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ORIGINAL RESEARCH ARTICLE

Microfouling development on artificial substrates deployed in the central Red Sea

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Summary Microfouling is the initial step in the growth of biofouling on hard substrata submerged in marine waters. In this study, microfouling development on nylon nets submerged in the central Red Sea coast of Saudi Arabia was analyzed during the winter and summer seasons for a period of 5 days each. The results showed a well-established biofilm community on nylon nets submerged for 24 h, with bacteria and diatoms being the primary colonizers. Protein was the major organic component of the biofilm that developed on the nylon nets during the winter and summer seasons. *Navicula* spp., *Nitzschia* spp., *Cylindrotheca* spp., and *Pluerosigma* spp. were the dominant diatom species settled on the nylon nets. *Pseudoalteromonas shioyasakiensis*, *Planomicrobium* sp., *Vibrio harveyi* and *Pseudoalteromonas rubra* were the dominant bacteria isolated from the nylon nets. While the abundance of bacteria showed a positive correlation with the nutrient concentration of the biofilm during both winter and summer seasons, diatom density exhibited a significant positive relationship with the biofilm nutrients during the winter season only. The results also revealed significant seasonal variations in the abundance of microfouling organisms and accumulation of nutrients on nylon nets.

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1. Introduction

Microfouling development (also known as 'Biofilms) will occur within a few hours after the submersion of an artificial substratum in the sea (Dobretsov, 2009; Huggett et al., 2009; Satheesh and Wesley, 2010; Siboni et al., 2007; Wang et al., 2012). After the initial biofilm formation, the larval forms of marine organisms and macroalgal spores will settle on such surfaces (see reviews: Salta et al., 2013; Satheesh et al., 2016). Biofilm development is a process that generally

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consists of the formation of a conditioning film and the settlement of microorganisms and phytoplankton communities (Bhosle et al., 2005; Wahl, 1989). The conditioning film is composed mainly of dissolved organic materials from the surrounding medium, such as proteins and carbohydrates (Bhosle et al., 2005). Conditioning films may alter the surface properties that enable the attachment of microorganisms, primarily bacteria (Bhosle et al., 2005). After settlement, bacterial communities produce extracellular polymeric substances, which embed the cells in the biofilm and act as a glue for the firm attachment on surfaces (Stoodley et al., 2002). Photosynthetic organisms, particularly diatoms, are the secondary colonizers on surfaces in the sea (Anil et al., 2006) and considered the earliest photoautotrophs, along with cyanobacteria, to input energy in the biofilms (Nagarkar et al., 2004; Roeselers et al., 2008; Rossi and De Philippis, 2015).

Biofilm development on hard substrata in the sea, including natural surfaces, has substantial ecological significance (Sawall et al., 2012). For instance, these biofilms are important for the recruitment of benthic organisms by providing conditioned surfaces for larval settlement and metamorphosis (Dobretsov, 2009; Patil and Anil, 2005; Sneed et al., 2014; Wang et al., 2012; Whalan and Webster, 2014; Wieczorek and Todd, 1998). Biofilms are also considered to be the main source of primary production in coastal ecosystems (Thompson et al., 2004) and provide many ecosystem services, such as nutrient recycling and degradation of pollutants (Decho, 2000; Passarelli et al., 2015). Biofilm development on hard substrata may show temporal and spatial variation (Jenkins and Martins, 2010) due to changes in physical, chemical and biotic factors of the aquatic environment (Donlan, 2002; Guo et al., 2017; McElroy et al., 2016). Nutrient availability is one of the major controlling factors for the development of biofilms on hard substrata (see review: Costerton et al., 1995).

Due to their sensitivity to environmental conditions, biofilms are considered indicators for assessing the health of the environment (Baragi and Anil, 2016; Passarelli et al., 2015). As biofilm structure responds to changes in environmental conditions, it may also affect the settlement of marine invertebrate larvae (Hung et al., 2005). Many previous studies have reported on the community structure, temporal variation of conditioning film and microfouling assemblage development on different substrata submerged in marine waters (Bhosle et al., 2005; Dang and Lovell, 2000; Mitbavkar and Anil, 2008; Rampadarath et al., 2017; Satheesh and Wesley, 2010; Siboni et al., 2007; Watson et al., 2015; Wesley and Satheesh, 2009). Studies on spatial and temporal changes in biofilm community structure will improve our knowledge of biofilm community ecology in marine waters. Further, few studies in the literature relate to biofilm development on artificial substrata submerged in the Red Sea (Abdul Azis et al., 2001; Saeed et al., 2000; Zhang et al., 2014). These previous studies were mainly focused on desalination plant water intake systems. Hence, in this study, microfouling development and the seasonal changes of the biofilm community structure were assessed in the central Red Sea by submerging nylon nets. Nylon nets were selected as artificial substrates due to their wide applications in cage aquaculture. The following questions were addressed in this study: (1) How long will it take for a biofilm community to develop on artificial substratum deployed in central Red Sea coastal waters? (2) Does the biofilm community structure show any seasonal variations? (3) Do the nutrients adsorbed on the substratum have any effect on microfouling organisms, such as bacteria and diatoms? (4) Does diatom settlement on artificial substratum show temporal succession with submersion duration?

2. Material and methods

2.1. Preparation and submersion of nylon nets

Test substrata (15 cm × 15 cm) were prepared using PVC frames and nylon nets used for cage farming (black colour). Nets were submerged at a depth of 2 m at Obhur Creek (near King Abdulaziz University marine station) of the central Red Sea (north of Jeddah) region of Saudi Arabia (N21°42.562′ E039°05.764′). The PVC frames were submerged in seawater during the winter (March 2016) and summer (August 2016) seasons. The nets were washed in distilled water, dried and rinsed with 70% alcohol before submersion in the seawater (Rao, 2003; Satheesh and Wesley, 2010). Nylon nets (in replicate, $n = 5$) were retrieved from the sea after 1, 2, 3, 4 and 5 days of submersion. The nets were rinsed with filtered (Millipore, 47 μm) and sterilized (autoclaved) seawater immediately after retrieval from the sea and placed in conical flasks containing 50 ml of filtered and sterilized seawater. The conical flasks were stored in a coolbox and transported to the laboratory. In the laboratory, the biofilm that developed on the nets was removed by two methods. In the first method, the biofilm was removed using a sterile nylon brush (Wesley and Satheesh, 2009) and the scrapped biofilm assemblage was dispersed into the sterile seawater stored in the conical flask. In the second method, the nets were stored in a conical flask with a known volume of filtered seawater and the flasks were agitated by placing them in a shaker for 1 h at 300 rpm to disperse the microfouling assemblage developed on the nets. After that, the nylon nets were removed from the conical flask and the microfouling assemblage dispersed seawater was collected. The biofilm samples removed from the nylon nets using these two methods were then combined for further analysis. This microfouling assemblage dispersed water was divided into two parts; one part was used for the analysis of bacteria and diatoms and the other part was used for the analysis of nutrients.

