

Picophytoplankton community from tropical marine biofilms

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ABSTRACT

Although the importance of picophytoplankton (< 3 µm) as the major primary producers is well established in the oceanic and coastal marine environment, occurrence of this community in the marine biofilms is not explored. Considering their small size and high abundance which is next to bacteria, the initial heterotrophic colonizers, it was hypothesized that picophytoplankton are the first autotrophic colonizers in marine biofilms. In this study biofilms were developed by immersing glass slides in the tropical waters of the Dona Paula Bay up to a period of 192 hours. The presence of picophytoplankton and the temporal variations in community structure was investigated flow cytometrically after every 24 h. Biofilms were also developed in the laboratory by immersing the glass slides in fresh natural seawater and the sequence of appearance of the microorganisms was assessed with high frequency samplings. Field observations showed the presence of three groups of picophytoplankton, two prokaryotes, *Prochlorococcus*-like organisms (*PRO*), *Synechococcus* (*SYN*) and the picoeukaryotes (PEUK). Nanoeukaryotes, which are mostly represented by diatoms were also monitored. In the total biofilm community, prokaryotes were dominant throughout the study period wherein contribution of *SYN* was highest (50%) in the earlier stages which were later overtaken by *PRO*-like cells. The contribution of PEUK and nanoeukaryotes was always below 20%. Picophytoplankton contribution to the total photosynthetic biomass was > 60% in the initial period of biofilm formation, both in terms of numbers and biomass with PEUK as the major contributors. However, after 2 days of incubation, their contribution to total chlorophyll declined thus revealing that although their numbers were increasing, picophytoplankton were succeeded by nanoeukaryotes in terms of biomass. Laboratory experiments revealed that heterotrophic bacteria and picophytoplankton appeared within five minutes in the biofilms which were followed by the nanoeukaryotes after 5 hours. This study shows that picophytoplankton are the pioneer autotrophic colonizers in the tropical marine biofilms, suggesting an essential role in the biofilm food web dynamics, especially in the initial stages.

Keywords: Flow cytometry; Marine biofilms; Picophytoplankton; Tropical bay

1. Introduction

Diatoms and dinoflagellates are more commonly regarded as the dominant phytoplankters in estuaries and lagoons, reaching bloom proportions in regions with requisite bioavailable nutrients and light (Paerl, 1988; Kennish, 1990). Diatoms are also recognized for their vital role in marine biofilms where they form a major part of the biomass and are considered to be the initial autotrophic colonizers in coastal regions (Cooksey et al., 1980; Callow 2000). Diatom metabolic activities provide the sole carbon and energy source for heterotrophic bacteria in biofilms where both types of organisms are present (Murray et al., 1986). Both these organisms, due to their higher numbers in the ambient waters have a higher surface encountering probability as a result of which they form important components in the initial phases of biofilm formation (Cooksey et al., 1984). Biofilms have at least three clearly defined roles in marine ecology: i) as major sources of primary production on the shore for microphagous herbivores (Hawkins et al. 1992; Sommer 1999); ii) as primary attachment sites for macroalgal propagules which settle and germinate on it (Wahl 1989; Steinberg et al. 2002); and iii) as sources of settlement cues for a variety of marine invertebrates and algae and may promote cellular metamorphosis in the process of biofouling (Dobretsov and Qian 2006). All these aspects highlight the importance of biofilms in the marine environment.

With epifluorescence microscopy and flow cytometry, unicellular picophytoplankton (cells < 3 μ m; Chen et al., 2011), which includes cyanobacteria (*Prochlorococcus* and *Synechococcus*) and eukaryotic algae (picoeukaryotes) have been recognized as vital components of the phytoplankton community in oceanic (Vaulot et al., 1990; Campbell et al., 1998) and estuarine ecosystems (Ray et al., 1989; Shang et al., 2007). Their contribution to the total photosynthetic biomass is known to be significantly high in the open oceans and also in the coastal regions (Campbell et al., 1998; Moran et al., 2007). The higher abundance of picophytoplankton increases their surface encountering capabilities as compared to diatoms. Although, picocyanobacteria have also been observed in mats and biofilms in hot springs (Ferris et al., 1996; Ward et al., 1998; Miller and Castenholz, 2000) and hypersaline ponds (Garcia-Pichel et al., 1998), the role of picophytoplankton community in marine biofilms is not well addressed.

In the process of biofilm formation, within minutes of immersing a clean surface in water it adsorbs a molecular 'conditioning' film consisting of organic material (Bakker et al. 2003). For some years, it was held that there was an obligatory order in the succession of organisms that followed the adsorption of the conditioning film (Marzalek et al. 1979). Bacteria were considered to be the initial colonizers (Corpe 1970; Sieburth 1979; Bhosle et al. 1989), followed by diatoms, other algae, and

invertebrate larvae. However, some studies have shown that although the presence of the bacterial film may facilitate the attachment of diatoms, it is not a prerequisite (Horbund and Freiberger 1970; Cooksey et al. 1980). These initial biofilm organisms are known to produce exopolymers that assist the process of adhesion and movement (Molino and Wetherbee 2008). So, the observed pattern of biofilm community development in natural systems (bacteria preceding algae) is more likely to be a reflection of the average abundance and the availability of the different colonizing forms at the moment of immersion of a new substratum (Wahl et al. 1989; Gawne et al. 1998). In view of this, considering the cell size, cell availability and cell abundance, since picophytoplankton are next to bacteria, it was hypothesized that picophytoplankton are the first autotrophic colonizers in biofilms contributing significantly to the biofilm photosynthetic biomass in the initial stages thereby playing an important role in the biofilm food web dynamics and probably in the production of exopolymers similar to bacteria and diatoms (Patil and Anil 2005c). This investigation was carried out at the Dona Paula Bay where extensive studies on biofilm diatom communities have been conducted (Patil and Anil, 2005a,b; Mitbavkar and Anil, 2007; 2008) but reports on picophytoplankton from the biofilms are lacking. The study addressed the following (1) the succession of organisms in the early phases of biofilm formation and (2) their contribution to the photosynthetic biomass so as to better understand the base of the food web in biofilms which support the higher trophic levels.

2. Materials and methods

2.1 Field experiments

2.1.1. Environmental parameters

During each of the experiments, water temperature was monitored at the study site. Salinity was measured every day with an autosal. Samples (500 ml) were collected for nutrient (nitrate, phosphate and silicate) analysis.

