J. Indian Inst. Sci., Sept.–Oct. 1999, **79**, 371–389.

Some observations on biofilm formation in two different aquatic systems

T. S. RAO*, V. P. VENUGOPALAN*, R. P. GEORGE** AND K. V. K. NAIR†
*Water & Steam Chemistry Laboratory, **Corrosion Science & Technology Division, Metallurgy & Materials Group, Bhabha Atomic Research Centre Facilities, IGCAR Campus, Kalpakkam 603 102, Tamil Nadu
†National Institute of Ocean Technology, Narayanapuram, Pallikaranai Post, Chennai 601 302, Tamil Nadu
Fax: +91 4114 40396 & 40397, email: subbarao@igcar.ernet.in

Abstract

Time course studies on biofilm formation were carried out in two different aquatic systems, viz. freshwater impoundment and in an estuary. Various physical, chemical and biological parameters were analyzed. In the freshwater system biofilm formation was also studied both in photic and aphotic conditions. Biofilm thickness increased from 52 to 128 μm in the photic environment and from 17 to 30 μm in the aphotic environment during 24–120 h of exposure. Biofilm biomass, chlorophyll a and other biochemical constituents were higher under illuminated conditions. Diatom numbers, as could be expected, were higher on the photic panels. In the estuarine biofilm, thickness, turbidity and biomass showed similar trends of temporal distribution. The biofilm nutrient levels were 100–1000 times higher than those in the ambient water. In the freshwater, biofilm nutrients concentration factors were consistently higher in aphotic than in photic conditions. Diatom numbers and hexose sugar content of the biofilm showed very good correlation ($r = 0.97$ and $p = 0.0001$). Anaerobic bacteria such as nitrate and sulfate reducers were observed within 24 h of biofilm formation. Denitrification phenomena was observed up to 96 h of biofilm growth.

Keywords: Biofilm, freshwater, photic, aphotic, estuarine, denitrification.

1. Introduction

Biofilms develop on all solid surfaces exposed to aquatic environments. Generally, biofilm is a complex functional consortium made up of microorganisms and extracellular polymer matrix, organic detritus and inorganic solids.[1] Investigations by many workers have highlighted problems involved in the study of biofilm formation, such as difficulties in duplicating biofilm development under laboratory conditions.[2, 3] Considerable amount of literature on biofilm characterization in freshwater systems has also been reported.[4–8] Most of the above-mentioned studies have attempted to characterise biofilms with respect to limited number of parameters such as microfauna associated with biofilms or biochemical composition. In the present study, a more comprehensive approach was taken to characterise biofilm in terms of their physical, chemical, biochemical and microbiological parameters. In order to have a comparative understanding of biofilm formation, biofilms developed in photic (naturally illuminated condition) and aphotic (like the cooling-water conduits) conditions in a freshwater system were studied. Furthermore, an estuarine environment was also chosen to explicate the influence of saline environment on biofilm development.

*For correspondence.

2. Materials and methods

Plexiglass panels ($15 \times 10 \times 0.3$ cm and $7 \times 3 \times 0.3$ cm) were suspended using a stainless steel frame at a depth of 1.5 m in the Sadras–Edaiyur estuary. Before immersion, the panels were washed thoroughly, dried and rinsed with 70% ethyl alcohol. They were retrieved after 24, 48, 72, 96 and 120 h of immersion to study the sequential development of biofilm. Samples of biofilm as well as of ambient water were simultaneously collected for analyses. In the laboratory, the retrieved panels were carefully rinsed with filtered (0.2 μm, Millipore) and autoclaved (120°C for 10 min) estuarine water. Wet biofilm thickness was determined microscopically (Nikon Optiphot) using smaller coupons.[9] Thickness measurements were made at about 25 random points in order to get a reasonably accurate mean value. The biofilm was scraped from the larger panels using a sterile nylon brush and the material was dispersed in 100 ml of filter-sterilised estuarine water. Water-quality parameters were analyzed as per standard methods.[10] The scraped biofilm suspension was filtered (0.2 μm, Millipore), the filtrate made up to a known volume using demineralized water and used for nutrient (nitrite, nitrate, phosphate and silicate) analysis, carried out using standard methods.[10] Biofilm nutrient values are expressed as mg atom per liter of biofilm volume to facilitate comparison with ambient water whose values are expressed as μg at l^{-1}. Filtered estuarine water (blank) was also analyzed for all the relevant parameters and the values were subtracted from biofilm values. Biofilm volume was calculated by multiplying the mean wet biofilm thickness by area.