2.2. Analysis of biofilm nutrients

The nutrient content (nitrate, nitrite, phosphate, carbohydrate and protein) analysis was carried out to analyze the temporal variability of biofilm nutrients. For an estimation of nutrients, the methods described by Wesley and Satheesh (2009) were followed. In brief, the microfouling sample dispersed seawater was filtered through a membrane filter (0.47 μm) before nutrient analysis. Total carbohydrate was determined by the phenol-sulphuric acid method (DuBois et al., 1956) using glucose as a standard. The protein content of the biofilm sample was measured by the Lowry et al. (1951) method using bovine serum albumin as is standard. For the estimation of nitrate, nitrite and phosphate the methods given by Venugopalan and Paulpandian (1989) were used.

2.3. Analysis of diatoms

The diatoms attached to the nylon nets were analyzed under a microscope (LEICA DMI 3000B) and the number of phytoplankton cells (cm^{-2} area of nylon nets) were documented using a haemocytometer. For identification of diatom species, the keys provided by Hasle and Syvertsen (1997) were used. The chlorophyll-*a* (biomass of phototrophs) in microfouling was measured after filtering the known volume of samples through GF/C glass filter (0.47 μm) paper. The filter papers were stored at −20°C before extraction using 90% acetone. The extraction was carried out overnight in a refrigerator and the chlorophyll content was measured using a spectrophotometer as previously described (Venugopalan and Paulpandian, 1989). The O.D was measured at three different wavelengths such as 630 nm, 645 nm, and 665 nm. The chlorophyll-*a* was calculated using the following formula:

$$\text{Chlorophyll-}a = 11.6\,\text{O.D}_{665} - 1.31\,\text{O.D}_{645} - 0.14\,\text{O.D}_{630}.$$

2.4. Isolation and identification of bacteria

Cultivable bacteria settled on the nylon nets were cultured using Zobell marine agar. Approximately 1 ml of dispersed biofilm was diluted (10-fold) using filtered and sterilized seawater and 0.1 ml was spread on Zobell marine agar plates. The Petri dishes were incubated at 28 ± 2°C for 48 h and the total viable colonies were counted. Bacterial colonies were selected based on colony morphology and purified by streaking on marine agar plates. The purified bacterial colonies were used for identification based on 16S rRNA gene sequencing. The bacterial genomic DNA was isolated using the InstaGene™ matrix genomic DNA isolation kit as per the protocol accompanying the kit. The isolated DNA was amplified using 16S rRNA universal primers 27F:AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT. An MJ Research Peltier Thermal cycler was used for the amplification of the DNA. PCR reactions were conducted under the following PCR conditions: Initial denaturation at 94°C for 2 min and then 35 amplification cycles at 94°C for 35 s, 55°C for 60 s and 72°C for 60 s. Final extension was at 72°C for 10 min. The DNA fragments were amplified for approximately 1400 bp using a positive control (*E. coli* genomic DNA) and a negative control in the PCR. The PCR products were purified using a Montage PCR clean-up kit (Millipore). Purified PCR products were sequenced using ABI PRISM® Big DyeTM terminator cycle sequencing kits with AmpliTaq® DNA polymerase (Applied Biosystems). Single-pass sequencing was performed using 16S rRNA universal primers 785F: GGATTAGATACCCTGGTA and 907R: CCGTCAATTCMTTTRAGTTT. Sequencing reactions were performed on an ABI 3730xl sequencer (Applied Biosystems). The resulting sequences were aligned and analyzed using NCBI blast for the identification of the bacteria. The program MUSCLE 3.7 was used for the alignment of sequences (Edgar, 2004) and the aligned sequences were cured using Gblocks 0.91b. The Tree Dyn 198.3 program was used for the construction of a phylogenetic tree (Dereeper et al., 2008).

2.5. Analysis of environmental parameters

All of the water quality parameters, such as salinity, temperature, pH, dissolved oxygen and the concentration of nutrients such as nitrite, nitrate and phosphate were analyzed at monthly intervals throughout the study using standard methods described elsewhere (Satheesh and Godwin Wesley, 2008) and the mean values for each season were calculated.

2.6. Scanning Electron Microscopic analysis (SEM) of microfouling assemblage development on nylon nets

Nylon nets were prepared as above and submerged in the Creek waters during July 2016 (summer season, experiment was conducted between 10-7-2016 and 15-07-2016) for the microfouling assemblage development. A portion of the nylon nets retrieved from the seawater each day was fixed in 2% glutaraldehyde (which was prepared in phosphate buffered saline) for 2 h. The net samples were then dehydrated through a series of ethanol gradients, starting with 20% and ending with 100% (20, 40, 60, 80 and 100%). The nets were submerged in each percentage of alcohol for approximately 30 min. Then, the nylon nets were dried by placing them on double-sided carbon tape. All samples were sputtered with 15 nm thick gold layers (JEOL JFC-1600 Auto fine coater). Finally, the nylon nets were examined with a Scanning Electron Microscope (FEI, Quanta FEG-450). The microscope was operated at an accelerating voltage of 10 kV.

2.7. Data analysis

The difference in the microfouling assemblage between two seasons (considering the abundance of bacteria and diatoms and the concentration of nutrients) was examined using a two-way ANOVA (analysis of variance), with time (days) and season (winter and summer) as factors. Correlation analysis (correlation coefficient) was also carried out using the abundance data of bacteria (CFU on culture plates) and diatoms against nylon net submersion duration [days] in winter and summer seasons. Additionally, the role of biofilm nutrients on microfouling community settlement was determined by correlation analysis between nutrient concentration in the biofilms and the abundance of microfouling communities (bacteria and diatoms) as variables. The two-way ANOVA and correlation coefficient analysis were carried out using the SPSS program. The relative abundance of dominant diatom species was calculated using the formula $(n/N) \times 100$, where '*n*' indicates the number of individuals of a particular species and '*N*' is the total number of diatoms in the biofilm.

3. Results

3.1. Environmental parameters of the creek waters

Environmental parameters of the creek waters during the study period are presented in Table 1. In general, all the parameters analyzed in this study showed higher values in the summer season except for dissolved oxygen, which was high during winter.

Table 1 Environmental parameters of the Obhur Creek during winter and summer seasons (mean ± SD).

