the conditions they experience in the natural environment. These diatoms are the dominant forms in both micro-phytobenthic communities and the seawater biofilms (developed over a range of substrates) of the southwest monsoon-influenced study region, i.e., Dona Paula Bay (Mitbavkar and Anil 2002, 2007, Patil and Anil 2005, 2015). These diatoms get transported from the surface to deeper sediments through tides, waves, or currents in the intertidal regions. It was found that these diatoms remained viable even up to a depth of 15 cm under dark conditions. The present study confirmed that *Amphora* and *Navicula* could survive for more than 35 days without undergoing any significant transformation in the morphology (except size) and photosynthetic activity (Table 1b; Figs. 7). These diatoms can able to survive for up to 5 years in the dark at 5⁰C (Anil et al. 2007) and also has alternative mechanisms (e.g., resting cell formation, nitrate reduction, use of stored energy) to remain viable under prolonged darkness (Anderson 1975, Kamp et al. 2011, McMinn and Martin 2013, Schaub et al. 2017). However, microscopic analysis revealed changes in the cell morphology (mainly the dimensions) and EPS production of *Amphora* and *Navicula* in the dark. For instance, (i) dark-adapted cells were relatively smaller in size than that 12h light: dark photocycle adapted cells (Table 1b), and (ii) the less EPS secretion in the dark than light: dark-adapted cells (Suppl. Fig. 5). Generally, diatom attachment to sediment grains or substratum is mediated through EPS secretion. The low EPS secretion indicates that the cells are loosely attached, which can be easily transported to

different places by physical forcing. Hence, it is presumed that (i) when the dark-adapted diatoms are brought back to the surface by physical forcing, they will quickly contribute to the micro-phytobenthic biomass, and primary production and (ii) quick response of dark-adapted diatoms to light suggests that the vegetative cells could proliferate at ports in the coastal zone on a global scale after injection into high light environments from the darkness of a ship's ballast water tank (Katayama et al. 2015). Such response is because the dark incubated biofilms possess micro-algal cells that can remain viable under prolonged darkness and resume growth immediately upon exposure to suitable light conditions. The time taken for the dark-adapted biofilms to grow is dependent on the dark history, i.e., the longer the dark adaptation, the time required for growth is relatively longer.

A study conducted in the Korean ports demonstrated that bloom-forming diatoms and pennate diatoms appear to be the species most likely to become successfully established in the studied regions (Kang et al., 2010). The biofilms of *Amphora* and *Navicula* are known to facilitate macrofouling development by offering positive cues for the settlement of the macrofouling organisms, e.g., barnacles, dominant fouling organisms (Patil and Anil 2005). Therefore, the survival potential of *Amphora* and *Navicula* observed in this study could be important in the functioning of the intertidal ecosystem as they contribute significantly to the biogeochemical process or can be a potential biofouling threat. Further, *Amphora coffeaeformis* is considered a potential producer of the neurotoxin domoic acid (Dhar et al. 2015), responsible for causing amnesic shellfish poisoning through human consumption of contaminated mussels (Shimizu et al. 1989). These recordings show that the species which survived in the experiments could significantly influence the ecosystem if successfully transported from one bioregion to another. The transport of ballast water in overseas traffic seems to play a key role in the unintentional movement of non-indigenous aquatic species across their geographic borderlines. Hence such diatoms can easily be transported along with the ships' ballast water and begin to colonize in the new areas.

This study concludes that not all fouling diatoms can survive under dark conditions. Of the 25 diatoms recorded, only *Amphora* followed by the *Navicula* community could remain viable and photosynthetically healthy under continuous dark conditions without undergoing asexual reproduction. Even the secretion of EPS (essential for cell attachment) was less in darkness. However, these diatoms further improved in their photosynthetic efficiency due to resumption of growth after re-exposure to normal light: dark photocycle but, the duration of the lag phase increased with a long dark history. Nevertheless, eurytolerant nature and high dark survival capability (with its

quick response to light) of *Amphora* and *Navicula* indicated that they have the potential to colonize benthic-ecosystems thus impacting the benthic and fouling community. Since *Amphora* and *Navicula* are dominant in the microphytobenthic and fouling communities, this investigation will have implications in the studies relevant to the ecology of microphytobenthos in intertidal regions; ships ballast water management and the development of antifouling strategies.

Acknowledgments

The authors are grateful to the Director, CSIR-National Institute of Oceanography (Goa, India), and Dr. AC Anil for their support and encouragement. We are also thankful to the two anonymous reviewers for their efforts. This paper is NIO contribution number

Compliance with ethical standards

Conflict of interest No conflicts of interest have been declared by the authors.

References

- Anderson OR (1975) Ultrastructure and cytochemistry of resting cell formation in *Amphora coffaeiformis* (Bacillariophyceae). *J Phycol*, 11: 272–281.
- Anderson OR (1976) Respiration and photosynthesis during resting cell formation in *Amphora coffaeiformis*. *Limnol Oceanogr*, 21: 453–456.
- Anil AC, Mitbavkar S, DeSilva MS, Hegde S, DeCosta PM, Meher SS, Banerjee D (2007) Effect of aging on the survival of benthic diatom propagules. *J Exp Mar Biol Ecol*, 343(1); 37-43.
- Bates SS (2000) Domoic acid-producing diatoms: Another genus added. *J Phycol*, 36: 978–983.
- Battin TJ, Buttirini A, Sabater F (1999) Immobilization and metabolism of dissolved organic carbon by natural sediment biofilms in a Mediterranean and temperate stream. *Aquat Microb Ecol*, 19:297–305
- Berges JA, Falkowski PG (1998) Physiological stress and cell death in marine phytoplankton: Induction of proteases in response to nitrogen or light limitation. *Limnol Oceanogr*, 129-135
- Breznak JA, Cooksey KE, Eckardt FEW, Filipz, Fletcher M, Gibbons RJ, Gude H, Hamilton WA, Hattori T, Hoppe HG, Matthyse AG, Savage DC, Shilo M (1985) Activity on Surfaces, Microbial adhesion and aggregation. Berlin: Springer, 203–221.
- Carlton, J.T., Ruiz, G.M. (Eds.) (2003) *Invasive Species: Vectors and Management Strategies*. Island Press, Washington, USA.
- Carlton JT, Newman WA, Pitombo FB (2011) Barnacle Invasions: Introduced, Cryptogenic, and Range Expanding Cirripedia of North and South America. In B.S. Galil et al. (eds.), *In the Wrong Place - Alien Marine Crustaceans: Distribution, Biology and Impacts, Invading Nature - Springer Series in Invasion Ecology* 6, DOI 10.1007/978-94-007-0591-3_5,

- Carney KJ, Delany JE, Sawant SS, Mesbahi E (2011) The effects of prolonged darkness on temperate and tropical marine phytoplankton, and their implications for ballast water risk management. *Mar Poll Bull*, 62: 1233-1244
- Costas E, Gonzalez R, Lo'pez-Rodas V, Huertas IE (2013) Mutation of microalgae from antifouling sensitivity to antifouling resistance allows phytoplankton dispersal through ships' biofouling. *Biol Invasions* 15:1739–1750
- de Brouwer, J., Wolfstein, K., Ruddy, G. *et al.* (2005) Biogenic Stabilization of Intertidal Sediments: The Importance of Extracellular Polymeric Substances Produced by Benthic Diatoms. *Microb Ecol*, 49: 501–512. <https://doi.org/10.1007/s00248-004-0020-z>
- Dhar BC, Cimarelli L, Singh KS, Brandi L, Brandi A, Puccinelli C, Marcheggiani S, Spurio R (2015) Molecular Detection of a Potentially Toxic Diatom Species. *Int J Environ Res Public Health*, 12, 4921-4941; doi:10.3390/ijerph120504921
- Dehning I, Tizler M (1989) Survival of *Scenedesmus acuminatus* (Chlorophyceae) in darkness. *J Phycol*, 25: 509-515.
- Drake LA, Doblin MA, Dobbs FC (2007) Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm. *Mar Poll Bull*, 55: 333–341.
- Drake LA, Tamburri MN, First MR, Smith G, Johengen TH (2014) How many organisms are in ballast water discharge? A framework for validating and selecting compliance monitoring tools. *Mar Poll Bull*, 86: 122–128.
- First MR, Robbins-Wamsley SH, Riley SC, Drake LA (2018) Assessment of variable fluorescence fluorometry as an approach for rapidly detecting living photoautotrophs in ballast water. *J Sea Res*, 133: 53-59.
- Franklin D, Choi C, Hughes C, Malin G, Berges J (2009) Effect of dead phytoplankton cells on the apparent efficiency of photosystem II. *Mar Ecol Prog Ser*, 382: 35-40
- Gawne B, Wang Y, Hoagland KD, Gretz MR (1998) Role of bacteria and bacterial exopolymer in the attachment of *Achnanthes longipes* (Bacillariophyceae). *Biofouling* 13: 137–156
- Gollasch S, Lenz J, Dammer M, Andres HG (2000) Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. *J Plankt Res*, 22: 923–937.
- Gollasch S, Minchin D, David M (2015) The transfer of harmful aquatic organisms and pathogens with ballast water and their impacts. *Global maritime transport and ballast water management*. Springer, Netherlands, pp. 35–58.
- Gorbunov MY, Kolber ZS, Lesser MP, Falkowski PG (2001) Photo-synthesis and photoprotection in symbiotic corals. *Limnol Oceanogr*, 46(1): 75–85.
- Gorbunov MY, Falkowski PG (2004) Fluorescence Induction and Relaxation (FIRE) Technique and Instrumentation for Monitoring Photosynthetic Processes and Primary Production in Aquatic Ecosystems.