To study biofilm formation in freshwater, two sets of panels were used, one exposed to natural illumination and the other in a darkened chamber. The chamber was made of stainless steel frame ($40 \times 30 \times 30$ cm), the sides and top of which were sealed with 10-mm-thick plexiglass sheets and painted black; the bottom was open to facilitate free exchange of water. The panels were suspended from stainless-steel hooks attached to the top of the chamber. The panels were exposed in a freshwater reservoir which receives subsoil water from Palar river. Biofilm samples as well as samples of ambient water were simultaneously collected for analyses of various parameters as described earlier. Water-quality parameters of the reservoir water were analyzed as per standard methods.[11] The scraped biofilm suspension was filtered (0.2 um, Millipore) and the filtrate was again made up to a known volume (250 ml) and used for nutrient analysis (nitrite, nitrate, phosphate and silicate).

In general, methods followed by other workers[2, 3, 5, 12] were used for different biochemical and biological analyses. Pre-ignited (450°C, 4h) filter papers (Whatman GF/C) were used to filter samples for biomass and particulate organic carbon (POC) estimation. Diatoms were counted using haemocytometer. Counts of culturable heterotrophic bacteria were made using standard microbiological methods.[13] Sulfate-reducing bacteria (SRB) were assayed using the Postgate medium,[14] the inoculated plates were incubated in anaerobic jars, SRB colonies could be detected after 96 hours of incubation. *Pseudomonas* sp. were assayed using King's B medium.[15] Nitrate-reducing bacteria (NRB) were counted on a defined medium consisting of potassium nitrate with sodium citrate as carbon source and traces of glucose (0.01%) to stimulate growth; pH of the medium was adjusted to 7.5. Before inoculation, the dissolved oxygen was scrubbed with inert gas (argon). Culturable aerobic heterotrophic bacteria (CAHB) were detected using half-strength ZoBell medium enriched with glucose (0.1%) The bacterial isolates were identified as per standard procedures.[16]

Proteins and carbohydrates (as hexose sugars) were analyzed.[17, 18] Lipids were extracted and quantified following the procedure reported by Rao and Harbola.[19] Statistical precision of various biofilm parameters assayed in triplicate showed that the mean coefficient of variation was <10.

3. Results

Data on water-quality parameters and their range during the course of this study are presented in Table I. Table IIA provides the nutrient concentration both in photic and aphotic biofilm. Figures 1 and 2 provide the data on various biofilm parameters assayed. Each sub-chart indicates comparative trends in both photic and aphotic environments. Table III gives a list of microflora identified on both sets of panels (photic and aphotic).

Biofilm thickness (Fig. 1a) on the photic panels increased from 52 μm after 24 h to 128 μm by 120 h, whereas on the aphotic panels the increase in thickness was much less. Similarly, biomass (Fig. 1b) of the photic biofilm was >3.5 times from 24 to 120 h, whereas in the aphotic biofilm the increase was only marginal. Total dissolved solids (TDS) in the photic biofilm showed a steady state during the period 24 to 120 h (Fig. 1c). However, aphotic biofilm showed a continuous accumulation of TDS. Diatom numbers (Fig. 1d) in aphotic biofilm was undetected up to 96 h following immersion; however, by the end of 120 h of growth the

Table I
Water-quality features of the freshwater reservoir during the course of the study