Parameters	Winter	Summer
Temperature [°C]	26.6 ± 0.44	30.2 ± 0.68
Dissolved oxygen [mg L^{-1}]	5.66 ± 0.31	4.92 ± 0.24
pH	8.00 ± 0.10	8.3 ± 0.09
Salinity	40 ± 0.89	41 ± 0.63
Nitrite [μg l^{-1}]	0.0007 ± 0.0003	0.003 ± 0.002
Nitrate [μg l^{-1}]	0.7014 ± 0.0243	1.62 ± 0.35
Phosphate [μg l^{-1}]	0.0072 ± 0.0014	0.012 ± 0.005

3.2. Nutrient concentration of biofilm

In the microfouling assemblage developed on nylon nets, the concentration of nutrients such as nitrate, nitrite, phosphate, carbohydrate and protein showed variation with submersion time during winter and summer (Fig. 1). The nutrient concentration on nylon nets was high during the summer season. Among the nutrients, protein concentration was highest in both winter and summer seasons, followed by carbohydrate concentration (Fig. 1). Two-way ANOVA results showed significant seasonal variation in the concentration of nutrients on nylon nets (Table 2). While nitrate, nitrite and phosphate concentrations showed significant variation in relation to submersion period, protein and carbohydrate concentrations did not show a significant difference between submersion days (Table 2).

3.3. SEM analysis

Scanning electron microscope (SEM) analysis of the nylon nets submerged during the summer season is given in Fig. 2. SEM microphotographs showed progressive growth of microfouling assemblages on nylon nets in each submersion time period. The net panel submerged for 5 days was clogged with a microfouling assemblage. Bacteria and phytoplankton communities were clearly visible in the SEM microphotographs (Fig. 2).

3.4. Bacteria and photosynthetic organisms on nylon nets

The bacterial abundance (CFU on culture plates) showed an increase on nylon nets with submersion time during the winter ($r = 0.765$, $P < 0.001$) and summer seasons ($r = 0.847$, $P < 0.001$) (Fig. 3). The bacterial abundance on nylon nets showed a positive correlation during the winter and summer seasons with all of the nutrients analyzed in this study (Table 3). The two-way ANOVA revealed a significant difference in bacterial colonies on nylon nets in relation to submersion duration and season (Table 2). The 16S rRNA gene sequence results revealed the presence of four dominant bacterial strains (based on the number of colonies in each observation) on nylon nets during the study period (Fig. 4). The identified strains were *Pseudoalteromonas shioyasakiensis* (NCBI GenBank accession number: KY224086), *Planomicrobium* sp. (NCBI GenBank accession number: KY224087), *Vibrio harveyi* (NCBI GenBank accession number: KY266820) and *Pseudoalteromonas rubra* (NCBI GenBank accession number: KY266819).

The concentration of chlorophyll-*a* in the biofilm showed fluctuations with submersion duration and season. In winter, a chlorophyll-*a* content of 0.013 mg cm^{-2} was observed on nylon nets submerged for one day. However, the values declined after 2–4 days of submersion (Fig. 1). The nylon nets observed after 5 days of submersion during the winter season recorded the highest chlorophyll-*a* content, 0.038 mg cm^{-2}. In contrast, during the summer season, the chlorophyll-*a* content of nylon nets submerged for one day was 0.006 mg cm^{-2} and reached 0.015 mg cm^{-2} after 5 days of submersion. In general, the results of this study showed that the biomass of phototrophs in the biofilm developed on nylon nets was high during winter and low in summer (Fig. 1). The two-way ANOVA showed a significant variation in

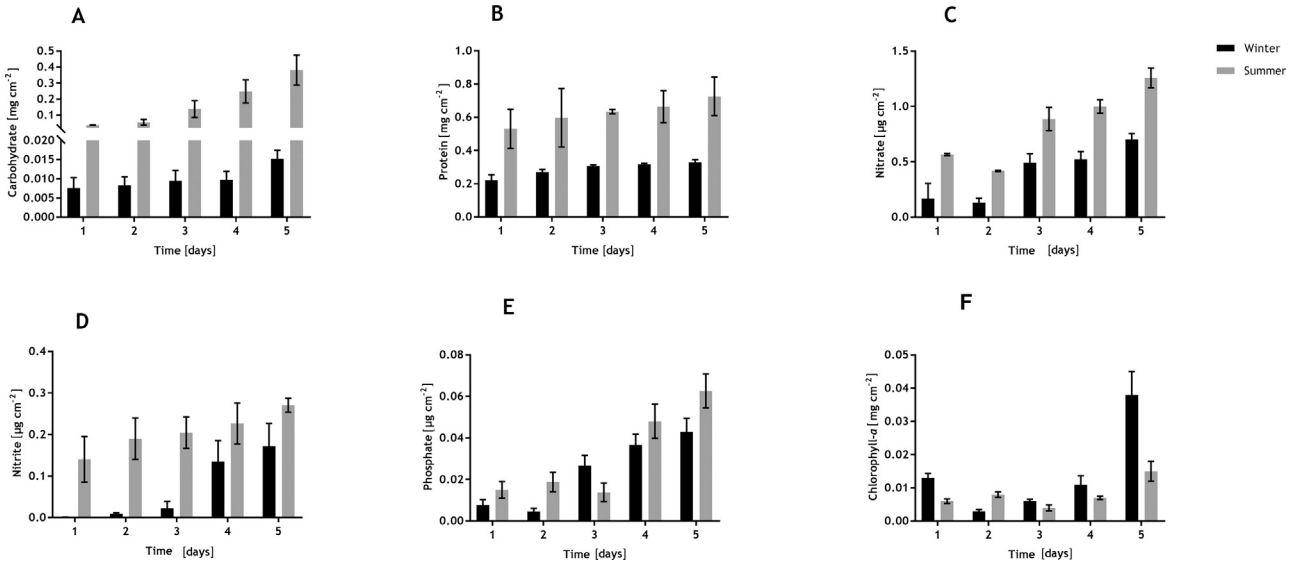

Figure 1 Seasonal changes in the concentration of nutrients and chlorophyll-*a* in the biofilm developed on nylon nets (mean ± SD, $n = 5$). Carbohydrate (a), protein (b), nitrate (c), nitrite (d), phosphate (e), chlorophyll-*a* (f).

Table 2 Two-way ANOVA (analysis of variance) of the abundance of bacteria and diatoms and concentration of nutrients in the biofilm developed on nylon nets. Nylon nets submersion duration (time, 5 days) and season (winter and summer) were considered as factors for ANOVA. Significant = $P < 0.05$. Not significant = $P > 0.05$.