2.1.2. Experimental protocol

This study was carried out three times, (1) from 2 May 2009 to 10 May 2009, (2) 15 December 2010 to 22 December 2010 and (3) 3 May to 10 May 2011 at the Dona Paula Bay located at the mouth of the Zuari estuary, Goa, ($15^{\circ} 27.5' N$, $73^{\circ} 48' E$), on the west coast of India. For biofilm development, 100 glass slides (7.6 x 2.2 cm) were fixed vertically to wooden frames and suspended at the sub-surface level (~ 1m below lowest low tide level) for a maximum period of eight days. Initially after 6 h and thereafter every 24 h till the eighth day, nine slides were removed. The

retrieved slides were transferred separately into 0.2 μm membrane filtered autoclaved seawater and brought to the laboratory. Each of the slides was then rinsed and scraped separately with a sterile scraper (BD FalconTM) into 15 ml of 0.2 μm membrane filtered autoclaved seawater. Out of nine scraped materials, three were fixed in paraformaldehyde (PFA; 0.2% final concentration) for picophytoplankton analysis in triplicate. These samples were then transferred to liquid nitrogen and subsequently stored at -80°C and analyzed within a week. Other three scraped materials (triplicate) were used for *in-vivo* fluorometric measurement of chlorophyll *a* using Turner fluorometer (Triology) from total sample. Subsequently, this sample was filtered through 3 μm Millipore filter paper and the filtrate ($< 3 \mu\text{m}$) was used to measure the chlorophyll *a* in order to estimate the relative contribution of picophytoplankton to total photosynthetic biomass. Total chlorophyll *a* concentration was also estimated through spectrofluorometric method from the remaining three scraped materials (Parsons et al., 1984).

2.2 Laboratory experiments

High frequency observations were carried out in the laboratory in order to ascertain the time of arrival of picoplankton in the marine biofilms. This experiment was conducted in June 2009, January 2011 and July 2011. Sixty glass slides were incubated in nutrient enriched seawater (f/2 media; Guillard and Rhyther, 1962) under 12h:12h light:dark condition at 27°C . After incubation, three slides were retrieved at every five minute interval from the incubation tub for the first half-an-hour. For the next one hour, three slides were retrieved after fifteen minutes interval. Subsequently, the slides were removed after 30 min, followed by one hour and two hour intervals until the completion of five hours incubation. The initial duration was changed to 15 minutes in the second experiment. This experiment duration was 48 h and that of the third experiment was 72 h. The retrieved slides were first rinsed with 0.2 μm membrane filtered autoclaved seawater so as to remove any unattached material and separately scraped in petriplates by adding 15 ml of autoclaved filtered seawater. The scraped material was preserved in PFA for picophytoplankton and heterotrophic bacterial analysis. Similarly, water samples from the incubation tub were also preserved at the same time.

2.3. Flow cytometric analysis

A BD FACSAriaTM II flow cytometer equipped with a laser emitting at 488 nm and a 70 μm nozzle was used for picoplankton analysis. Emitted light was collected through the following set of filters: 488/10 band pass (BP) for side scatter, 575/26 BP for orange fluorescence, 530/30 BP for green fluorescence and 695/40 BP for red fluorescence. Picophytoplankton groups could be

discriminated and enumerated according to their specific autofluorescence properties and light scatter differences (Fig. 1). For the heterotrophic bacterial counts, samples were quickly thawed and incubated in the dark for 30 min at 37⁰C after addition of SYBR–Green I (final concentration 10⁻⁴; Molecular probes) which was used as the nucleic acid stain (Marie et al., 1997). Fluorescent beads (2 μm for picophytoplankton and 1 μm for bacteria; “Fluoresbrite”, polysciences) were used as internal standards and for calibration of the above parameters. Flow cytometric data were collected and saved as listmode files.

3. Results

3.1. Picophytoplankton groups in the water column and biofilms

Three groups of picophytoplankton were observed, *Prochlorococcus*-like cells (*PRO*), *Synechococcus* (*SYN*) and picoeukaryotes (*PEUK*) (Fig. 1). *PRO*-like cells was identified based on their small side scatter (which is a proxy for cell size) as compared to the other groups, red autofluorescence and lack of orange fluorescence. Based on their larger side scatter and red autofluorescence and lack of orange fluorescence, *PEUK* were identified. *SYN* was identified based on their orange pigment fluorescence when excited with blue (488 nm) laser. Cells larger than the *PEUK* were grouped as nanoeukaryotes (> 3 – 20 μm).

3.2 Field experiments

3.2.1. Environmental parameters

The range of temperature, salinity, nutrients and chl *a* of the ambient waters during the study period is given in Table 1. During the first and the third experiments conducted in May, the temperature was higher as compared to that in December. The same trend was observed for salinity. Nitrate and phosphate concentrations were higher in May than in December and silicate concentrations were higher in December.

3.2.2. Chlorophyll in biofilms

In May 2009, both the *in-vivo* chlorophyll fluorescence of total and < 3μ fraction was low till day (D) 3 (959 and 335, respectively). Thereafter the values increased and on the final day of observation chl fluorescence of the total was 50,000 and that of < 3μ fraction was 7000 (Fig. 2A). Contribution to total chlorophyll by the < 3μ fraction was > 57% up to D2 and thereafter showed a decreasing trend from D3 (35%) to D8 (15%) (Fig. 2B). In December 2010, *in-vivo* chlorophyll

fluorescence of total and $< 3\mu$ fraction was low till D4. On D5 the highest fluorescence values were recorded for both fractions (20,000 and 17,000, respectively). Thereafter the chl fluorescence reduced but showed an increasing trend till D8 (14,000 and 6000, respectively) (Fig. 2C). Contribution to total chlorophyll by the $< 3\mu$ fraction ranged from 67% to 88%, with the highest recorded on D5. Thereafter, their contribution started decreasing and on D8 it was 42% of the total biomass (Fig. 2D). In May 2011, total *in-vivo* chlorophyll fluorescence ranged up to 3000 whereas $< 3\mu$ ranged up to 1900, both showing an increasing trend from the initial observation after 6h till D8 (Fig. 2e). Contribution to total chlorophyll by the $< 3\mu$ fraction was above 78% till D7 and reduced to 62% on D8. The highest contribution of 85% was observed on D4 (Fig. 2F).

From the relative mean chlorophyll fluorescence values of single cell of each group obtained from the flow cytometry statistics, the mean chlorophyll fluorescence values for each of the three picophytoplankton groups was calculated. It was observed that *SYN* and *PRO*-like cells contribution to the picophytoplankton chlorophyll biomass ranged from 8 to 27% and that of *PEUK* ranged from 73 to 92% during the experimental period (Fig. 3A, B).