Parameter	Unit	Mean	Range
PH	pH units	8.6	(8.4 – 8.9)
Specific conductivity	μ S cm^{-1}	295	(285 – 300)
Total alkalinity	ppm $CaCO_3$	102	(98 – 105)
P Alkalinity	ppm $CaCO_3$	9.2	(7.5 – 9.4)
Total hardness	ppm $CaCO_3$	75.4	(72 – 78)
Total suspended solids	mg l^{-1}	3.5	(0.4 – 6.9)
Total dissolved solids	mg l^{-1}	198	(170 – 200)
Dissolved oxygen	mg l^{-1}	7.9	(6.2 – 9.5)
Calcium	mg l^{-1}	20.4	(10 – 27)
Chloride	mg l^{-1}	42.5	(32 – 46)
Sulfate	mg l^{-1}	10.6	(8 – 12)
Phosphate	– mg at l^{-1}	0.09	(0.01 – 0.16)
Nitrite	– mg at l^{-1}	0.005	(0.002 – 0.009)
Nitrate	– mg at l^{-1}	0.015	(0.01 – 0.25)
Silicate	– mg at l^{-1}	8.2	(5 – 12)
Chlorophyll a	μg l^{-1}	25.7	(18 – 38.8)
Particulate organic carbon	μg C l^{-1}	1062	(980 – 1187)
Total protein	mg l^{-1}	5.4	(3.6 – 7.4)
Hexose sugar	mg l^{-1}	6.2	(3.5 – 8.2)
Total lipid	mg l^{-1}	54	(44 – 62)
Diatom count	cells ml^{-1}	250	(220 – 285)
Sulfate-reducing bacteria (SRB)	cfu ml^{-1}	2	(0 – 4)
CAHB	cfu ml^{-1}	5×10^5	$(4 – 6 \times 10^5)$

Table IIA
Nutrient concentrations in freshwater biofilm

Nutrients provided	Biofilm formation under	Duration of biofilm formation (h)					Concentration factor*
		24	48	72	96	120	
Phosphate Mg at P l^{-1}	Photic	0.40	0.55	0.65	0.72	0.82	40 – 80
	Aphotic	1.62	3.30	11.80	9.20	1.10	160 – 1180
Silicate mg at Si l^{-1}	Photic	3.40	9.30	11.00	12.50	15	1 – 3
	Aphotic	20.00	30.00	35.00	66.00	143	4 – 28
Nitrate mg at N l^{-1}	Photic	0.44	0.33	0.26	0 37	0.45	13 – 22
	Aphotic	1.85	2.20	2.60	3.30	5.20	92 – 250
Nitrite mg at N l^{-1}	Photic	0.200	0.300	0.560	0.800	1.20	12 – 60
	Aphotic	0.005	0.009	0.012	0.034	0.08	60 – 335

[a] as compared to ambient water.

diatom population was ca. 50 cells cm^{-2}. The diatom count in the photic biofilm showed a sigmoid pattern. The increase after 72 h was exponential. Cyanobacteria and diatoms were the major constituents in the photic biofilm (Table III). However, a few diatom species were found in the aphotic biofilm after 96 h of incubation. SRB were assayed to follow the onset of anaerobic conditions in the biofilm. The biofilm at 24 h reached a thickness of 18 µm on aphotic panels and at this stage the SRB number was 1.4 cfu cm^{-2}. In the photic panels, after 24 h, although the biofilm thickness was 52 µm, the SRB number was only 0.7 cfu cm^{-2}. SRB numbers on the aphotic panels were generally higher than those on the photic panels (Fig. 1e). The culturable aerobic and heterotrophic bacterial count (CAHB) showed a reduction of two orders of magnitude in the photic panels from 24 to 120 h, whereas on the aphotic panels the decrease was by four orders of magnitude (Fig. 1f). The heterotrophic bacteria were dominated by exopolymer-producing species such as *Pseudomonas aeruginosa* which accounted for about 60% of the culturable population. Large number of ciliated protozoans was observed on the aphotic panels after 72 h of biofilm growth.