Source of variation	df	Bacteria		Diatoms		Nitrate		Nitrite		Phosphate		Protein		Carbohydrate		Chlorophyll-*a*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Time [days]	4	34.06	0.000	211.16	0.000	117.19	0.000	11.7	0.000	52.13	0.000	1.74	0.160	2.064	0.104	254.64	0.000
Season	1	100.64	0.000	1054.3	0.000	441.26	0.000	181.01	0.000	12.90	0.001	526.17	0.000	216.95	0.000	155.060	0.000
Time*season	4	31.40	0.000	214.18	0.000	1.95	0.121	3.43	0.017	6.45	0.000	1.38	0.257	2.063	0.104	86.19	0.00

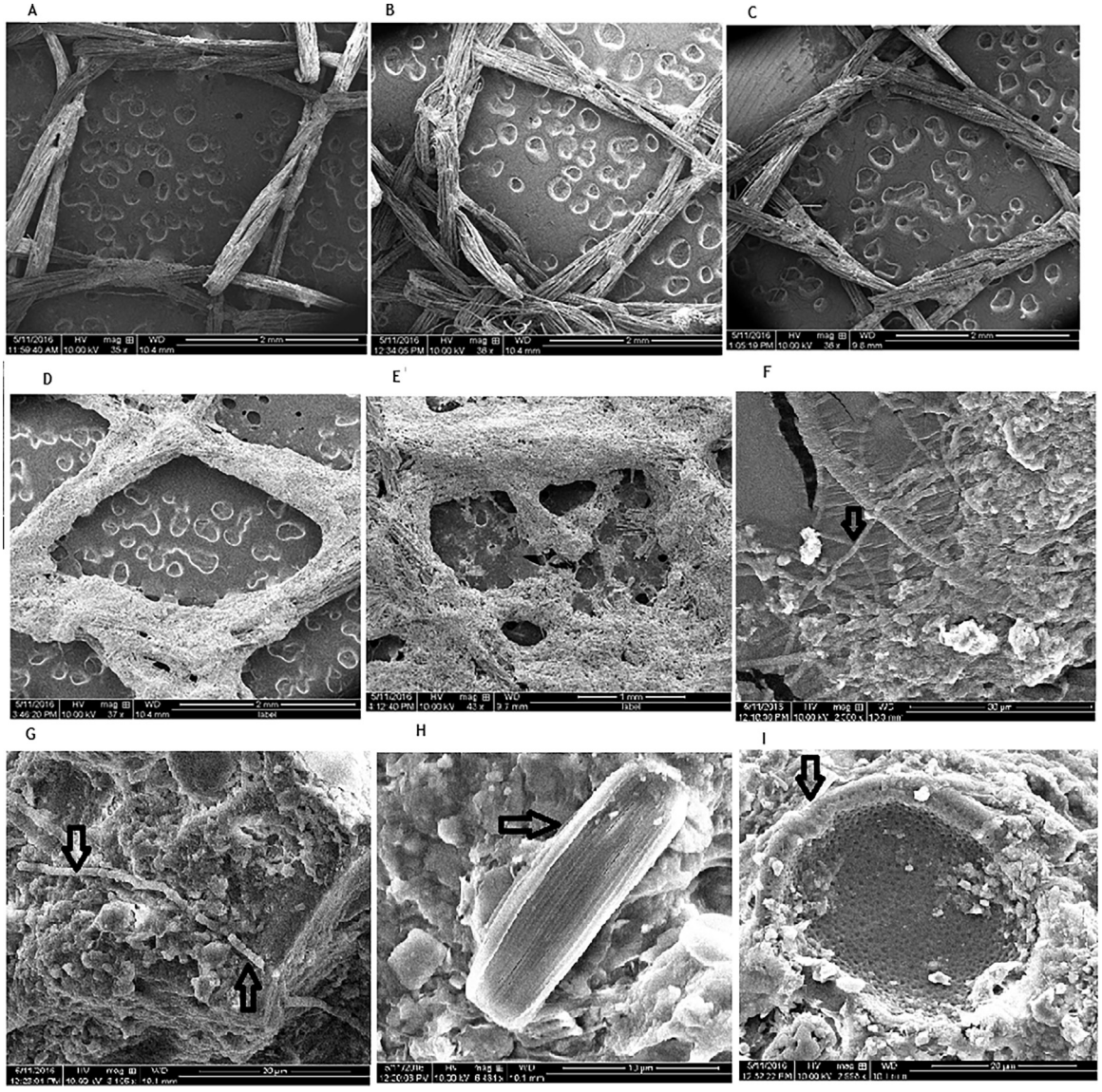

Figure 2 Scanning Electron Microscope photographs of biofilm development on nylon nets submerged in central Red Sea waters. Nylon net submerged for one day (a), nylon nets submerged for 2 days (b), nylon nets submerged for 3 days (c), nylon nets submerged for 4 days (d), nylon net submerged for 5 days (e), nylon net submerged for 24 h (one day) showing the settlement of bacteria (f and g), nylon net submerged for 24 h (one day) showing the settlement of diatoms (h and i).

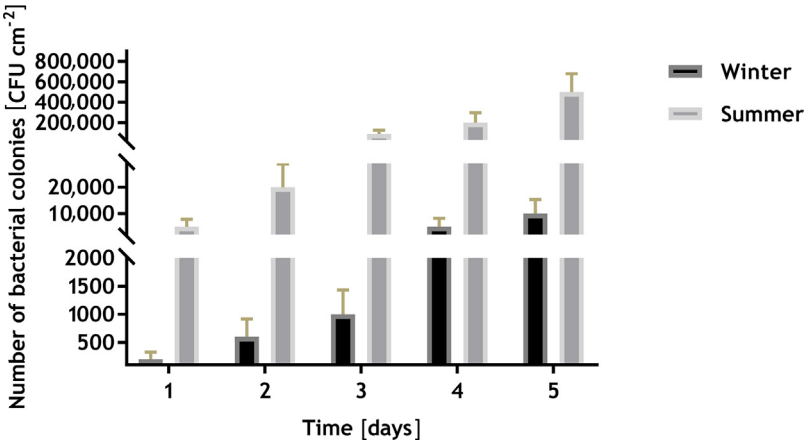

Figure 3 Colonization of bacterial communities on nylon nets submerged for 5 days during the winter and summer seasons in the central Red Sea (mean CFU ± SD, $n = 5$).

Table 3 Correlation between the abundance of microfouling organisms and concentration of nutrients in the biofilm. The abundance of diatoms and bacteria (bolded in the first column of the table) was correlated with the other parameters. $P < 0.05$ = significant, $P > 0.05$ = not significant.