- Greene RM, Geider RJ, Falkowskii PG (1992) Effect of iron limitation on photosynthesis on a marine diatom. *Limnol Oceanogr* 36: 1772-1782
- Gullard R, Ryther J (1963) Studies on marine planktonic diatoms. I. *Cyclotella* and *Husted* and *Detonula confervacea* Cleve. *Can J Microbiol*, 8: 229-239
- Jochem F J (1999), Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. *Mar Biol*, 135: 721-728.
- Kamp A, de Beer D, Nitsch JL, Lavik G, Stief P (2011) Diatoms respire nitrate to survive dark and anoxic conditions. *PNAS*, 108: 5649–5654. doi:10.1073/pnas.1015744108.
- Kamp A, Stief P, Knappe J, de Beer D (2013) Response of the Ubiquitous Pelagic Diatom *Thalassiosira weissflogii* to Darkness and Anoxia. *PLoS ONE* 8(12): e82605. doi:10.1371/journal.pone.0082605
- Kana TM, Giedler RJ, Critchley C (1997) Regulation of photosynthetic pigments in micro-algae by multiple environmental factors: a dynamic balance hypothesis. *New Phytol*, 137: 629–638.
- Kang J, Hyun B, Shin K (2010) Phytoplankton viability in ballast water from international commercial ships berthed at ports in Korea. *Mar Poll Bull*, 60: 230-237.
- Katayama T, Murata A, Taguchi S (2015) Responses of pigment composition of the marine diatom *Thalassiosira weissflogii* to silicate availability during dark survival and recovery. *Plankton Benthos Res*, 6(1): 1–11.
- Kraus GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Ann Rev Plant Physiol and Plant Mol Biol* 42: 13-349
- Kvernvik AC, Hoppe CJ M, Lawrenz E, Prasil O, Greenacre M, Wiktor JM, Leu E (2018) Fast reactivation of photosynthesis in arctic phytoplankton during the polar night. *J Phycol*, 54: 461–470
- Maranda L, Wang R, Masuda K, Shimizu Y (1990) Investigation of the source of domoic acid in mussels. In *Toxic Marine Phytoplankton*, Granéli E, Sundström B, Edler L, Anderson DM, Eds.; Elsevier Science Publishing Co.: New York, NY, USA, pp. 300–304.
- McMinn A, Martin A (2013) Dark survival in a warming world. *Proc R Soc B*, 280: 20122909.
- McQuoid MR, Hobson LA (1996) Diatom Resting Stages. *J Phycol*, 889-902.
- McQuoid MR, Godhe A, Nordberg K (2002) Viability of phytoplankton resting stages in the sediments of a coastal Swedish Fjord. *Eur J Phycol*, 37:191-201.
- Mitbavkar S, Anil AC (2002) Diatoms of the microphytobenthic community: population structure in a tropical intertidal sand flat. *Mar Biol* 41–57.
- Mitbavkar S, Anil AC (2007) Species interactions within a fouling diatom community: roles of nutrients, initial inoculum and competitive strategies. *Biofouling*, 23(2): 99–112.

- Mitbavkar S, Anil AC (2008) Seasonal variations in the fouling diatom community structure from a monsoon-influenced tropical estuary. *Biofouling*, 415-426.
- Muirhead JR, Minton MS, Miller WA, Ruiz GM (2014) Projected effects of the Panama Canal expansion on shipping traffic and biological invasions. *Divers Distrib*, 21: 75–87.
- Nymark M, Valle KC., Hancke K, Winge P, Andresen K, Johnsen G, Bones AM, Brembu T (2013) Molecular and Photosynthetic Responses to Prolonged Darkness and Subsequent Acclimation to Re-Illumination in the Diatom *Phaeodactylum tricorutum*. *PLoS ONE*, 8:1-19.
- Naik SM, Anil AC (2018) Influence of darkness on pigments of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta) *J Photochem Photobiol B: Biology* 186:17-22
- Palmisano AC, Sullivan CW (1982) Physiology of sea ice diatoms. II. Dark survival of three polar diatoms. *Can J Microbiol*, 29: 157–160.
- Patil, J.S., Anil, A.C., 2005a. Influence of diatom exopolymers and biofilms on metamorphosis in the barnacle *Balanus amphitrite*. *Mar. Ecol. Prog. Ser.* 301, 231-245.
- Patil JS, Anil AC (2005) Biofilm diatom community structure: Influence of temporal and substratum variability, *Biofouling*, 189–206.
- Patil JS, Jagadeesan V (2011) Effect of chlorination on the development of marine biofilms dominated by diatoms, *Biofouling*, 17: 241–254.
- Patil JS, Anil AC (2015) Efficiency of copper and cupronickel substratum to resist development of diatom biofilms. *Int Biodeter Biodegrad* 105: 203-214
- Patil JS, Anil AC (2018) Simulation experiments to elucidate variable fluorescence as a potential proxy for bulk microalgal viability from natural water, sediments and biofilms: Implication in ships ballast water management. *J Env Manag* 222: 242–249.
- Percival SL, Walker JT, Hunter PR (2000) *Microbiological aspects of biofilms and drinking water*. CRC Press, Boca Raton, FL
- Perkins KJ, Andrewartha JM, McMinn A, Cook SS, Hallegraeff GM (2010) Succession and physiological health of freshwater microalgal fouling in a Tasmanian hydropower canal. *Biofouling* 26 (6): 637 — 644
- Peters E (1996) Prolonged darkness and diatom mortality. II. Marine temperate Species. *J Exp Mar Biol Ecol*, 207: 43-58.
- Peters E, Thomas, DN (1996) Prolonged darkness and diatom mortality. I. Marine Antarctic Species, *J Exp Mar Biol Ecol*, 207: 25-41.
- Pippo F Di, Bohn A, Congestri R, De Philippis R, Albertano P (2009) Capsular polysaccharides of cultured phototrophic biofilms. *Biofouling*, 20: 495-504.
- Qian PY, Lau SC, Dahms HU, Dobretsov S, Harder T (2007). Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. *Mar Biotechnol*, 9:399-410

- Round FE (1971) Benthic marine diatoms. *Oceanogr Mar Biol Ann Rev.* 83- 139.
- Ruiz GM, Carlton JT, Grosholz ED, Hines AH (1997) Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *Am Zool* 37: 621–632.
- Ruiz, G.M., Fofonoff, P.W., Steves, B.P., Carlton, J.T., 2015. Invasion history and vector dynamics in coastal marine ecosystems: a North American perspective. *Aquat. Ecosyst. Health Manag.* <http://dx.doi.org/10.1080/14634988.2015.1027534>.
- Schaub I, Wagner H, Graeve M, Karsten U (2017) Effects of prolonged darkness and temperature on the lipid metabolism in the benthic diatom *Navicula perminuta* from the Arctic Adventfjorden, Svalbard. *Polar Biol*, 40:1425–39.
- Smayda TJ, B. Mitchellnes (1974) Dark survival of autotrophic planktonic marine diatoms. *Mar Biol*, 25: 195-202.
- Shimizu Y., Gupta S., Masuda K., Maranda, L., Walker, C.K., Wang, R. (1989). Dinoflagellate and other microalgal toxins: chemistry and biochemistry, *Pure and Applied Chemistry* 61: 513-516.
- Staats N, Stal LJ, Mur L.R. (2000) Exopolysaccharide production by the epipelagic diatom *Cylindrotheca closterium*: effects of nutrient conditions. *J Exp Mar Biol Ecol*, 13–27.
- Steinman AD (1996) Effects of grazers on freshwater benthic algae. In: Stevenson RJ, Bothwell ML, Lowe RL (eds) *Algal ecology*. Academic Press, San Diego, p 341–373
- Tomas CR (1997) *Identifying marine phytoplankton*, Academic Press. pp 35-77.
- Wieczorek SK, Todd CD (1998). Inhibition and facilitation of settlement of epifaunal marine invertebrate larvae by microbial biofilm cues. *Biofouling*. 12:81-118.