3.2.3. Cell abundance in water column

Picophytoplankton and nanoeukaryotes were observed in the ambient waters throughout the study period. In May 2009, during the initial observation *SYN* was the most dominant picophytoplankton (10×10^3 cells ml^{-1}) followed by *PEUK* and *PRO*-like cells (3×10^3 cells ml^{-1} and 2×10^3 cells ml^{-1}). Nanoeukaryote abundance was 12×10^3 cells ml^{-1} . Subsequently, *PRO*-like cells and *PEUK* were the most abundant groups (0.4×10^3 cells ml^{-1}). This trend continued up to the end of the incubation period (Fig. 4A). In December 2010, *SYN* was the most dominant group (54.0×10^3 to 159.0×10^3 cells ml^{-1}) followed by *PEUK* (8.2×10^3 to 31.0×10^3 cells ml^{-1}). Nanoeukaryotes were next in abundance (0.5×10^3 to 3.8×10^3 cells ml^{-1}) whereas *PRO*-like cells were the least abundant (undetectable levels to 0.4 cells ml^{-1}) (Fig. 4B). In May 2011, *SYN* was dominant up to D3 (55×10^3 to 188×10^3 cells ml^{-1}) which was subsequently overtaken by *PEUK* that remained dominant till the end of the incubation period (5.0×10^3 cells ml^{-1} to 208.0×10^3 cells ml^{-1}). Nanoeukaryotes were more dominant than *SYN* after D6 (128×10^3 cells ml^{-1}). *PRO*-like cells were the least abundant group (0.2×10^3 cells ml^{-1} to 4×10^3 cells ml^{-1}) (Fig. 4C).

3.2.4. Cell abundance in biofilms

The water column picophytoplankton community was reflected in the biofilm along with the nanoeukaryotes. In May 2009, after 6 h *SYN* and *PRO*-like cells were the dominant groups (0.3×10^3

and 0.4×10^3 cells cm^{-2} , respectively) followed by PEUK (0.08×10^3 cells cm^{-2}) and nanoeukaryotes (0.04×10^3 cells cm^{-2}). This trend continued up to D4 after which *PRO*-like cells were overtaken by PEUK. At the end of the incubation period (D8) PEUK was the dominant group (253×10^3 cells cm^{-2}) followed by *SYN* and *PRO*-like cells (157×10^3 and 163×10^3 cells cm^{-2}). The nanoeukaryotes also started increasing at a faster pace after D4 and were close to the abundance of the three picophytoplankton groups at the end of the incubation (Fig. 5A). In December 2010, in the initial observation after 6 h, *SYN* was the dominant group (0.6×10^3 cells cm^{-2}) followed by *PRO*-like cells (0.3×10^3 cells cm^{-2}), nanoeukaryotes (0.1×10^3 cells cm^{-2}) and PEUK (0.06×10^3 cells cm^{-2}). In the one day old biofilms, *SYN* (0.2×10^3 cells cm^{-2}) was overtaken by *PRO*-like cells (0.4×10^3 cells cm^{-2}). This was observed till the end of the experimental duration (8 days) wherein the former was 111.0×10^3 cells cm^{-2} and latter was 404.0×10^3 cells cm^{-2} . All the groups showed an increasing trend till D4. A high increase in abundance was observed on D5 for all the groups after which the community stabilized. The picoeukaryotes and nanoeukaryote abundance was almost similar (80×10^3 cells cm^{-2} and 40×10^3 cells cm^{-2}) (Fig. 5B). In May 2011, after 6 h, *SYN* and *PRO*-like cells showed similar abundance (0.3×10^3 cells cm^{-2}) followed by PEUK (0.052×10^3 cells cm^{-2}) and nanoeukaryotes (0.047×10^3 cells cm^{-2}). After one day, *SYN* abundance was higher (1.6×10^3 cells cm^{-2}) than *PRO*-like cells (0.8×10^3 cells cm^{-2}). Subsequently, from D3 till the end of the experiment, *SYN* was overtaken by *PRO*-like cells and was the most abundant group (1.5×10^3 to 60×10^3 cells cm^{-2}) with *SYN* as the second dominant till the end of the incubation period (1.6×10^3 to 13.0×10^3 cells cm^{-2}). After D3 nanoeukaryotes abundance showed a high increase and by the end of the incubation period, PEUK and nanoeukaryote abundance were almost similar (11.0×10^3 cells cm^{-2}) (Fig. 5C).

3.3. Laboratory experiments

Through flow cytometric analysis, heterotrophic bacteria, picophytoplankton and nanoeukaryotes could be differentiated from the surrounding water and the biofilms.

3.3.1 Cell abundance in water column

In June 2009, *SYN* cell abundance was the highest and ranged from 298×10^2 to 1280×10^2 cells ml^{-1} . This was followed by PEUK (61×10^2 to 204×10^2 cells ml^{-1}). *PRO*-like cells (26×10^2 to 172×10^2 cells ml^{-1}) and nanoeukaryotes were the least abundant groups. Heterotrophic bacterial abundance ranged from 4776×10^2 to $11,474 \times 10^2$ cells ml^{-1} (Fig. 6A). In January 2011, *SYN* cell abundance was the highest and ranged from 300×10^2 to 1200×10^2 cells ml^{-1} . This was followed by PEUK (27×10^2 to 126×10^2 cells ml^{-1}). *PRO*-like cells (3×10^2 to 32×10^2 cells ml^{-1}) and

nanoeukaryotes (8×10^2 to 30×10^2 cells ml^{-1}) were the least abundant groups (Fig. 7A). In July 2011, *SYN* cell abundance was the highest and ranged from 33×10^2 to 84×10^2 cells ml^{-1} . This was followed by nanoeukaryotes (10×10^2 to 23×10^2 cells ml^{-1}) while PEUK (5×10^2 to 17×10^2 cells ml^{-1}) and *PRO*-like cells (4×10^2 to 15×10^2 cells ml^{-1}) were the least abundant groups. Heterotrophic bacterial abundance ranged from 6705×10^2 to $44,461 \times 10^2$ cells ml^{-1} (Fig. 8A).

3.3.2. Cell abundance in biofilms

In June 2009, within five minutes of incubation, heterotrophic bacteria as well as picophytoplankton were observed in the scraped material from the incubated slides (Fig. 6B). Heterotrophic bacteria were highest in abundance (9×10^4 cells cm^{-2}) followed by *SYN* (0.2×10^3 cells cm^{-2}). *PRO*-like cells appeared only after 30 minutes. Cell abundance of *SYN*, *PRO*-like cells and PEUK did not exhibit much variation up to 75 min of incubation subsequent to which they showed increase. Heterotrophic bacteria showed an increase in cell numbers after 2 h of incubation. At the end of the incubation period, heterotrophic bacterial numbers were the highest (17×10^4 cells cm^{-2}) followed by that of *PRO*-like cells (0.4×10^3 cells cm^{-2}), *SYN* (4.5×10^3 cells cm^{-2}) and PEUK (1.2×10^3 cells cm^{-2}). In January 2011, within fifteen minutes of incubation, all three picophytoplankton groups were observed in the biofilms (Fig. 7B). Nanoeukaryotes were not observed. Amongst the picophytoplankton, *SYN* was the most abundant (131 cells cm^{-2}) followed by PEUK (39 cells cm^{-2}) and *PRO*-like cells (17 cells cm^{-2}). *SYN* exhibited a lag phase for 2 h and grew exponentially only after 3 h. In case of *PRO*-like cells and PEUK lag phase was observed up to 3 h and increased exponentially thereafter. Nanoeukaryotes appeared in the biofilms only after 5 h and showed exponential growth after 12 h. At the end of the incubation period, *PRO*-like cells dominated the picophytoplankton community (4.2×10^3 cells cm^{-2}) followed by *SYN* (3.6×10^3 cells cm^{-2}) and PEUK (1.4×10^3 cells cm^{-2}). Nanoeukaryotes were the least abundant (2.1×10^3 cells cm^{-2}). In July 2011, within five minutes of incubation, heterotrophic bacteria (107×10^2 cells cm^{-2}) as well as picophytoplankton were observed in the scraped material from the incubated slides (Fig. 8B). *SYN* abundance was highest (24 cells cm^{-2}) followed by *PRO*-like cells (29 cells cm^{-2}) and PEUK (3 cells cm^{-2}). *SYN* and *PRO*-like cells exhibited exponential growth after 2 h and 1 h, respectively. PEUK abundance did not show a definite trend, although their numbers increased after 6 h it subsequently declined till the end of the incubation period. Nanoeukaryotes appeared only after 3 h and showed an exponential increase in abundance after 6 h. Heterotrophic bacterial abundance increased after 3 h. At the end of incubation period heterotrophic bacteria was the most abundant (17×10^5 cells cm^{-2})