Winter		*P*	Summer		*P*
Parameters	Correlation coefficient (*r*)		Parameters	Correlation coefficient (*r*)	
Total number of diatoms vs			**Total number of diatoms vs**		
Total number of bacteria	0.824	0.000	Total number of bacteria	−0.103	0.623
Nitrite	0.383	0.044	Nitrite	0.152	0.467
Nitrate	0.897	0.005	Nitrate	−0.115	0.492
Phosphate	0.810	0.000	Phosphate	0.083	0.694
Protein	0.685	0.007	Protein	−0.115	0.557
Carbohydrate	0.673	0.000	Carbohydrate	−0.115	0.218
Chlorophyll-*a*	0.704	0.000	Chlorophyll-*a*	0.038	0.066
Total number of bacteria vs			**Total number of bacteria vs**		
Nitrite	0.444	0.026	Nitrite	0.523	0.007
Nitrate	0.819	0.000	Nitrate	0.619	0.000
Phosphate	0.688	0.001	Phosphate	0.829	0.000
Protein	0.532	0.002	Protein	0.619	0.005
Carbohydrate	0.574	0.006	Carbohydrate	0.619	0.007
Chlorophyll-*a*	0.593	0.000	Chlorophyll-*a*	0.935	0.003

chlorophyll-*a* content in biofilm between winter and summer seasons (Table 2).

3.5. Settlement of diatoms on nylon nets

The diatom communities in the biofilm on the nylon nets consist of 13 species submerged during the winter season and 9 species in summer (Fig. 5, Table 4). Season-wise analysis of diatom communities showed the abundance of *Navicula* spp., *Cylindrotheca* spp., *Licmophora* spp. and *Nitzschia* spp. on nylon nets submerged during the winter season (Fig. 5, Table 4). During the summer season, *Nitzschia* spp., *Pleurosigma* spp., *Navicula* spp., *Concinodiscus* spp., *Synedra* spp. and *Gyrosigma* spp. were the abundant diatoms. Diatoms such as *Cylendrotheca* spp., *Diploneis* spp., *Amphiprora* spp. and *Cymbella* spp. were not observed on the nylon nets during the summer season (Table 4).

Diatom abundance was higher during winter than summer (Fig. 6). During the winter season, *Navicula* spp. was the most abundant diatom, with a maximum of 1221 individual cm^{-2} after 4 days (Fig. 5). *Cylindrotheca* spp. was abundant during the later stages of microfouling development on nylon nets (Fig. 5). During the summer season, *Nitzschia* spp. and *Pluerosigma* spp. were more dominant than the other species on nylon nets, with a maximum of 135 and 85 individual cm^{-2}, respectively, after 3 days (Fig. 5). In general, diatom settlement (total numbers) on nylon nets showed an increase with submersion time in both winter ($r = 0.896$, $P < 0.001$) and summer ($r = 0.227$, $P > 0.05$). Though phytoplankton abundance increased with submersion time during summer, this increasing trend was not observed on panels submerged for 4 and 5 days (Fig. 6). The relative abundance of dominant diatom species, such as *Navicula* spp., *Nitzschia* spp., *Cylindrotheca* spp. and *Pluerosigma* spp. is provided in Fig. 7. The

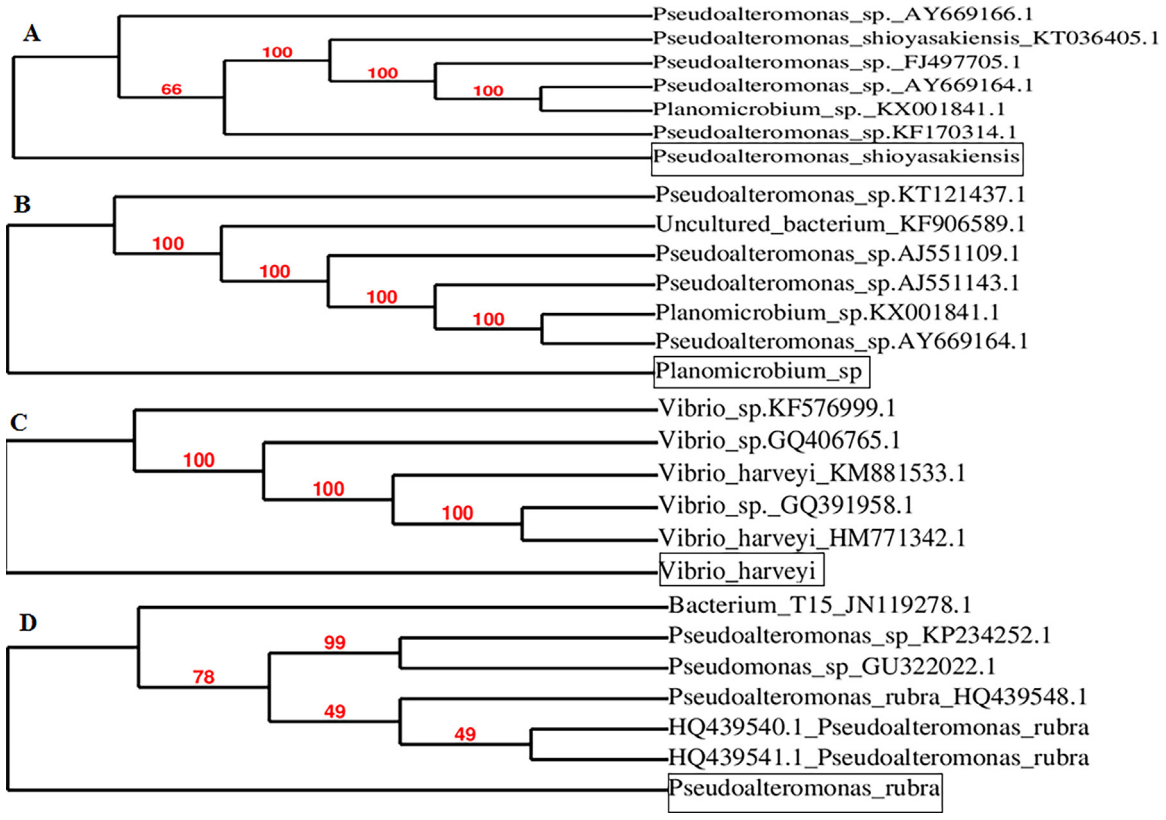

Figure 4 Phylogenetic tree of bacterial strains colonized on nylon nets submerged in the central Red Sea. The phylogenetic tree was constructed based on 16S rRNA gene sequences. *Pseudoalteromonas shioyasakiensis* (KY224086) (a), *Planomicrobium* sp. (KY224087) (b), *Vibrio harveyi* (KY266820) (c), *Pseudoalteromonas rubra* (KY266819) (d). The strains without accession numbers in the figure are recorded during this study.