The concentration of nutrients in the biofilm was many times higher than that in ambient water (Table IIA). The data also showed that, in general, nutrients accumulated more in the aphotic biofilm. Silicate levels (Fig. 2d) in the photic biofilm showed a gradual increase with

Table IIB
Nutrient concentration factors* in the estuarine biofilm

Parameter	24 h	48 h	72 h	96 h	120 h
Phosphate	3500	4900	7000	7900	7900
Silicate	2363	3200	6500	3100	1600
Nitrate	162	205	136	85	120
Nitrite	330	982	1258	641	264
Ammonia	112	175	300	562	425

*as compared to ambient water.

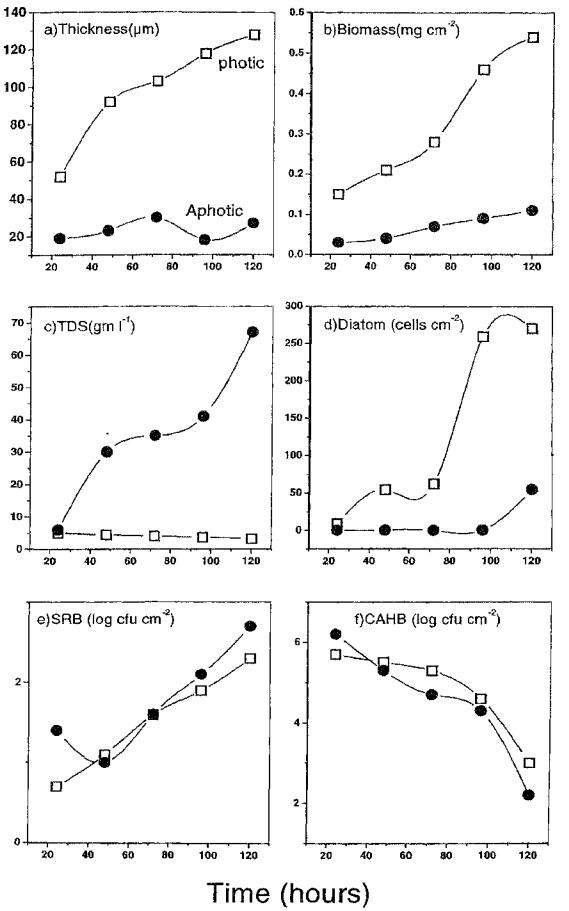

FIG. 1. Multiple chart showing the variation in the biofilm parameters (both in photic and aphotic conditions) in a freshwater impoundment, a) thickness, b) biomass, c) total dissolved solids (TDS), d) diatom count, e) SRB count and f) culturable aerobic heterotrophic bacteria (CAHB).

time. However, increase of silicate levels on aphotic panels was more pronounced. In the 24-h-old photic biofilm, a silica level of 3.4 mg at Si l^{-1} was recorded, whereas the aphotic panel had 20 mg at Si l^{-1}. Phosphates (Fig. 2c) also showed a similar trend, but decreased after 72 h in the aphotic biofilm. Nitrate levels were higher compared to the photic biofilm (Fig. 2b). However, during the 24 to 120 h of biofilm growth, nitrite levels in the aphotic biofilm (Fig. 2a) remained almost steady in photic biofilm and a linear increase in nitrite concentration was seen with time.

Particulate organic carbon (Fig. 2e) showed a steady increase from 24 to 120 h in the photic biofilms. The POC content in the aphotic biofilm was less than half of that in the photic biofilms (after 120 h). Although the chlorophyll a (Fig. 2f) values were relatively low in the initial stages in the aphotic biofilm, a ten-fold increase was observed in photic biofilm by the end of 120 h. Total protein and carbohydrate contents (Fig. 2g and h) increased with time on the photic panels. On the contrary, in the aphotic biofilm, the carbohydrate and protein values decreased with time. Lipid content (Fig. 2i) of the biofilm gradually increased in both the biofilms; however, the increase was marginal in the aphotic biofilm, while the photic biofilm had two-fold higher lipid values.