picoplankton. Amongst the picophytoplankton, *PRO*-like cells dominated the community (455 cells cm^{-2}).

4. Discussion

It is well known that natural biofilms are usually dominated by diatoms (Brandini et al., 2001). However, the occurrence of picophytoplankton which are the most dominant autotrophic forms in the marine environment is less studied in biofilms. Except for a report from a tropical estuary of Singapore where contribution of *SYN* to the periphytic community ranged from 60 to 80% during a five day incubation period (Nayar et al., 2005), there is no other published literature on picophytoplankton in marine biofilms. The reason for this could be the small size of these cells which are not visible through light microscopy and hence missed during the routine observations of biofilms.

The observation of higher abundance of picophytoplankton in the water column compared to that of the nanoeukaryotes which mostly comprises of diatoms is in par with the earlier reports including those in tropical regions (Pan et al. 2007, Wang et al. 2010). Similar trend was reflected in the biofilms. However, with increase in incubation period the replacement of the initial dominant group, *SYN* by the other less dominant picophytoplankton groups, in spite of their lower abundance in the water column shows that the rate of recruitment of cells from the surrounding environment was overtaken by the rate of cell multiplication of the attached cells in the biofilms. Such an observation was also reported in case of the field diatom community (Cooksey et al., 1984) wherein after an initial colonization period of 48 h, logarithmic increase in the number of cells on glass or stainless steel surfaces in the sea took place only in daylight hours which was attributed to cell multiplication.

Picophytoplankton contribution to the total photosynthetic biomass was substantial in the initial stages of biofilm formation. Earlier studies have shown that usually *PEUK* are the dominant contributors to the total photosynthetic biomass in the water column due to their comparatively larger size (Mitbavkar et al., 2009). Similar results were obtained from the biofilms which suggests that this group can play a major role in the trophic dynamics of biofilms in the early stages of biofilm formation. However, with increase in the incubation period, decline in their contribution to the total photosynthetic biomass in spite of their increasing abundance, suggests that in terms of biomass picophytoplankton were succeeded by nanoeukaryotes. Earlier studies conducted in the same study area on the fouling diatom community did not find a 1:1 relation between biofilm chlorophyll *a* and

diatom cell numbers (Patil and Anil, 2005b; Mitbavkar and Anil, 2007, 2008). This disparity could be partly due to the non-inclusion of picophytoplankton in these studies.

Presence of picophytoplankton and nanoeukaryotes in the 6 h old biofilms made it pertinent to understand the sequence of colonization of these microorganisms along with the heterotrophic component, bacteria in the initial phases of biofilm formation. Laboratory time-series incubation experiments with high frequency sampling revealed that both, heterotrophic as well as autotrophic picoplankton recruitment on the immersed substrata takes place within a very short time, as early as within 5 minutes whereas nanoeukaryotes appeared after 5 h. Zobell and Allen (1935) through their pioneer research in biofouling showed that heterotrophic bacteria were the first to settle on glass slides. Marshall (1988) through *in situ* colonization experiments showed that bacteria are the first colonizers followed by sessile diatoms. Time-series studies on settlement by Acs et al., (2000) have shown that after 3 h from immersion, only coccoid bacteria were found on the substratum but after 6 h the first algae (*Diatoma vulgare*) was observed. In their study the first sampling was after three hours but however, the bacteria must have settled on the substratum much earlier as we observed in our study. In case of diatoms, studies carried out in the Biscayne Bay, USA at different times showed diatoms on coupons after 10 d (Marzalek et al. 1979) and only after a few hours (Cooksey et al. 1984). This temporal difference between the 2 studies could be due to the differences in enumeration techniques wherein the former counted diatoms after preparation for electron microscopy, a method which would be insensitive to small numbers and the latter used either chlorophyll fluorescence or direct light microscopy. So, the disparity in the results obtained in the earlier studies could be due to the time interval of test panel retrieval for observation and also the enumeration techniques employed. The surface encountering probability which depends on the availability and abundance of the different colonizing forms at the moment of immersion of new substrata in the ambient waters is another factor that can influence the sequence of colonization and cell abundance in biofilms (Wahl et al. 1989).

Although the presence of picoplankton was detected after five minutes, the actual increase was observed after a lag of few hours. The initial attachment of bacteria to surfaces is considered to occur in two stages: the reversible attachment phase and the irreversible attachment phase (Marshall et al., 1971; Lawrence et al., 1987). Marshall (1988) reported that cells became irreversibly attached to surfaces only after a period of unstable attachment, during which time cells revolved around the axis of attachment and frequently emigrated from the attachment site rather than becoming irreversibly attached. Benthic algal abundance was also found to increase after 14 hours of incubation (Acs et al.,

2000). Studies on the adhesion of a primary population of microorganisms to marine surfaces have shown that clean surfaces placed in the sea sorb bacteria within hours while development of a critical population size to yield growth may take days or weeks (Hendricks, 1974). Zobell (1943) suggested that when a microbe attaches to a surface, its metabolic activity is altered. So, the delay in increase in cell abundance could be due to the time the cells take for irreversible attachment and later for multiplication of these attached cells.

In conclusion, this study revealed that in tropical waters picophytoplankton which dominates the coastal waters are the initial autotrophic colonizers in the marine biofilms much before the arrival of diatoms as was thought till now. An interesting observation was that *PRO*-like cells, which are usually reported to be abundant only in the oceanic regions, were present both in the ambient waters and the biofilm in significant numbers. Picophytoplankton contribution was > 60% in the initial period of biofilm formation, both in terms of numbers and biomass which was later succeeded by larger sized nanoeukaryotes in terms of biomass. These findings have implications in the studies related to food webs in marine biofilms. Further studies should investigate whether these picophytoplankton, similar to the diatoms, have any role in attracting the larvae of higher organisms in the process of biofouling.