Figure legends

Fig.1: Schematic presentation of the experimental design to elucidate the effect of varying period of darkness on the development of microalgal biofilms using (a) seawater and (b) fouling diatom cultures (*Amphora coffeaeformis* and *Navicula distans*) followed by their growth responses after several days (a. 10 days for seawater and b. 5 to 9 days for cultures) of incubation under 12 h light: dark conditions. The experiments with seawater and cultures were performed in acrylic tanks and multiwells respectively. The seawater microalgal biofilms on glass slides (a) and fouling diatom cultures with f/2 nutrient enrichment in multiwells (b) developed under 12h light: dark conditions was considered as the experimental control. Additionally, diatom cultures were incubated with (filtered sea water) and without f/2 nutrient enrichment i.e. f/2, f/2 -N-Si, f/2-P-Si in multiwells to elucidate the effect of micronutrient on growth and biofilm formation. The experiment with seawater was repeated twice and in each experiment (both a and b setup) measurements were done from triplicate setup. Please see method section for details.

Fig.2: (a and c) Influence of 12h light:dark cycle and continuous dark on the accumulation of chlorophyll *a* and pheopigments in the biofilms and (b and d) chlorophyll and pheopigment accumulation in dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle.

Fig.3:(a and c) Influence of 12h light:dark cycle and continuous dark on the variable chlorophyll fluorescence (F_v) and photosynthetic efficiency (F_v/F_m) of micro-algae in the biofilms and (b and d) variable chlorophyll fluorescence (F_v) and B'photosynthetic efficiency (F_v/F_m) of micro-algae in dark adapted biofilms after 10 days of incubation under 12h light:dark cycle.

Fig.4: Bubble plot showing the (a & a') Influence of 12h light:dark cycle on the growth of different micro-alga species in the biofilms, (b & b') influence of continuous dark on the growth of different micro-alga species in the biofilms and (c & c') abundance of different micro-algal species in dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle. *: no data due to dense cover by algal filament and therefore the data for experiment 1 and 2 are presented separately. All the data presented are $\log(X+1)$ transformed and the maximum size of the bubble corresponds to data value of 6.

Fig.5: Effect of different nutrient sources on the growth of *Amphora coffeaeformis* and *Navicula distans* grown under (a) 12h light:dark cycle (b) three days continuous dark followed by incubation under 12h light:dark cycle, (c) five days continuous dark followed by incubation under 12h light:dark cycle and (c) (b) seven days continuous dark followed by incubation under 12h light:dark cycle. f/2 -P-Si : Media without phosphate and silicate, f/2 -N-Si : Media without nitrate and silicate, f/2 : media containing nitrate, phosphate and silicate and SW: media without nutrient enrichment.

Fig.6: Effect of different nutrient sources on the variable chlorophyll fluorescence of *Amphora coffeaeformis* and *Navicula distans* grown under (a) 12h light:dark cycle (b) three days continuous dark followed by incubation under 12h light:dark cycle, (c) five days continuous dark followed by incubation under 12h light:dark cycle and (c) (b) seven days continuous dark followed by incubation under 12h light:dark cycle. f/2 -P-Si : Media without phosphate and silicate, f/2 -N-Si : Media without nitrate and silicate, f/2 : media containing nitrate, phosphate and silicate and SW: media without nutrient enrichment.

Fig.7: Effect of different nutrient sources on the photosynthetic efficiency of *Amphora coffeaeformis* and *Navicula distans* grown under (a) 12h light:dark cycle (b) three days continuous dark followed by incubation under 12h light:dark cycle, (c) five days continuous dark followed by incubation under 12h light:dark cycle and (c) (b) seven days continuous dark followed by incubation under 12h light:dark cycle. f/2 -P-Si : Media without phosphate and silicate, f/2 -N-Si : Media without nitrate and silicate, f/2 : media containing nitrate, phosphate and silicate and SW: media without nutrient enrichment.

Table 1. (a.) List of micro-algal species recorded under different conditions during the experiments. * - species recorded under the respective conditions and # indicates that the species present till the last day of the experiments under the respective condition. DTL –dark to light condition.

Sno	Species	Expt 1			Expt 2		
		Light	Dark	DTL	Light [§]	Dark	DTL
	Centrics						
1	<i>Coscinodiscus radiatus</i>	*#	*		*	*	*
2	<i>Skeletonema costatum</i>		*	*	*		*
3	<i>Gunardia</i>		*	*	*		
4	<i>Chaetoceros</i>	*	*	*			
5	<i>Ditylum brightwelli</i>		*	*	*		
6	<i>Melosira nummuloides</i>						
	Pennates						
7	<i>Amphora coffaeiformis</i>	*#	*	*#	*		*
8	<i>Amphora costata</i>	*	*				*
9	<i>Cylindrotheca closterium</i>	*#		*	*		*
10	<i>Cymbella gastroides</i>	*#	*				
11	<i>Diploneis</i>			*			
12	<i>Navicula distans</i>	*#	*	*#	*		*
13	<i>Navicula sp.2</i>	*#	*	*	*	*	*
14	<i>Navicula sp.3</i>	*	*	*	*		*
15	<i>Navicula sp.4</i>	*#	*	*	*		*
16	<i>Navicula sp.5</i>	*	*#	*	*		
17	<i>Navicula sp.6</i>	*	*	*	*		
18	<i>Nitzschia longissima</i>	*		*			
19	<i>Nitzschia panduriformis</i>	*		*	*		*
20	<i>Nitzschia sp.3</i>	*#		*	*		
21	<i>Nitzschia sp.4</i>	*	*		*		
22	<i>Pleurosigma angulatum</i>	*#	*	*	*		
23	<i>Thalassionema nitzschoides</i>	*	*	*	*		*
24	<i>Thalassiothrix sp.</i>			*			
25	Unidentified pennate diatom	*#					*

(b) Table showing the morphology dimensions of *Amphora* and *Navicula* grown under 12 h light: dark photocycle and prolong darkness.

Species	Media	Light		3 days Dark		5 days Dark		7 days Dark	
		cell size (µm)		cell size (µm)		cell size (µm)		cell size (µm)	
		length	breadth	length	breadth	length	breadth	length	breadth
Amphora	f/2-P-Si	18±4	4±1	15±2	4±1	15±3	4±1	16±3	4±1
	f/2-N-Si	17±3	4±1	16±3	4±1	15±3	4±1	14±5	4±1
	f/2	19±4	3±1	17±2	4±1	16±3	4±1	15±5	4±1
	sw control	19±4	3±1	17±2	3±1	14±4	3±1	15±3	4±1
Navicula	f/2-P-Si	18±2	5±2	18±2	4±2	17±3	4±2	17±2	8±2
	f/2-N-Si	19±3	5±2	18±2	4±1	18±2	4±1	16±2	9±2
	f/2	18±2	4±2	16±2	4±2	18±1	4±2	16±2	8±2
	sw control	18±2	3±1	16±2	4±1	18±1	5±2	17±2	8±2