The data on water quality of the Edaiyur estuary during the study period are presented in Table IV. The estuarine water is rich in nutrients and the primary productivity was relatively high (5.0 mg C m^3). Amongst the bacterial population, the counts of SRB and NRB were relatively high. Figure 3 (A to D) illustrates the variations in various biofilm parameters assayed in the estuarine biofilm.

3.1. *Physical parameters* (Fig. 3A)

All the three parameters (biomass, thickness and turbidity) showed similar curve trajectories. Linear regression analyses between biofilm thickness and biomass gave a correlation coefficient $r = 0.9587$ and p value < 0.0001 which is considered significant. The correlation coefficient (r) between turbidity and biomass was 0.9273 (p value was 0.01).

3.2. *Biological parameters* (Fig. 3B)

The CAHB counts showed a decreasing trend after 72 h of biofilm growth. SRB showed a gradual increase with time, while the NRB count increased up to 72 h and then decreased by the end of 120 h. The denitrifying bacteria observed during the course of this study were *Alcaligens* sp., *Bacillus* sp., *Micrococcus denitrificans,* and *Pseudomonas* sp. Diatom population reached a maximum by 48 h and then onwards showed a decreasing trend.

3.3. *Chemical parameters* (Fig. 3C)

Temporal variations in nutrient distribution were observed in the biofilm. Silicate levels in the biofilm were very high and showed a steep increase in the initial phase of the biofilm growth (up to 72 h), followed by a decrease towards the end of 120 h. Phosphate content showed a marginal increase during the course of study (24 to 120 h). Almost all the nutrients showed a decreasing trend by the end of 120 h of biofilm growth, except nitrate which showed a slight

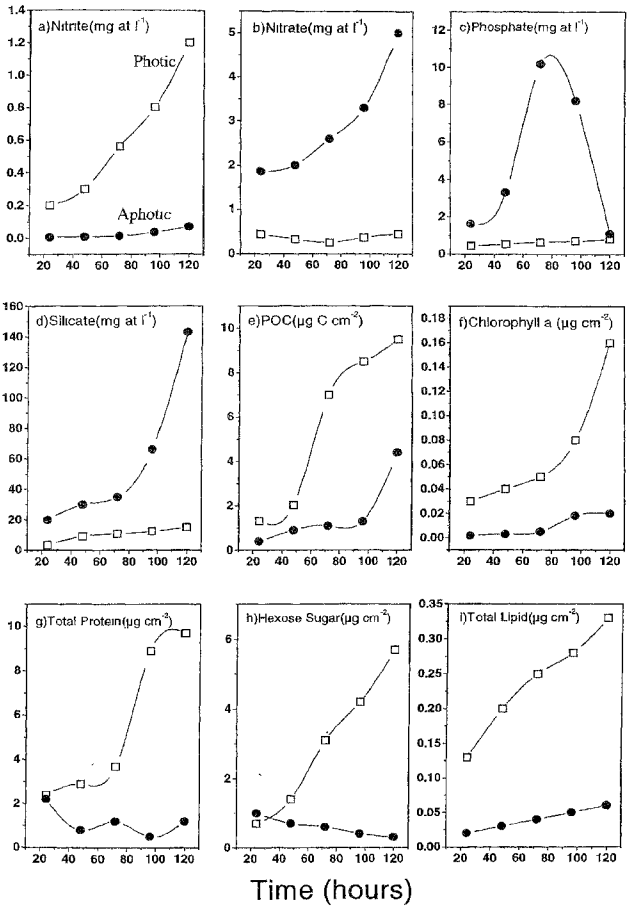

FIG. 2. Multiple chart showing the variation in the biofilm parameters (both in photic and aphotic conditions) in a freshwater impoundment. a) nitrite, b) nitrate, c) phosphate, d) silicate, e) particulate organic carbon (POC), f) chlorophyll a, g) total protein, h) hexose sugar, and i) total lipid.