Acknowledgements

The authors are grateful to Dr Satish Shetye, Director, National Institute of Oceanography and Dr. A.C. Anil, scientist, for their support and encouragement. This work was carried out with partial support from Department of Science and Technology, India. This is NIO contribution #.

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Legend to Figures

Fig. 1. Examples of flow cytometry cytograms. Data of samples taken from 5 day old biofilm in the field. (A) Phycoerythrin orange fluorescence vs. chlorophyll red fluorescence (B) side scatter (a proxy for cell size) vs. chlorophyll red fluorescence.

Fig. 2. Total chlorophyll *a* (mg m^{-2} ; dashed line) and *in vivo* fluorescence (a.u.) in May 2009, December 2010 and May 2011 of $< 3\mu$ fraction and total sample (A, C, E) and the percentage chlorophyll contribution of $< 3\mu$ fraction (■) and $> 3\mu$ fraction (■) to the total (□) (B, D, F) from the biofilms of the Bay.

Fig. 3. Temporal variation in chlorophyll red fluorescence (a.u.) of *Synechococcus* (■), *Prochlorococcus*-like (□) and picoeukaryote (■) groups (A) and percentage contribution of each group to the total red fluorescence (B) obtained from flow cytometric analysis of biofilms of the Bay.

Fig. 4. Temporal variation in *Synechococcus*, *Prochlorococcus*-like, picoeukaryote and nanoeukaryote abundance (cells mL^{-1}) in the surface waters during May 2009 (A), December 2010 (B) and May 2011 (C).

Fig. 5. Temporal variation in *Synechococcus*, *Prochlorococcus*-like, picoeukaryote and nanoeukaryote abundance (cells mm^{-2}) recorded in May 2009 (A), December 2010 (B) and May 2011 (C) from biofilms of the Bay.

Fig. 6. Temporal variation in *Synechococcus*, *Prochlorococcus*-like, picoeukaryote, nanoeukaryote and heterotrophic bacterial abundance recorded in the surrounding water (A) and biofilms (B) under laboratory conditions in June 2009.

Fig. 7. Temporal variation in *Synechococcus*, *Prochlorococcus*-like, picoeukaryote and nanoeukaryote abundance recorded in the surrounding water (A) and biofilms (B) under laboratory conditions in January 2011.

Fig. 8. Temporal variation in *Synechococcus*, *Prochlorococcus*-like, picoeukaryote, nanoeukaryote and heterotrophic bacterial abundance recorded in the surrounding water (A) and biofilms (B) under laboratory conditions in July 2011.

Table 1
Water temperature, salinity, nutrients and chlorophyll concentrations during the study period

Sampling date	2 May to 10 May 2009	15 Dec to 22 Dec 2010	3 May to 11 May 2011
Temperature ($^{\circ}\text{C}$)	31.6 – 32.5	28.5 – 29.5	30.7 – 31.4
Salinity	35.9 – 36.5	31.5 – 32.5	35.7
Nitrate (μM)	0.07 – 2.93	0.5 – 0.8	0.8 – 1.82
Phosphate (μM)	0.78 – 1.22	0.2 – 0.3	0.97 – 3.5
Silicate (μM)	5.13 – 10.31	13.2 – 14.7	1.3 – 10.2
Chlorophyll (mg m^{-3})	1.1 – 6.5	1.8 – 4.2	1.05 – 4.2