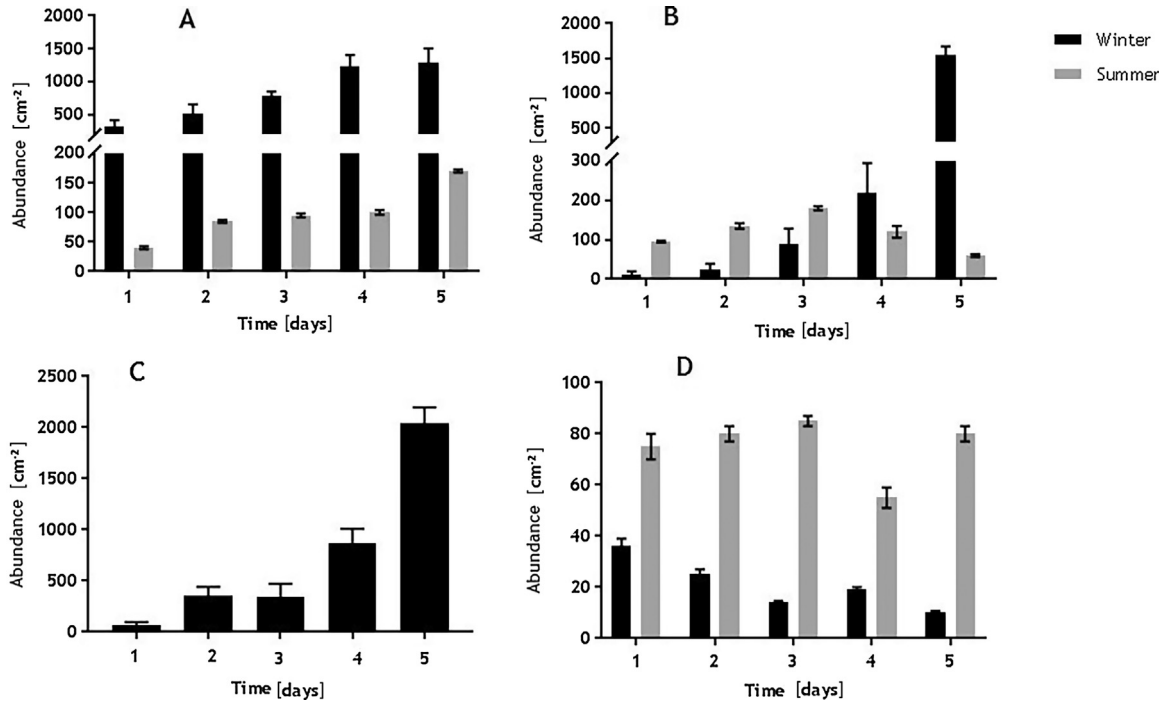

Figure 5 Abundance (mean ± SD, $n = 5$) of four dominant diatoms in the biofilm developed on nylon nets during the winter and summer seasons. *Navicula* spp. (a), *Nitzschia* spp. (b), *Cylindrotheca* spp. (c), *Pluerosigma* spp. (d).

Table 4 Density of diatom species which were not abundant in the biofilm developed on nylon nets during the winter and summer seasons (mean ± SD, $n = 5$).

Phytoplankton	Winter					Summer				
Submersion duration [days]→	1	2	3	4	5	1	2	3	4	5
Licmophora spp.	135 ± 70.92	233 ± 95.90	177 ± 53.86	175 ± 61.55	257 ± 24.73	0	11 ± 2.35	14 ± 4.18	20 ± 4.12	10 ± 1.87
Concinodiscus spp.	35 ± 10.04	75 ± 54.63	57 ± 12.17	91 ± 25.14	49 ± 18.59	30 ± 6.29	45 ± 5.87	60 ± 3.08	50 ± 5.87	40 ± 7.71
Cyclotella spp.	11 ± 13.61	4 ± 2.73	0	7 ± 2.76	0	0	0	0	4 ± 1.87	0
Pseudo-Nitzschia spp.	2 ± 4.97	2 ± 1.32	4 ± 1.56	9 ± 4.43	0	5 ± 1.87	6 ± 2.92	4 ± 1.23	9 ± 1.87	0
Gyrosigma spp.	13 ± 14.49	0	4 ± 1.66	56 ± 8.39	229 ± 28.98	25 ± 5.87	35 ± 5.87	30 ± 3.08	0	0
Diploneis spp.	2 ± 4.97	0	0	13 ± 2.04	53 ± 25.34	0	0	0	0	0
Synedra spp.	55 ± 49.18	71 ± 18.59	196 ± 34.21	442 ± 122.32	1457 ± 191.55	25 ± 3.08	30 ± 6.29	20 ± 2.74	15	10
Amphiprora spp.	43 ± 21.38	22 ± 7.86	73 ± 12.25	196 ± 81.88	465 ± 49.11	0	0	0	0	0
Symbella spp.	0	0	0	4 ± 1.10	13 ± 1.02	0	0	0	0	0
Amphora spp.	0	0	0	0	0	0	0	8	5	4

settlement of these diatom communities also showed a significant difference in relation to nylon nets submersion duration and season (Table 5). The relative abundance of *Navicula* spp. was as high as 36% and was 26% during the winter season (1-day-old nylon nets), while for *Nitzschia* spp., the relative abundance was 35.08% (after 3 days) in the summer season. The relative abundance of *Cylindrotheca* spp. showed a maximum of 24.38% (after 5 days) in winter, while this diatom was not observed during the summer season. *Pluerosigma* spp. showed a maximum relative abundance of 24.27% during the summer season. The relative abundance analysis showed a succession of diatom density, as *Navicula* spp. was dominant for up to 4 days while *Cylindrotheca* spp. was the dominant diatom on nylon nets submerged during the winter season.

The total density of diatoms showed significant variation in relation to submersion time ($F = 211.16$, $P < 0.01$) and season ($F = 1054.25$, $P < 0.01$) (Table 2). Correlation analysis revealed a positive correlation between the abundance of diatoms and bacteria ($r = 0.824$, $P < 0.001$) during the winter season. Additionally, the abundance of diatom on nylon nets showed a positive correlation with chlorophyll-*a* ($r = 0.704$, $P < 0.001$), and nutrients such as nitrate ($r = 0.897$, $P < 0.001$), nitrite ($r = 0.383$, $P < 0.05$), phosphate ($r = 0.810$, $P < 0.001$), protein ($r = 0.685$, $P < 0.001$) and carbohydrate ($r = 0.673$, $P < 0.001$) during the winter season (Table 3). However, the abundance of diatoms did not show any significant relationship with bacteria and nutrients during the summer season (Table 3).