increase. Nitrate levels in the biofilms increased up to 48 h, but thereafter decreased gradually. Nitrite concentration showed an initial increase up to 72 h and thereupon declined. Ammonia was detected in the biofilm by 24 h of biofilm growth. The concentration showed a linear increase up to 96 h, and then decreased. The progressive increase and detection of ammonia in the biofilm after 24 h of growth indicated that ammonia is produced *in situ* in the biofilm. Regression analysis of the nitrate reducers versus ammonia revealed significant correlation ($r = 0.97$ and $p = 0.0001$). This indicates that ammonia generation was probably caused by NRB and not by mere accumulation from the environment.

3.4. *Biochemical parameters* (Fig. 3D)

Chlorophyll a and lipid content showed only marginal variations during the study period. POC levels showed a linear increase from 24 to 120 h of biofilm growth. A significant number of protozoans were observed in the biofilm after 72 h of growth.

3.5. *Denitrification process* (Fig. 4)

NRB counts and nitrate, nitrite and ammonia values were plotted to understand the dynamics of nitrogen in the estuarine biofilm. At the end of 120 h, biofilm showed nitrification process which was evident by an increasing trend observed in nitrate concentration, whereas ammonia production showed a decreasing trend. This suggested that nitrification process was set in the biofilm after 120 h of growth. Table IIB shows the nutrient concentration factors in the biofilm in comparison with the ambient estuarine water. Phosphate concentrations were very high in the biofilm, followed by silicate, nitrite and ammonia.

4. Discussion

4.1. *Freshwater biofilm*

Biofilms are biologically active matrices consisting of cells, cell-derived and noncellular materials that accumulate at solid–water interfaces. Predicting the type, rate and extent of biofilm formation is useful in environmental studies, pollution abatement and design and operation of industrial equipment which are prone to biofouling.[20] The nature of the biofilm which develops in an aquatic environment depends on the characteristics of the water body. During the course of this study, temporal heterogeneities were observed in various physical, chemical and biological parameters.

Light plays an important role in the aquatic environment.[21] Biofilms growing on illuminated surfaces are expected to be different from those growing on surfaces under aphotic conditions (such as surfaces in a cooling circuit). Biofilm being a closely knit consortium of physiologically interacting multispecies entities, its chemistry is interlinked with its biological composition. Most of the studies on biofilm characterization so far have used surfaces immersed in well-lit sea water.[5, 12, 22] The results showed that aphotic biofilm had a slower growth rate as represented by lesser biomass and thickness and consisted of bacteria as predominant colonizers and later on heterotrophic diatoms to a lesser extent. On the other hand, biofilm developed in the photic environment had a much faster build up with high density of

Table III
List of algal species identified in the freshwater biofilm

(I) Cyanobacteria	*Microspora* sp.	*Nitzschia serrata*
Anabaena sp	*Oedogonium* sp.	*Nitzschia palea*
Calothrix sp.	*Pediastrum tetras*	*Opephora* sp.*
Gloecapsa rigreseus	*Selenastrum gracile*	*Rhoicospheria* sp.
Lyngbya spiralis	*Scenedesmus bifugatus*	*Synedra ulna**
Nostoc sp.	*Ulothrix* sp	(IV) Others
Oscillatoria sanctam	(III) Diatoms	*Dinobryon* sp *(chrysophyte)*
Phormidum sp.	*Achnanthes* sp.	*Tribonema* sp.
Phormidum fragile	*Amphora coffaeformis*	*(Xanthophyceae)*
Rivularia sp.	*Cocconeis* sp.	* also seen in aphotic biofilm
Spirulina subtilissuna	*Cymbela* sp.	
(II) Green algae	*Fragilaria* sp.	
Coelastrum microsporum	*Gomphonema* sp	
Chlorella vulgaris	*Licmophora* sp.*	
Chlorococcum sp.	*Navicula* sp.	
Coleochaete sp.		