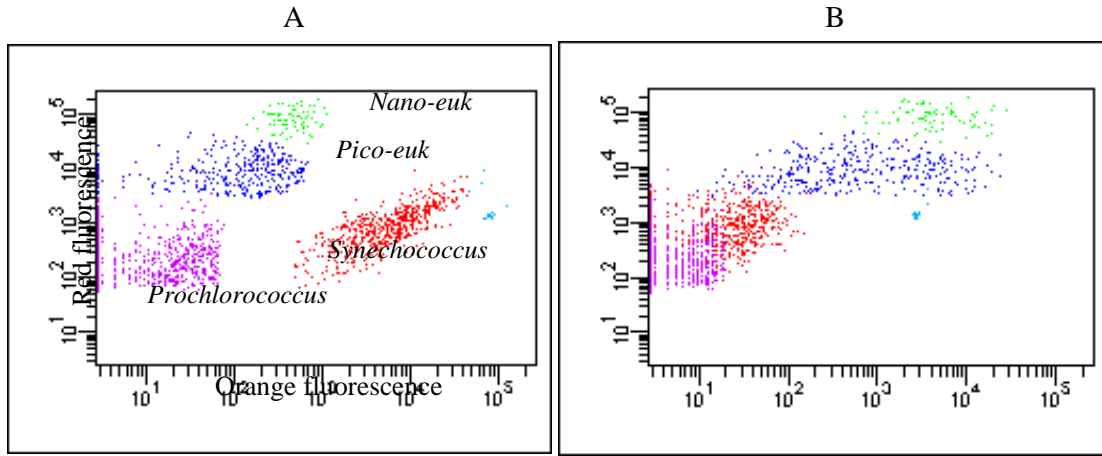


Fig. 1

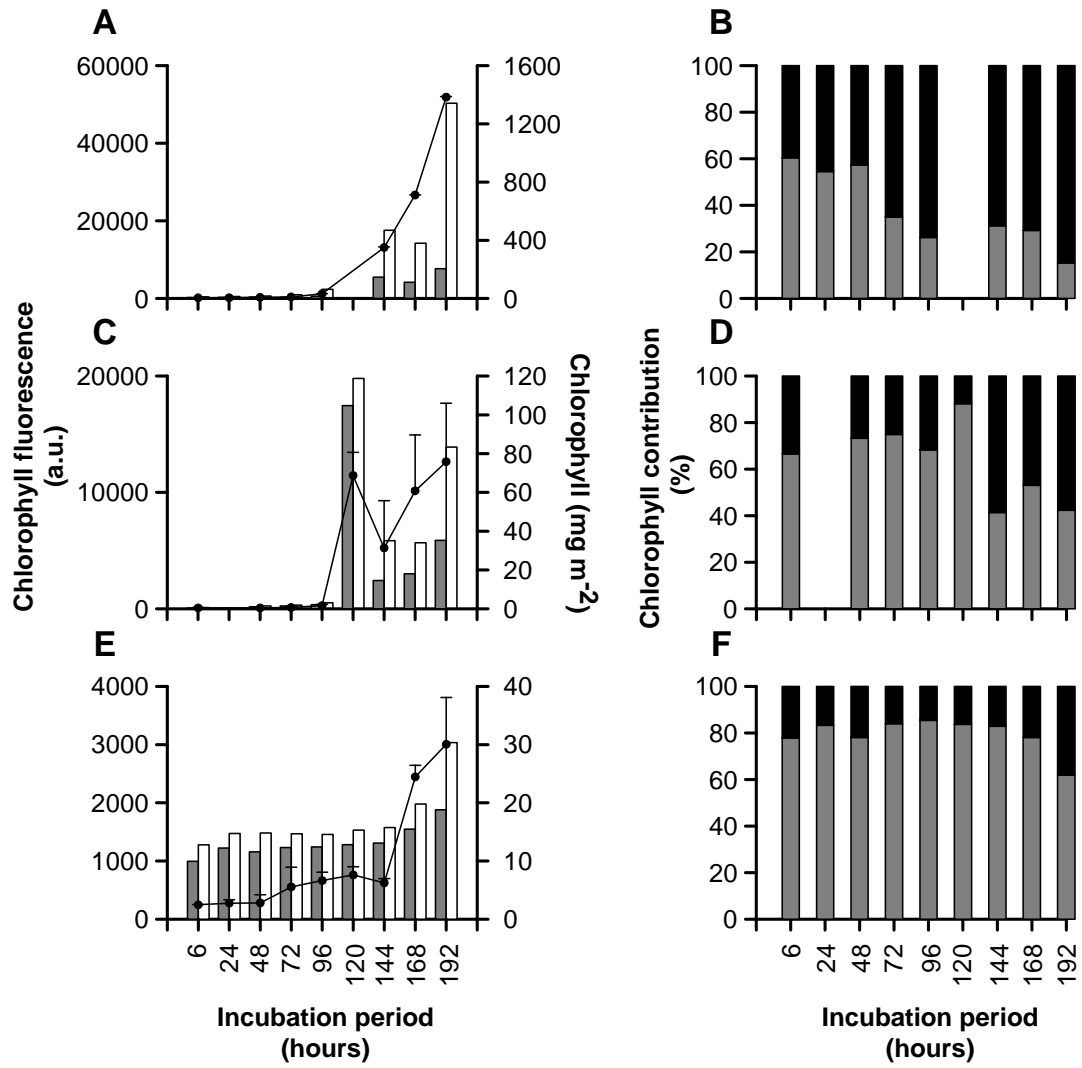


Fig. 2.

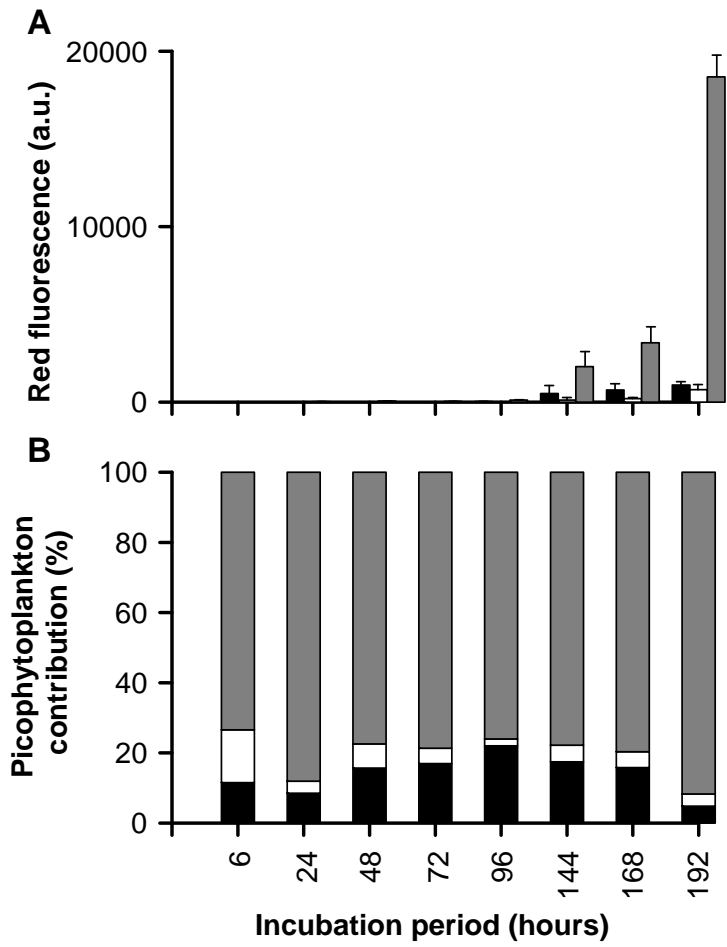


Fig. 3.

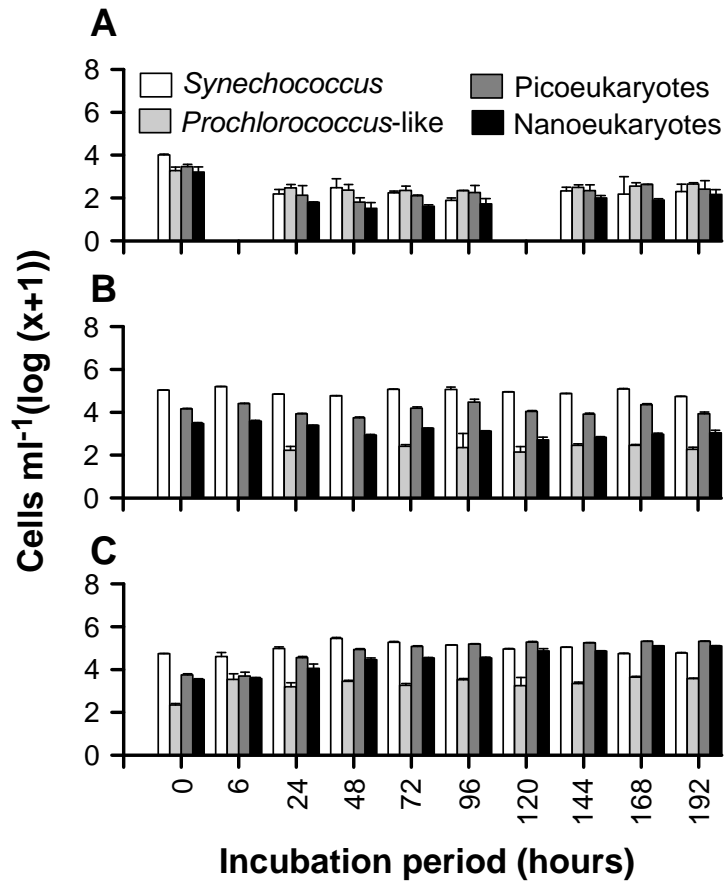


Fig. 4.