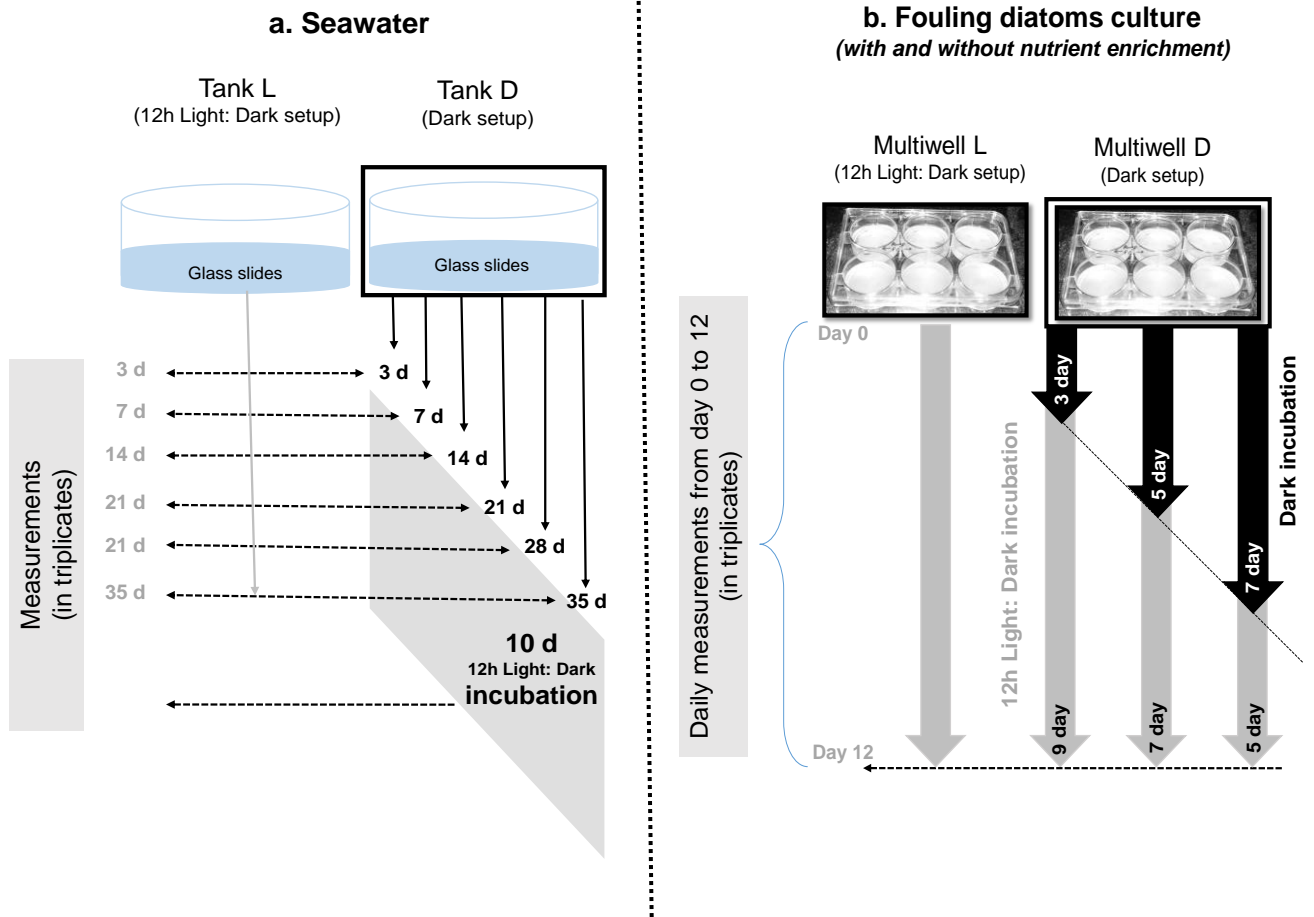


Fig.1

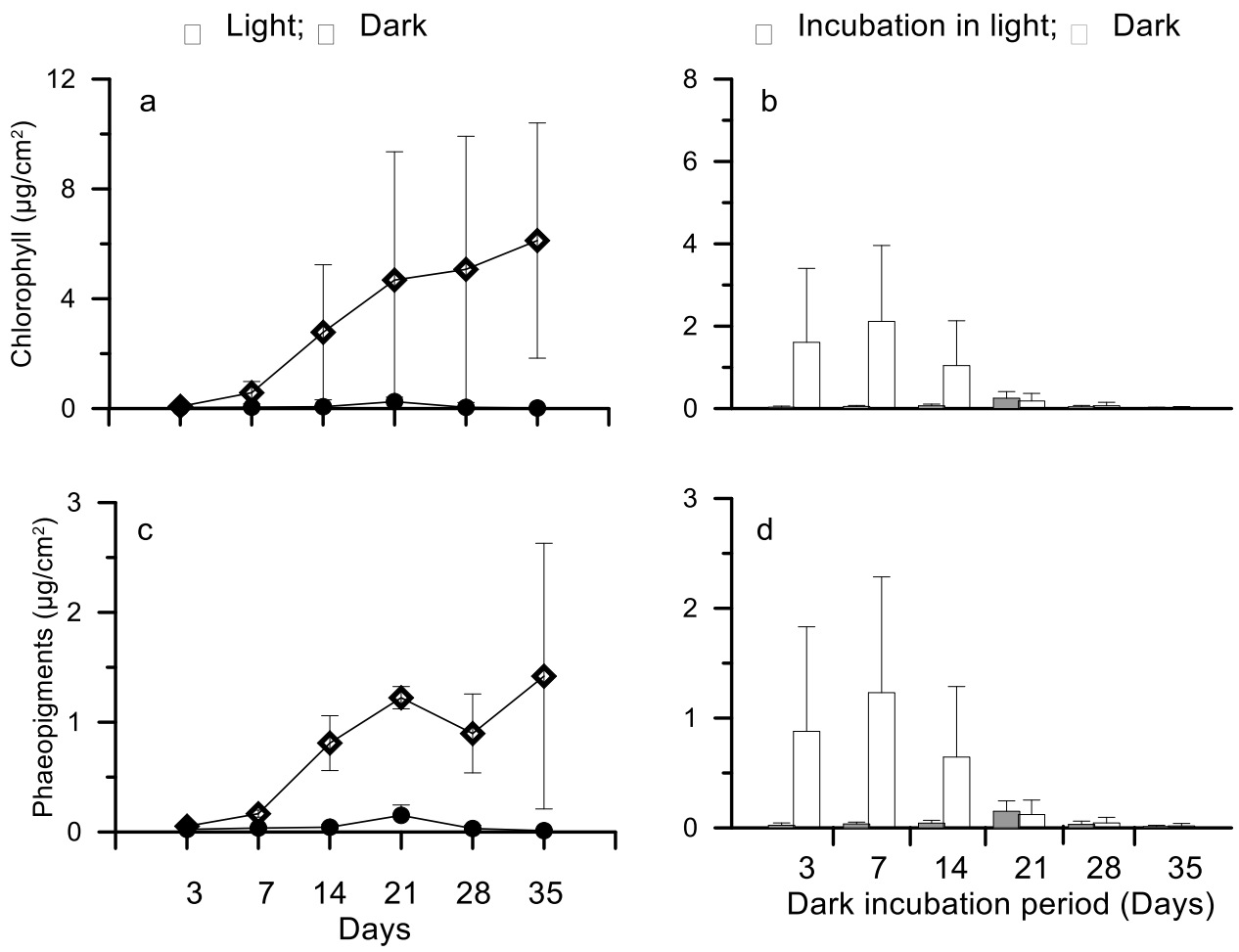


Fig.2:

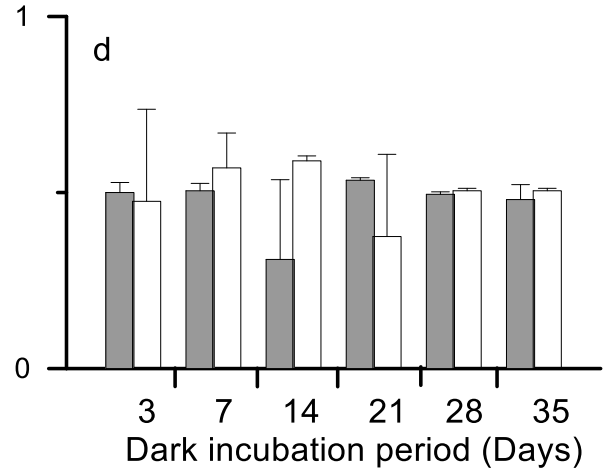
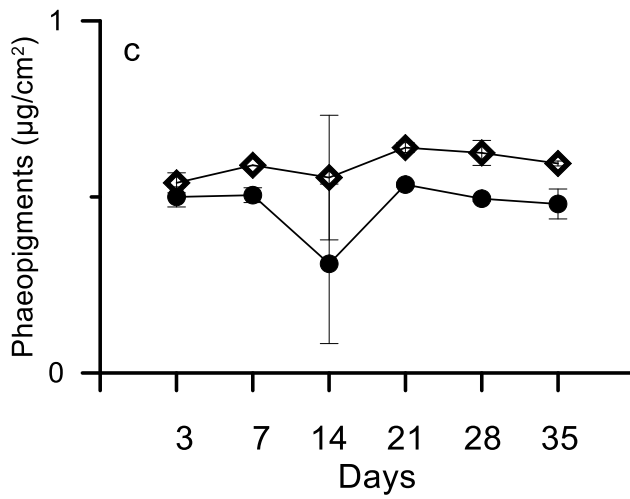
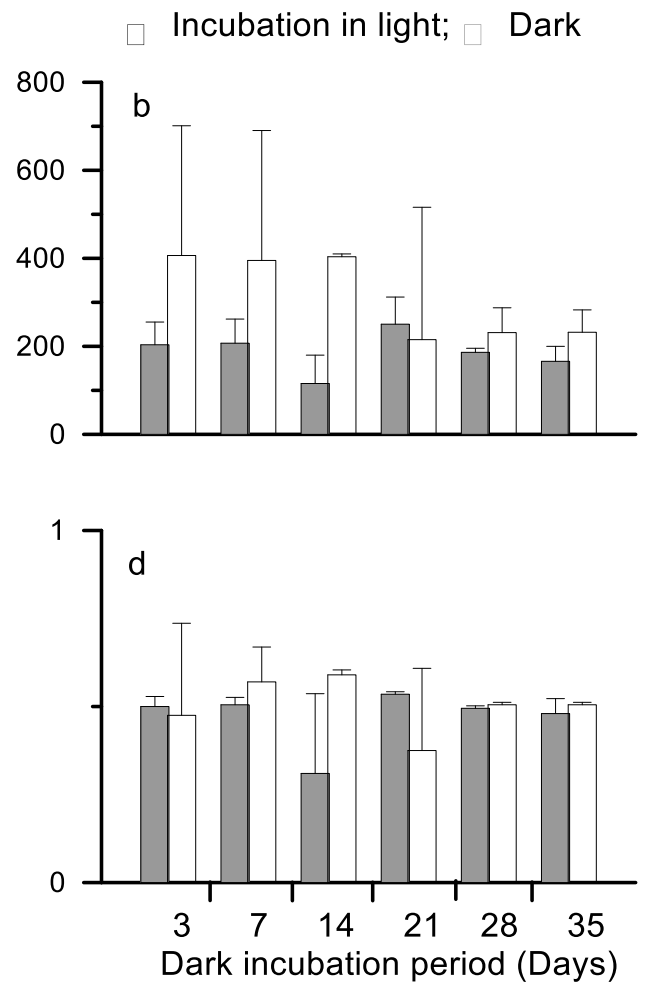
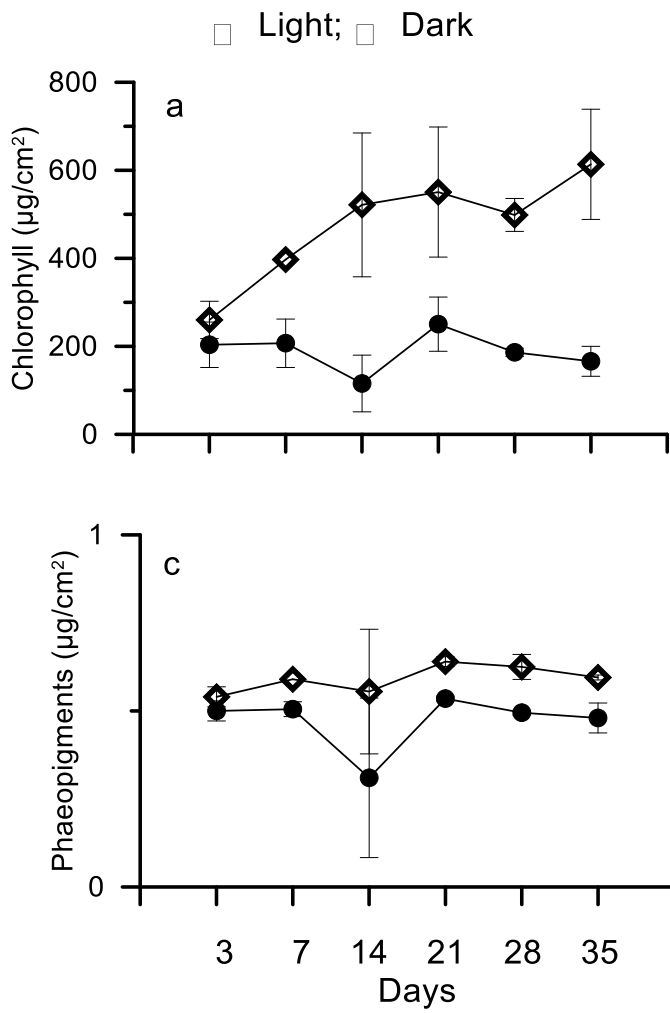