4. Discussion

The biofilm that forms on substrates submerged in marine waters consists of organic and inorganic materials from the surrounding environment (Bhosle et al., 2005; Jain and Bhosle, 2009), in addition to microorganisms. The results of the present study indicated that protein was the major organic component in the biofilm developed on nylon nets submerged in the central Red Sea. The carbohydrate concentration was very low during the winter season. The concentration of nitrate, nitrite and phosphate in the biofilm also showed seasonal variations. The observed seasonal variations may be mainly due to the concentration of these nutrients in the coastal waters. In these waters (nylon nets submersion medium), the nutrient concentrations (data was only available for nitrate, nitrite and phosphate) were high during the summer season. As the nutrient content of the Creek waters was high during summer, accumulation of nutrients on net panels also showed higher values during this period. Previous studies by Wesley and Satheesh (2009) also confirmed that the nutrient concentration of biofilms depends on the nutrient load of the submersion medium.

The biofilms are reported to induce the attachment of microorganisms and macroorganisms (Bakker et al., 2004; Freckelton et al., 2017; Hadfield, 2011; Jain and Bhosle, 2009; Li et al., 2014; Qian et al., 2007; Whalan and Webster, 2014; Yang et al., 2016) and paves the way for the development of a biofouling assemblage on hard surfaces (Bhosle et al., 1990; Hadfield et al., 2014). In this study, the concentrations of all of the nutrients showed significant positive correlations with bacterial colonies (CFU on culture plates)

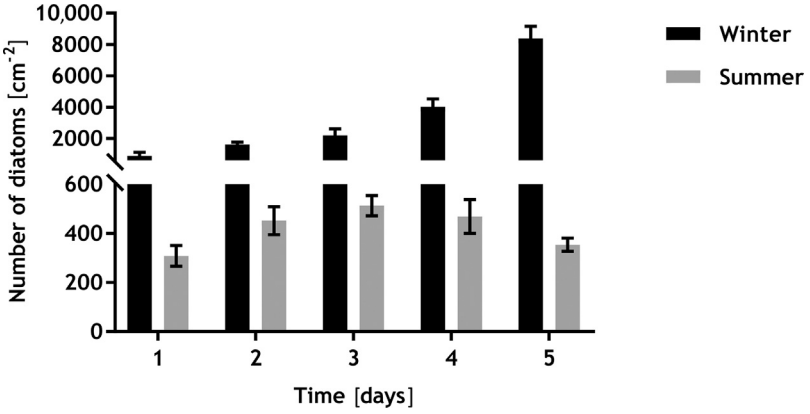

Figure 6 Total number (mean ± SD, $n = 5$) of diatoms settled on nylon nets during the winter and summer seasons.

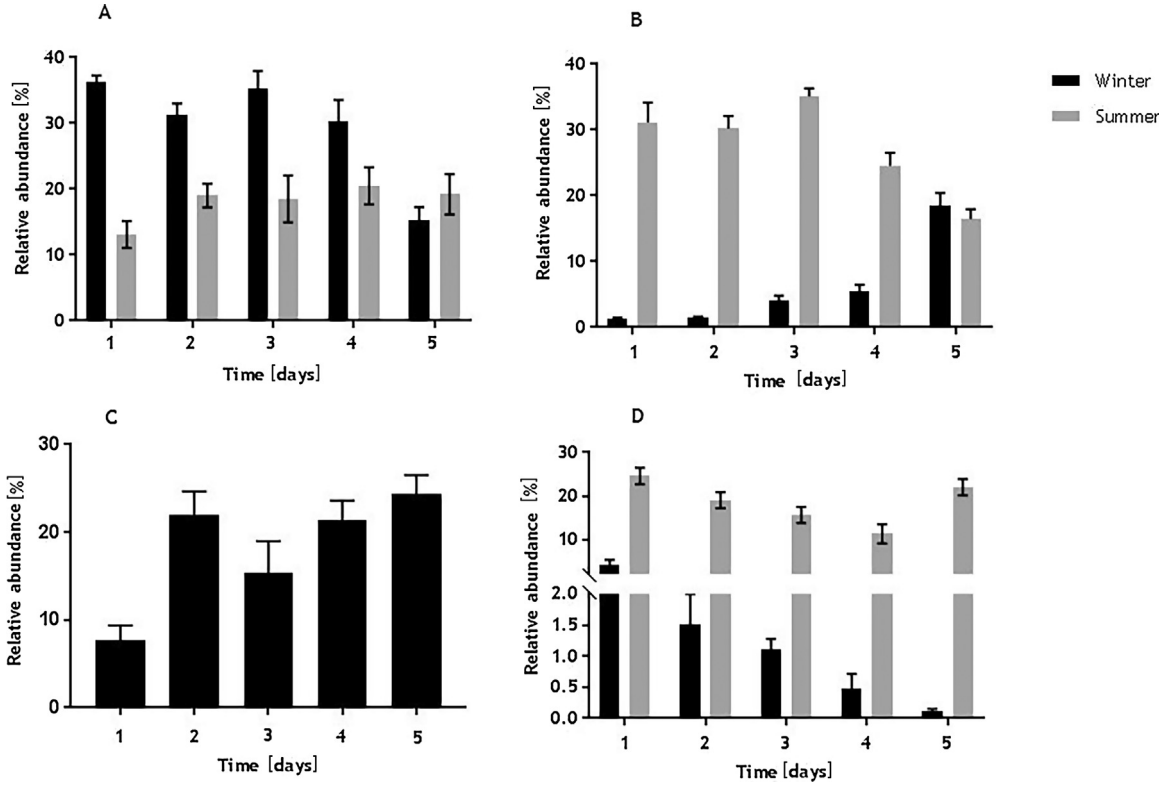

Figure 7 Relative abundance of dominant diatom species in the biofilm. *Navicula* spp. (a), *Nitzschia* spp. (b), *Cylindrotheca* spp. (c), *Pluerosigma* spp (d).

Table 5 Two-way ANOVA (analysis of variance) of the abundance of four dominant diatoms in the biofilm developed on nylon nets. Nylon nets submersion duration (time, 5 days) and season (winter and summer) were considered as factors for ANOVA. Significant = $P < 0.05$. Not significant = $P > 0.05$.