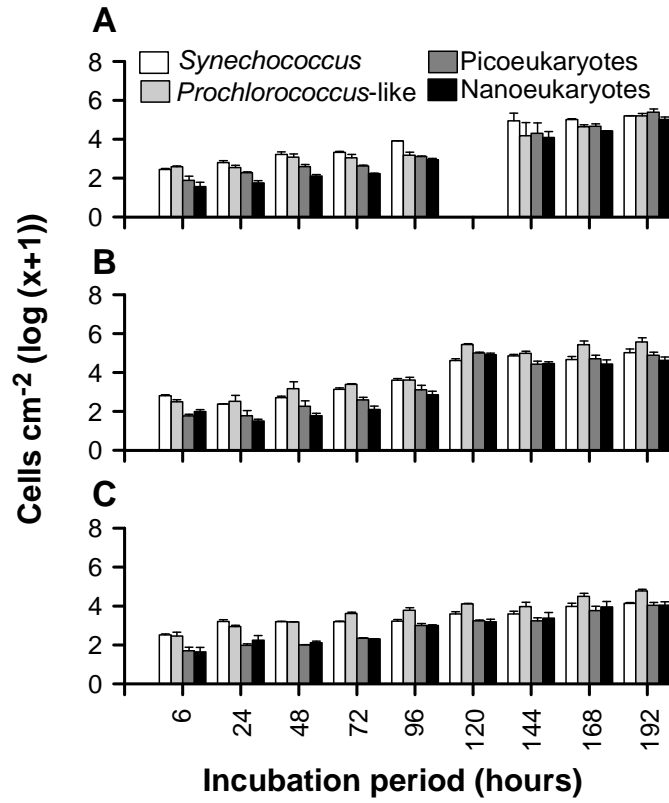
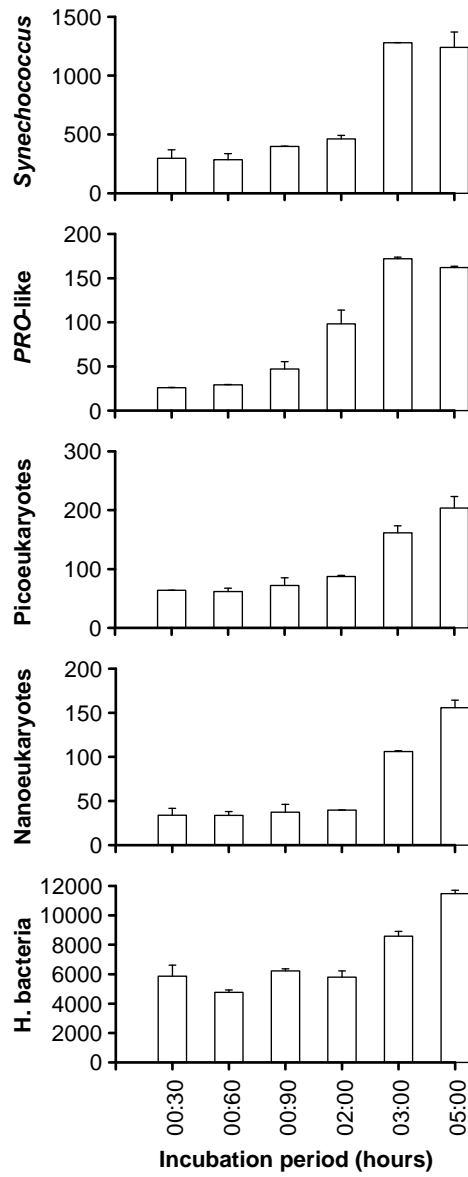


Fig. 5.