Fig.3

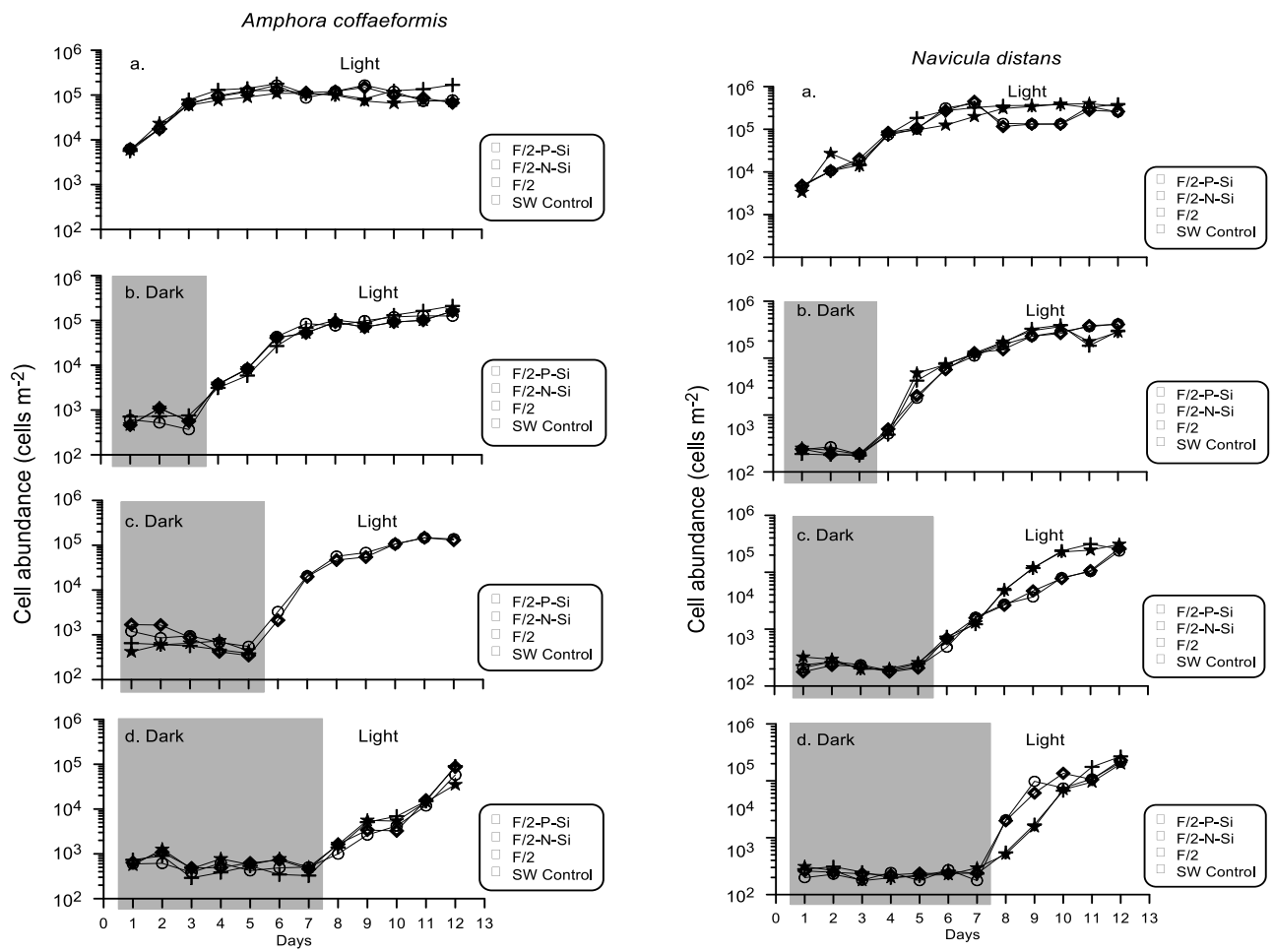


Fig.5

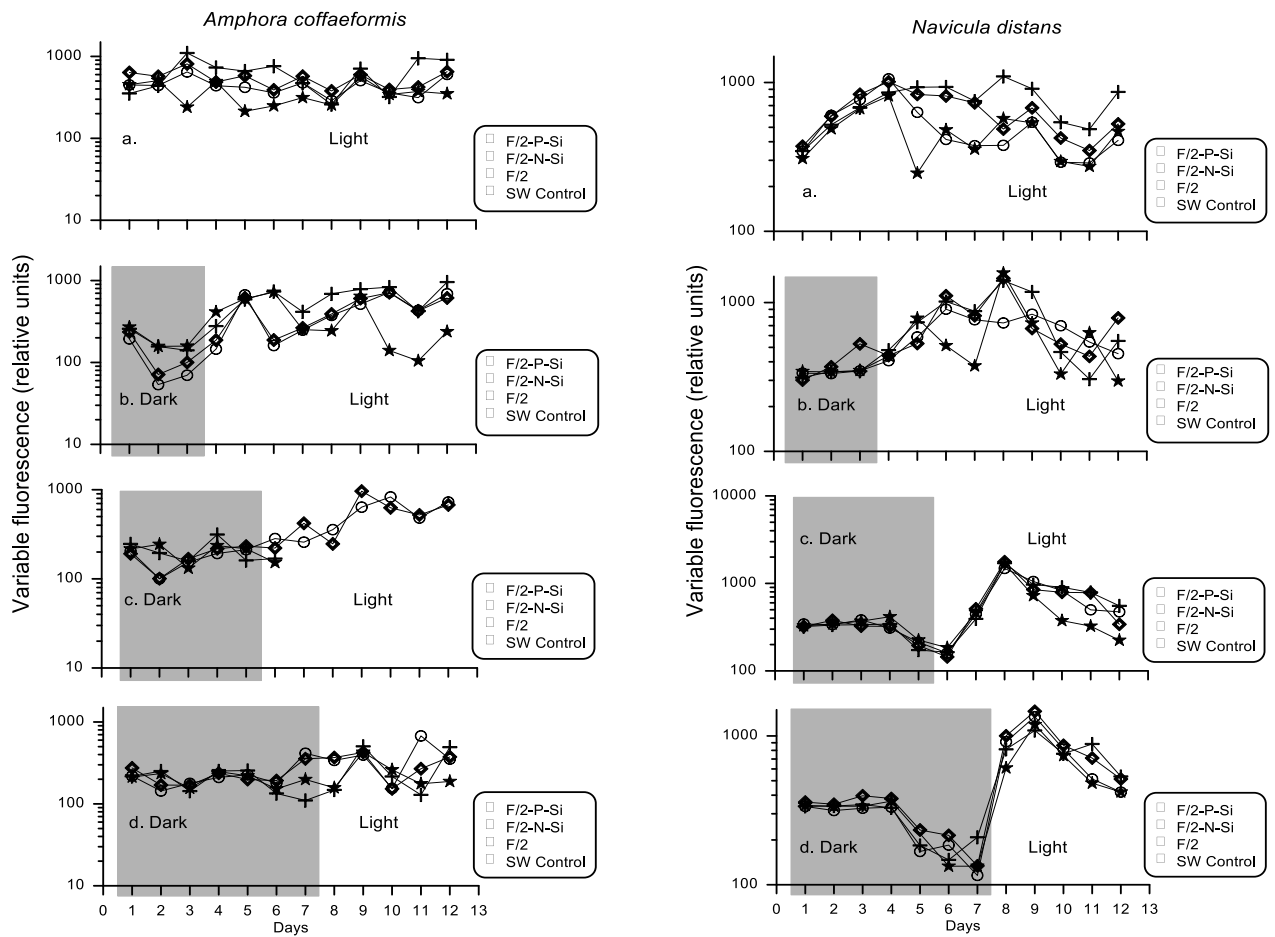


Fig.6

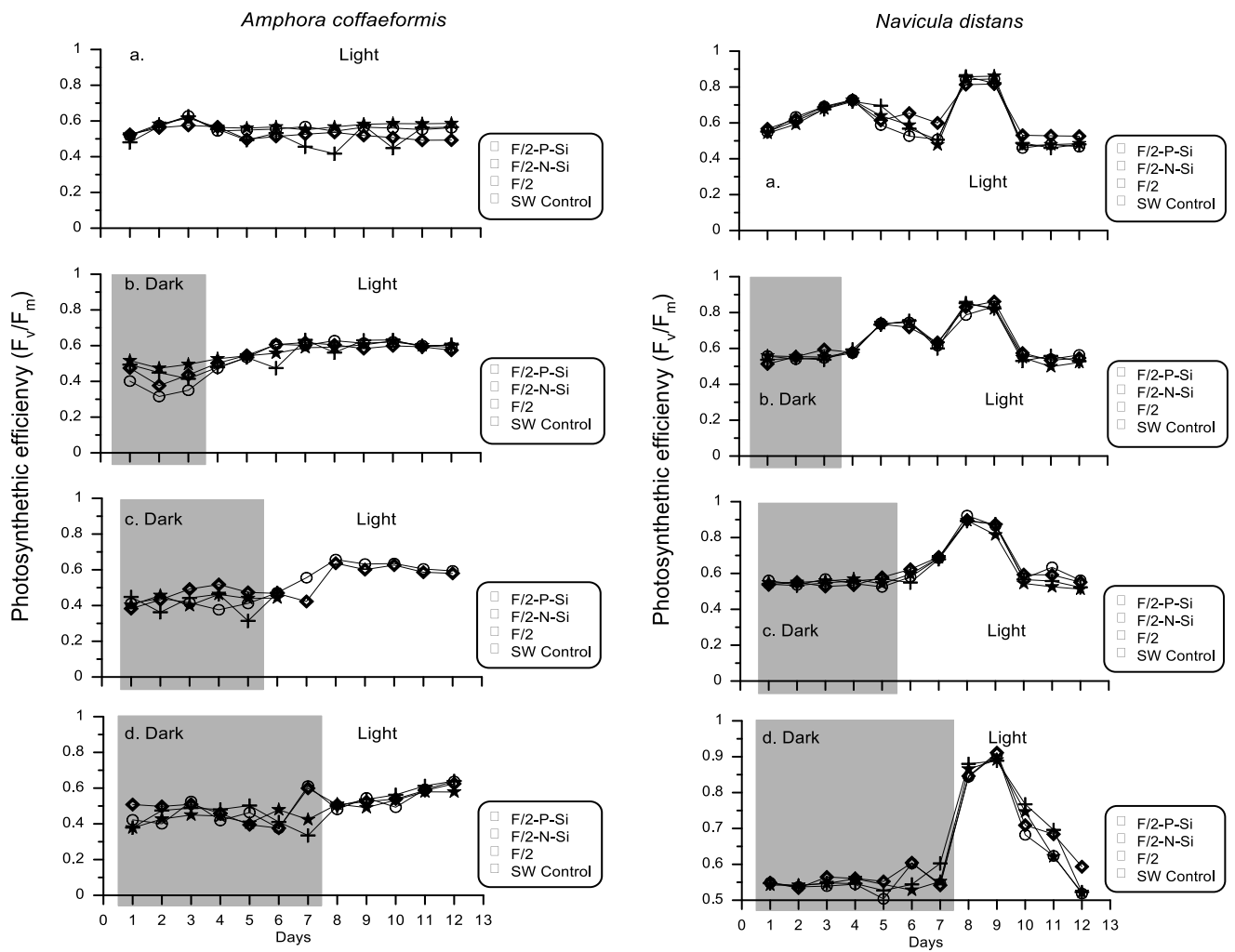
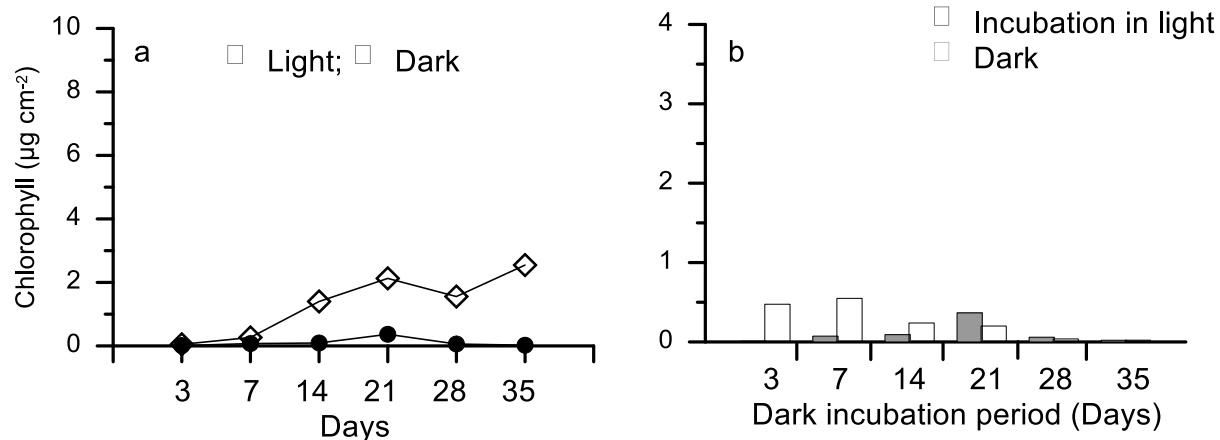
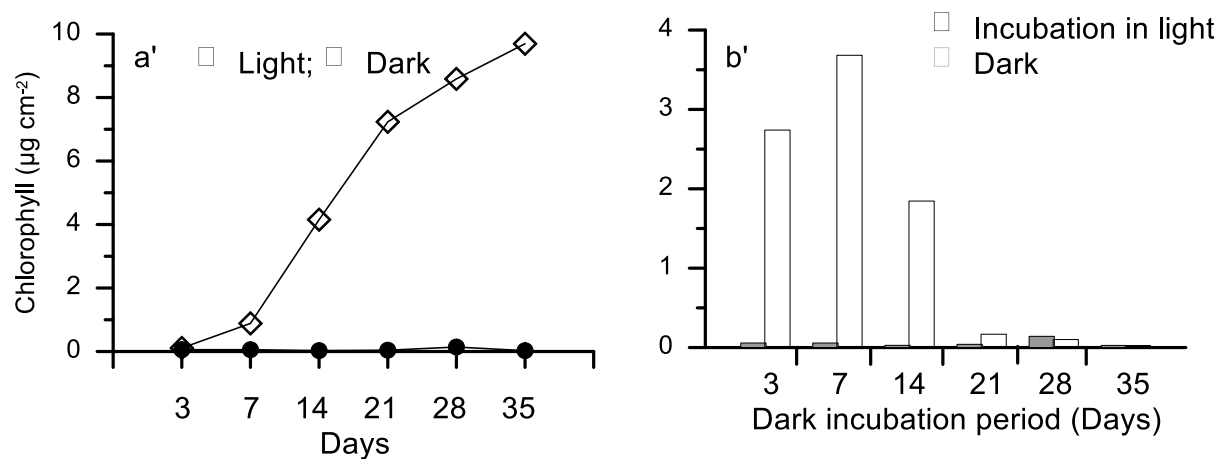