Source of variation	df	*Navicula* spp.		*Nitzschia* spp.		*Cylindrotheca* spp.		*Pleurosigma* spp.	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Time [days]	4	102.90	0.000	51.93	0.000	292.57	0.000	19.79	0.000
Season	1	3175.8	0.000	1396	0.000	9499.6	0.000	833.79	0.000
Time*season	4	133.05	0.000	49.54	0.000	292.57	0.000	67.18	0.000

during winter and summer. These adsorbed nutrients may change the surface properties that enable the adhesion of microorganisms (Bakker et al., 2004; Fletcher and Marshall, 1982). The correlation analysis revealed a positive relationship between nutrients and diatoms during the winter season. However, in the summer season, there was no significant relationship between nutrients and diatom abundance on nylon nets. This showed that the seasonal factor, rather than adsorbed nutrients, plays an important role in the settlement of diatoms on surfaces.

Scanning Electron Microscopy visualization method used in this study gave better insight into the temporal sequence of microfouling development on nylon nets submerged in the marine waters. The SEM microphotographs of nylon nets confirmed the findings of previous studies which reported the settlement of bacteria and diatoms on artificial substrates submerged for a period of 24 h in the seawater (Mejdandžić et al., 2015; Satheesh and Wesley, 2010; Siboni et al., 2007). Bacterial communities form an important part of the biofilms developed on hard substrata submerged in marine waters (see reviews: Hadfield, 2011; Salta et al., 2013). In this study, the total number of cultivable bacteria (bacterial colonies on culture plates) on nylon nets showed an increase with submersion time during the winter and summer seasons. Additionally, the abundance of bacteria in biofilm was greater during summer than winter. The bacterial communities attached to the nylon nets revealed the presence of 4 dominant species. The genus *Pseudoalteromonas* is commonly found in marine biofilms on living and non-living surfaces (see review: Satheesh et al., 2016). Another prominent bacterial strain, *Vibrio harveyi*, has also been reported in marine biofilms (Henares et al., 2012) and is commonly used as a model organism for studying cell-cell communication (quorum sensing) in biofilms (Henke and Bassler, 2004). While most studies have confirmed the dominance of Alphaproteobacteria on marine biofilms (Salta et al., 2013), the bacterial strains identified in this study were Gammaproteobacteria and Firmicutes. Further, Al-Awadhi et al. (2013) highlighted the variations in bacterial identities between culture-dependent and culture-independent methods. Hence, the observed variations in bacterial identities may be due to the culture-dependent method followed in this study. In general, Gammaproteobacteria and Alphaproteobacteria have been identified as the primary colonizing bacterial groups on artificial substrata submerged in marine waters (Chung et al., 2010; Dobretsov et al., 2013; Webster and Negri, 2006).

The results of the present study showed that diatoms constitute an important part of the microfouling assemblage developed on net panels submerged in the Obhur Creek waters of the central Red Sea. The abundance of most of the diatom species on nylon nets was higher during winter than summer. The diatom community was mainly dominated by pennate diatoms, such as *Navicula* spp. and *Nitzschia* spp., during this study. Several previous studies also reported the dominance of pennate diatoms in marine biofilms (Mitbavkar and Anil, 2000; Patil and Anil, 2005; Satheesh and Wesley, 2012; Wetherbee et al., 1998). The abundance of pennate diatoms on artificial substrata may be due to the existence of a raphe along the length of the frustules that helps the pennate diatoms to attach to solid surfaces (Anil et al., 2006). *Cylindrotheca* spp. and *Pluerosigma* spp. were also abundant in the biofilm developed on the nylon nets.

The abundance of organisms in biofilms is influenced by various factors. The results clearly showed seasonal changes in microfouling communities, such as bacteria and diatoms, on nylon nets submerged in the central Red Sea coast. Seasonal variations in micro- and macrofouling community settlement on hard surfaces have been reported from tropical coastal waters by many previous researchers (Satheesh and Godwin Wesley, 2008; Satheesh and Wesley, 2011, 2012; Sawall et al., 2012; Yang et al., 2015). There are several explanations for the observed seasonal variations in microfouling community development on nylon nets. Mainly, seasonal changes in the aquatic medium may affect the development of biofilm on hard surfaces (Donlan, 2002). Further, environmental parameters such as temperature and nutrient content of the coastal waters showed considerable variations between two seasons. Other factors such as water current and wave action may also affect the settlement of microfouling assemblages. In the central Red Sea, wave action was high during winter and low during summer (Fery et al., 2015; Ralston et al., 2013). The abundance of diatoms was high during winter and low during summer but the bacterial count and nutrient concentration of the biofilm revealed an opposite trend (high in summer and low during winter). Additionally, in summer, the wind direction is northwest, driving the surface water towards the south for approximately four months at a velocity of 15—20 cm per sec. During winter, the direction is reversed, resulting in the inflow of water from the Gulf of Aden into the Red Sea (Omer, 2010). Previous studies also noted that the settlement of diatoms on hard surfaces in marine waters is affected by water velocity, size, immigration and the reproductive rate of the organisms in a specific region (Johnson et al., 1997; Satheesh and Wesley, 2012).

The abundance of diatoms in the water column may also impact their settlement on substrata. In the present study, the distribution of diatoms in the study area was not considered. However, a review of available literature shows a pronounced seasonality in the distribution phototrophic organisms the Red Sea. For instance, Raitsos et al. (2013) revealed an elevated chlorophyll-*a* content during winter and a low content in summer in the Red Sea. Previous studies in Obhur Creek (the present study area) also revealed the presence of diatoms such as *Cosinodiscus* sp. and *Nitzchia closterium* throughout the year and the absence of *Navicula* spp. during summer (Khomayis and Al-Harbi, 2003). This indicates that the abundance of diatom species in the microfouling communities in this study may mainly depend on the diatom distribution in the surrounding coastal waters, along with the environmental factors discussed earlier.

Microfouling communities interact with each other, and there may exist a strong positive or negative relationship between the organisms. In this study, the abundance of bacteria and diatoms showed a strong positive relationship during the winter season and a weak negative correlation in the summer season. Understanding the interactions between organisms settled on artificial substratum is important, as these primary colonizers (like bacteria and diatoms) are believed to induce or inhibit the settlement of marine invertebrate larvae (Hadfield and Paul, 2001; Huang et al., 2012; Jin and Qian, 2005). Due to their settlement-inhibiting or -inducing effects on marine invertebrate larvae and macroalgal spores, the marine biofilms may also influence the

structure and functioning of benthic communities in marine ecosystems (Hadfield, 2011; Russell et al., 2013).

In conclusion, bacteria and diatoms were the primary colonizers on the artificial substratum submerged in the central Red Sea. While there was a definite correlation between nutrient concentration and bacterial abundance during both seasons, this relationship was not observed for diatoms. Additionally, the interaction between microfouling organisms, such as bacteria and diatoms, showed temporal variability, highlighting the role that environmental conditions play. The results of the present study also revealed a significant seasonal variation in the abundance of microfouling organisms and the accumulation of nutrients on nylon nets.

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