A. Water (x 10² cells ml⁻¹)



B. Biofilms (x 10² cells cm⁻²)

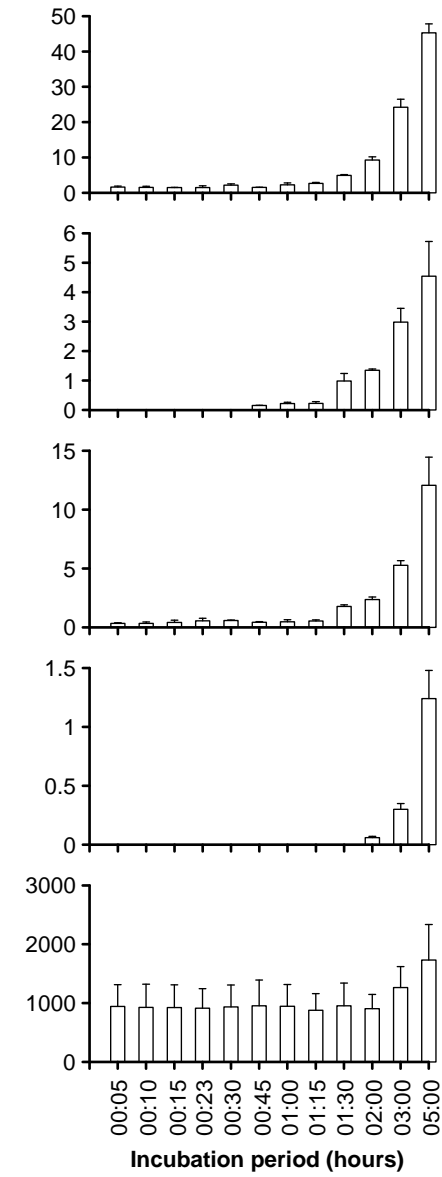


Fig. 6

A. Water (x 10² cells ml⁻¹)

B. Biofilms (x 10² cells cm⁻²)

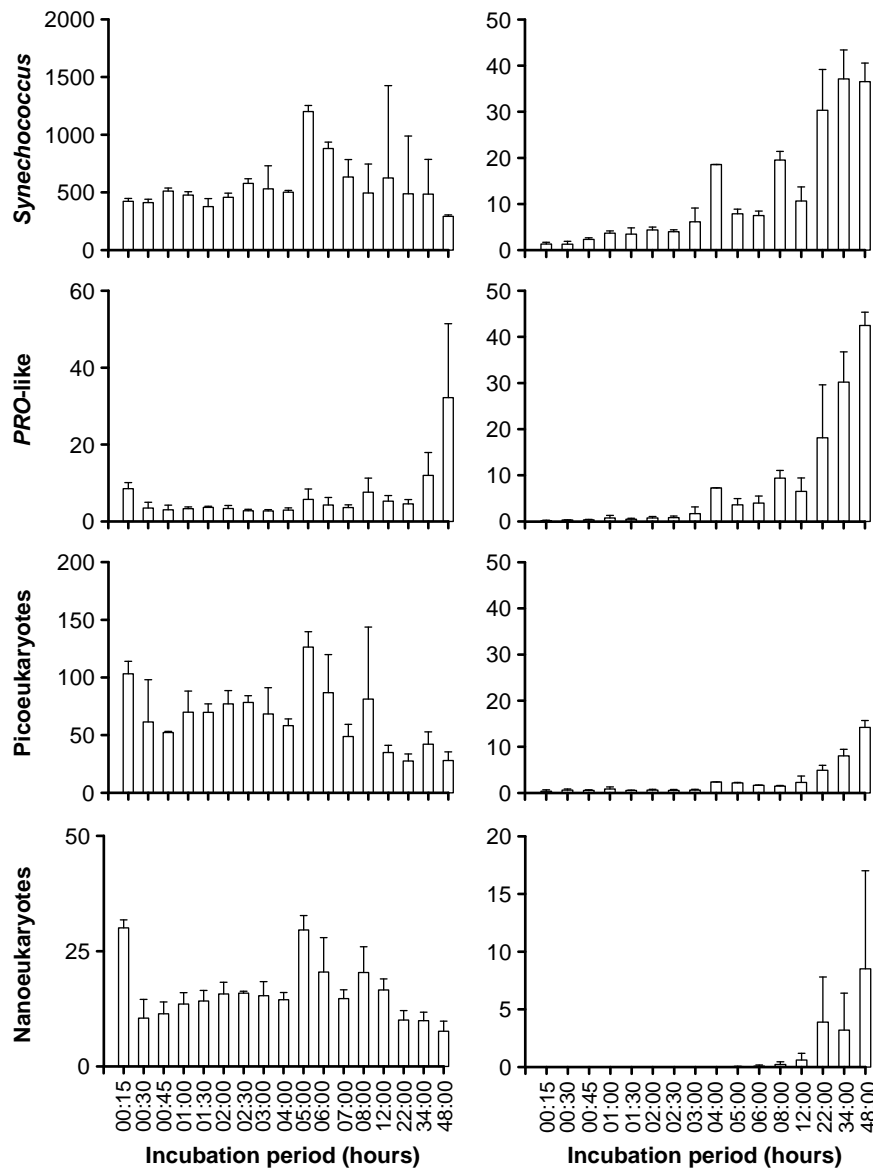
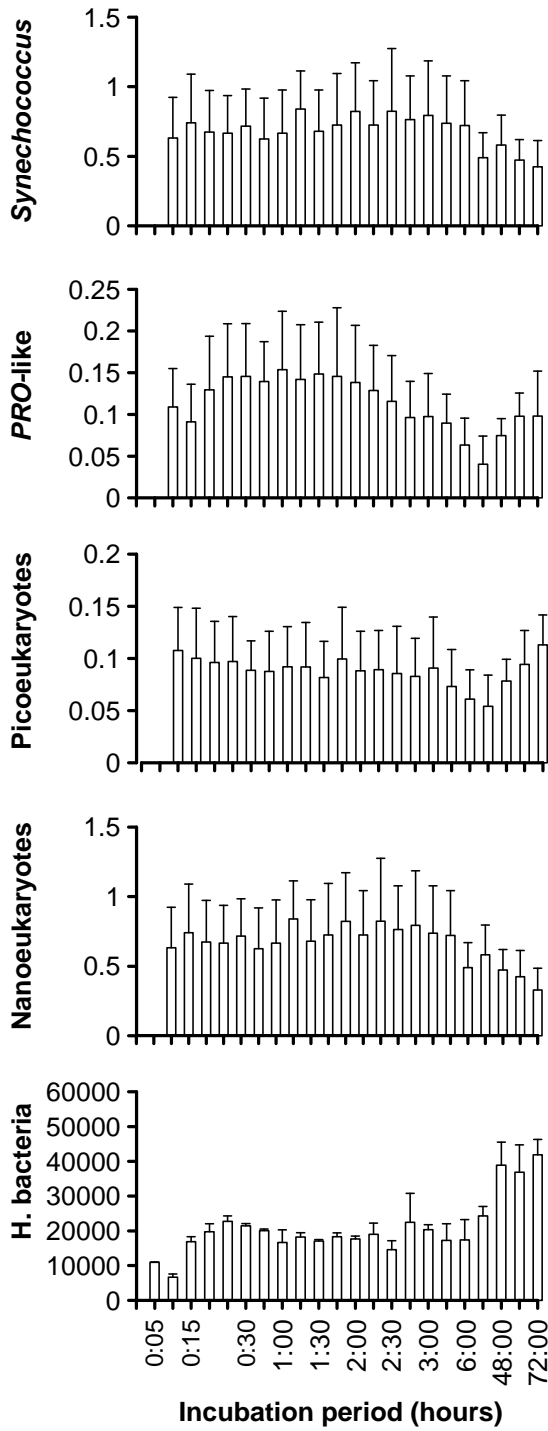


Fig. 7.

A. Water (x 10² cells ml⁻¹)



B. Biofilms (x 10² cells cm⁻²)

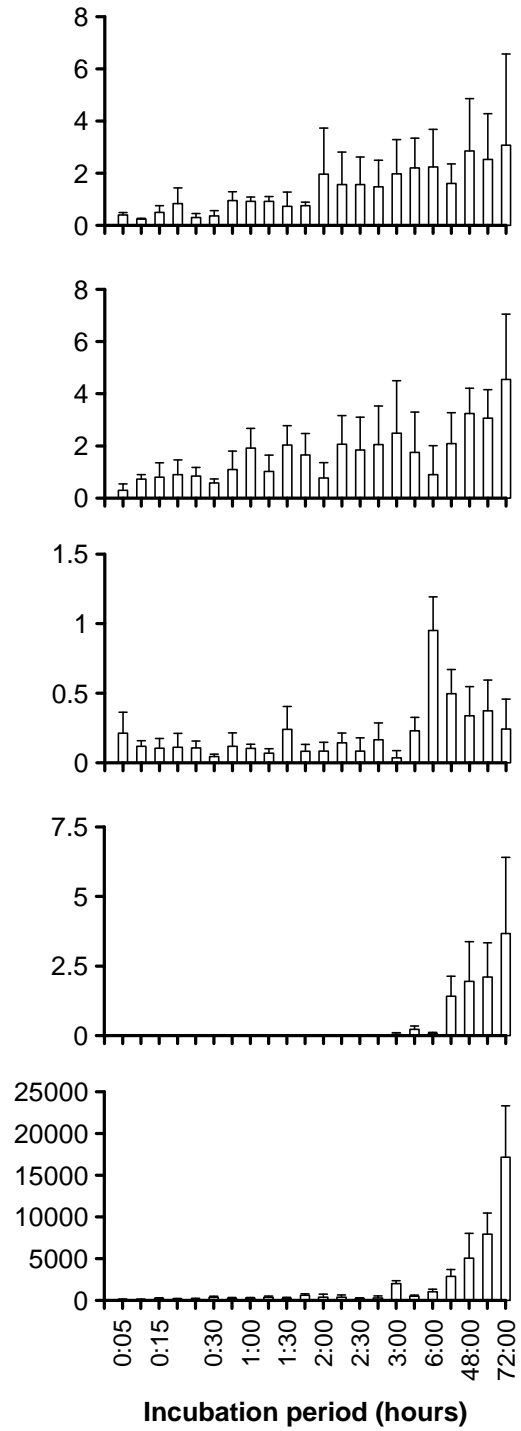


Fig. 8.