Fig.7

Experiment 1

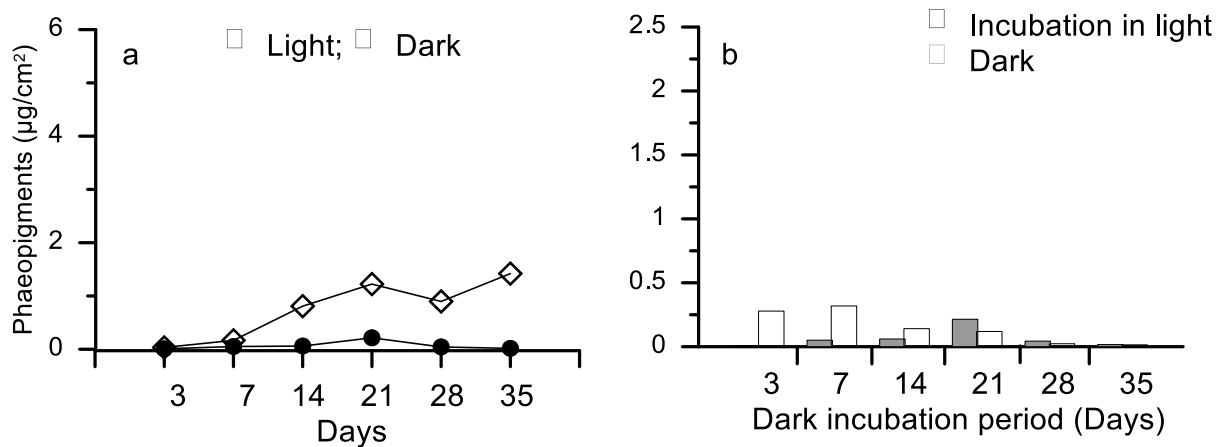


Experiment 2

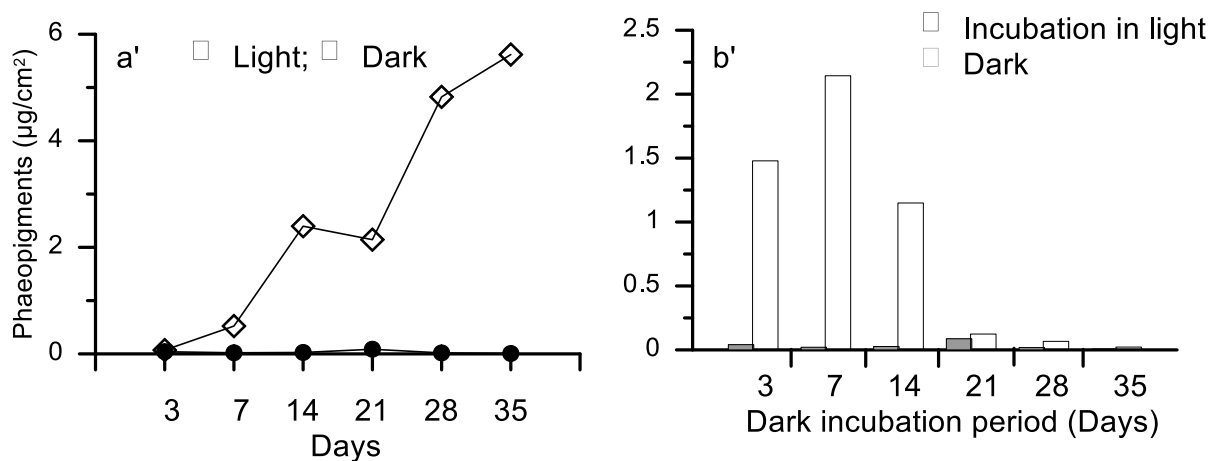


Supplementary Fig.1: (a & a') Influence of 12h light:dark cycle and continuous dark on the accumulation of chlorophyll *a* in the biofilms and (b & b') chlorophyll accumulation in dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle.

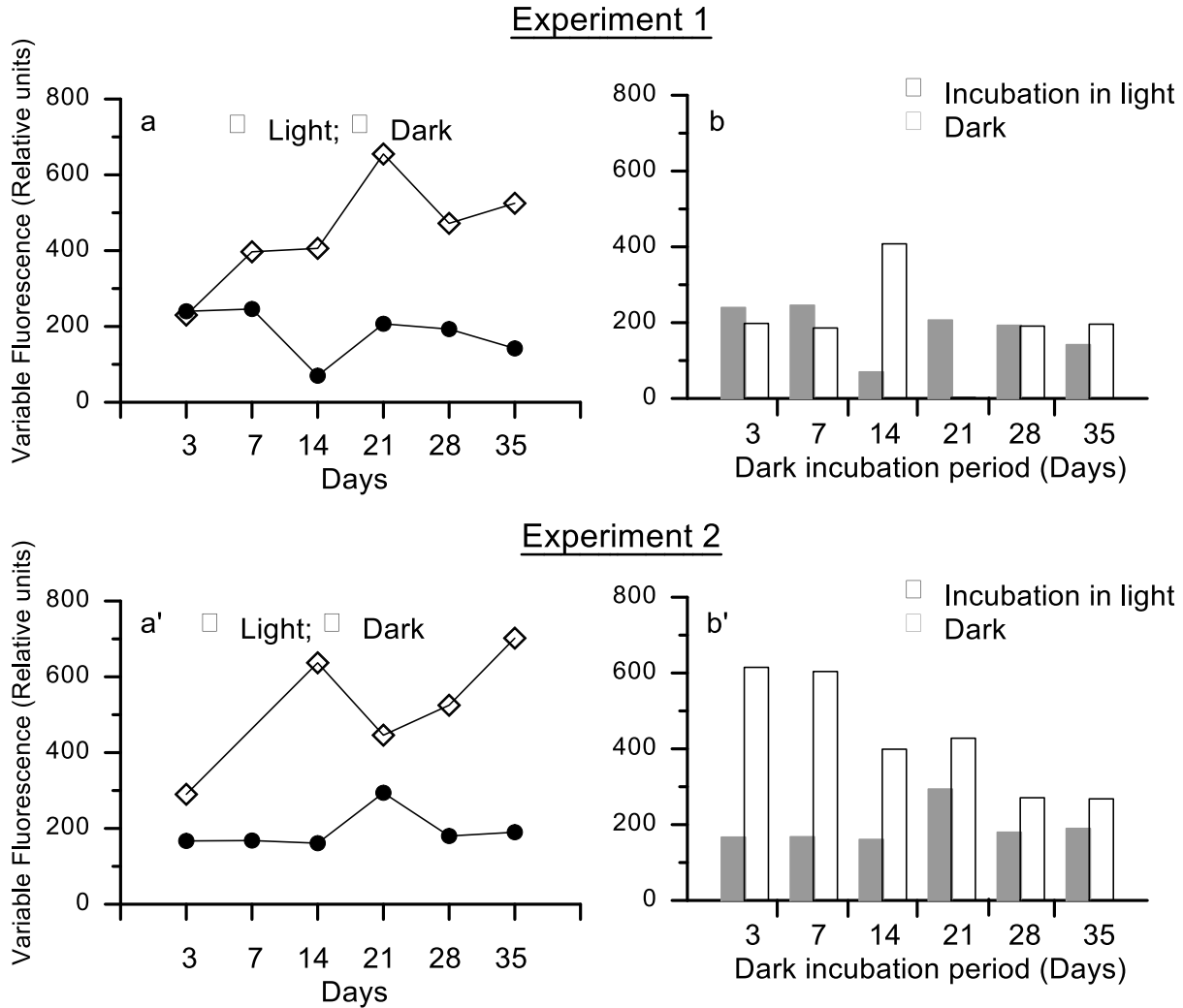
Experiment 1



Experiment 2

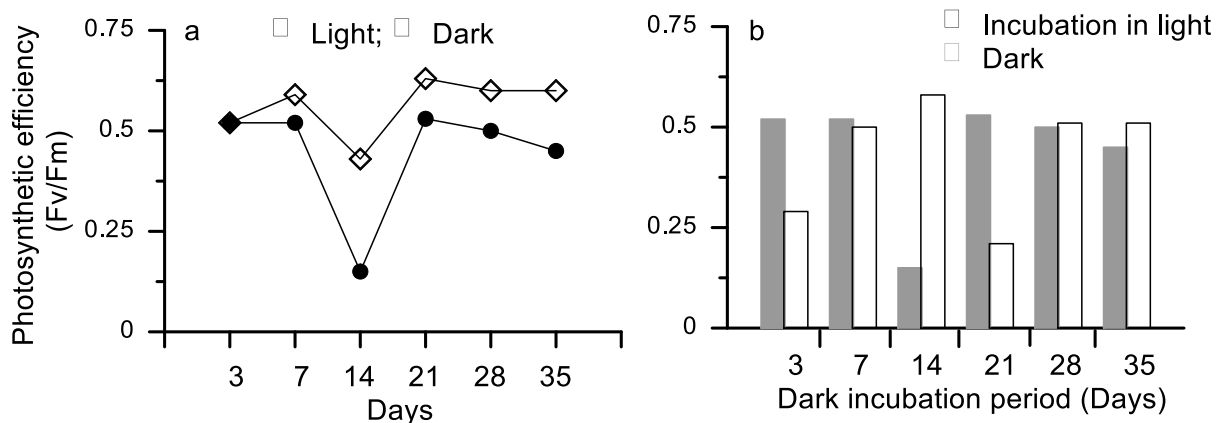


SupplementaryFig.2: (a & a') Influence of 12hlight:dark cycle and continuous dark on the accumulation of phaeopigments in the biofilms and (b & b') phaeopigments accumulation in dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle.

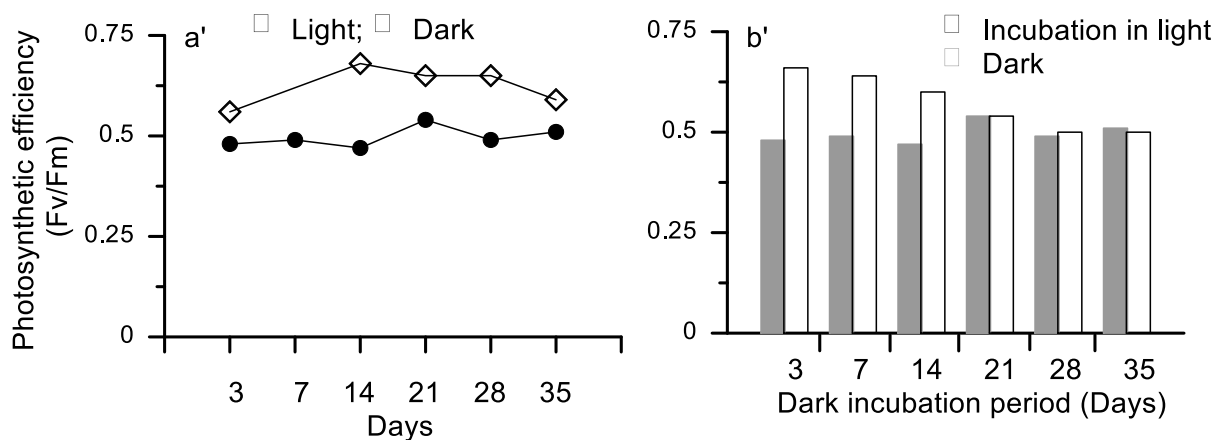


Supplementary Fig. 3: (a & a') Influence of 12h light:dark cycle and continuous dark on the variable chlorophyll fluorescence (F_v) in the biofilms and (b & b') variable chlorophyll fluorescence (F_v) of dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle.

Experiment 1

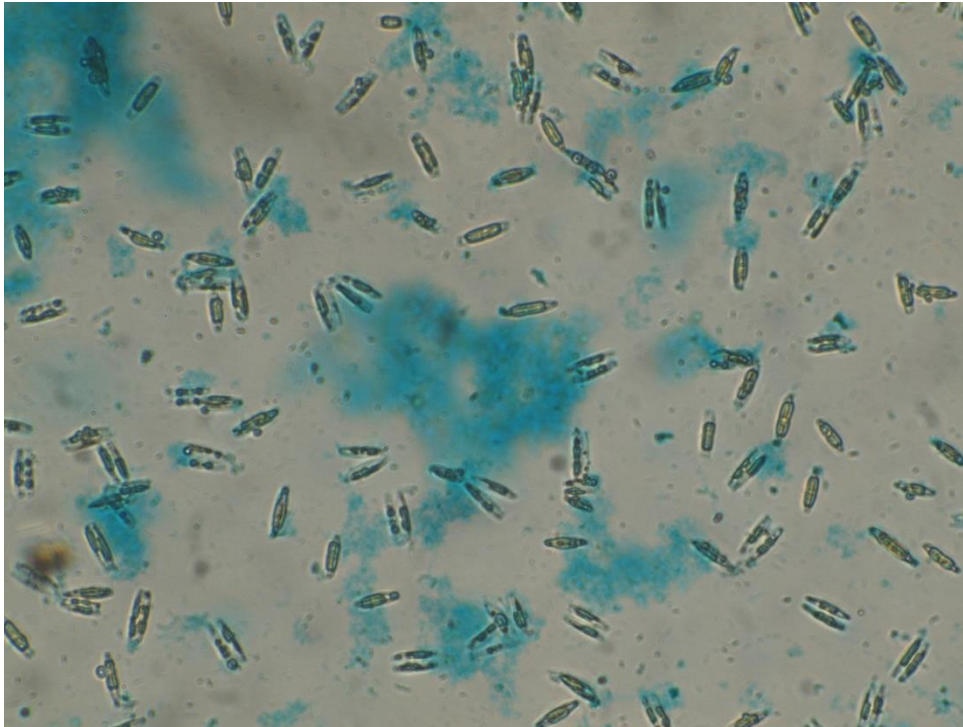


Experiment 2

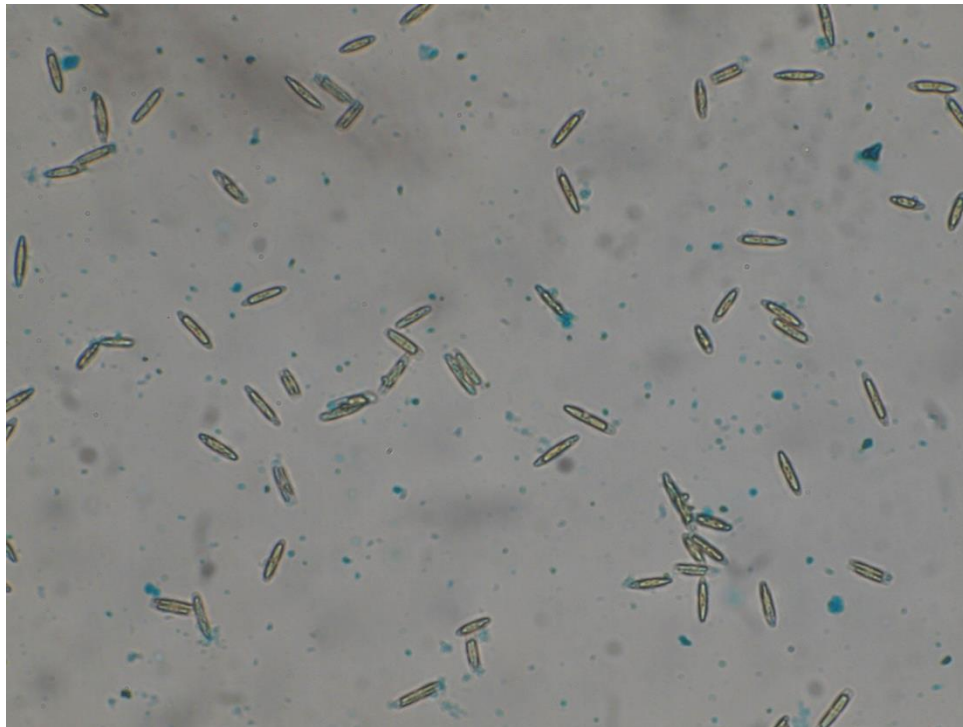


SupplementaryFig.4: (a & a') Influence of 12hlight:dark cycle and continuous dark on the photosynthetic efficiency (Fv/Fm) of micro-algae in the biofilms and (b & B')photosynthetic efficiency (Fv/Fm) of micro-algae in dark adapted biofilms and after 10 days of incubation under 12h light:dark cycle.

(b) *Navicula* under 12hlight:dark cycle.



(a) *Navicula* under continuous dark.



Supplementary Fig.5: Alcian blue staining of *Navicula* (a) grown under 12hlight:dark cycle and (b) continuous dark condition.