algae and was characterized by a wide spectrum of organisms (bacteria, diatoms, filamentous algae, cyanobacteria, see Table III). It was observed that bacteria were predominant at the beginning of biofilm development in both illuminated and aphotic environments. The high POC content of 9.40 μg C cm^{-2} after 120 h in the photic biofilm could be attributed to increase in the number of algal settlers (see chlorophyll a values, Fig. 2f). Consistent with the literature, the results suggest that bacteria are initial colonizers of surfaces. The initial phase of biofilm growth (24 to 48 h) did not show any major changes in bacterial population colonizing the substratum. The succession pattern observed was; bacteria (initial colonizers), rapidly followed by a monolayer of adnate diatoms, Cyanophyceae, unicellular Chlorophyceae and multicellular and filamentous Chlorophyceae.

The CAHB observed during the course of this study contained species known to produce copious quantities of extracellular polysaccharides (in the present study, *Pseudomonas aeruginosa* amounted to 60% of the exopolymer-producing bacteria observed) and the relatively high hydrophobic surface provided by plexiglass could possibly be one of the reasons for such settlement. The SRB numbers both in the dark and illuminated biofilm indicated that thickness of the biofilm was correlated with the development of anaerobic environment. The SRB numbers were more on the aphotic biofilm, owing to reduced oxygen tension in the biofilm. The numbers, however, are marginally lower when compared to another freshwater lake, which was studied at Kota, Rajasthan, in north India.[23] This lake water, which is being used to cool a pressurized heavy-water reactor, showed SRB numbers ranging from 3.5 to 5.3 cfu cm^{-2} in the biofilm during a 24 to 120 h of exposure period.[23] Studies by Jones and Lock[22] have shown that the biofilm community that developed on illuminated surfaces consisted of both phototrophs and heterotrophs. Autotrophic production of oxygen would discourage SRB growth from proliferating in the photic biofilm. On the other hand, dark-grown biofilm are more likely to have greater SRB numbers as shown by the present data. Moreover, SRB being heterotrophic in nature, can utilize small-chain organic molecules and hence probably proliferate well on the aphotic panels. The absence of photosynthetic organisms can initiate early onset of an-

aerobic condition in the aphotic biofilm. This observation is very important in the context of biocorrosion of heat-exchanger surfaces where development of anaerobic conditions could lead to proliferation of SRB. Microscopic observations showed diatoms also in significant numbers in the aphotic biofilm after 96 h. These were probably heterotrophic diatoms, which are capable of growing in the absence of sunlight.[24] The presence of large amount of nutrients in the aphotic biofilm and the release of bacterial exudates during grazing by protozoans could have created favorable conditions for diatom to multiply on heterotrophic mode of nutrition. The high-reactive silica values in the aphotic biofilm apparently point to lack of utilization by diatoms, unlike in the case of photic biofilm where they are rapidly used up by the diatoms.

This study indicates that biofilm development in the early stages is nonhomogenous, an observation similar to that of Dexter *et al.*[25] as the biofilm develops more cells and their exopolymers accumulate and fill the free spaces. Costerton *et al.*[26] have reported that the presence of large network of exopolymers in the biofilm as well as extensive water channels are responsible for the water-holding capacity of biofilms. Apparently, this gelatinous matrix may also act as a trap for nutrients. Literature reports indicate the possibility of biofilm acting as a sink for nutrients and the solid–liquid interface as a zone of nutrient accumulation.[20, 27] But, there are no reports on the quantification of nutrients or their concentration factors in biofilms. The present data (Table IIA) provide evidence of nutrient enrichment in the biofilm. It is reported that the stereochemistry of the exopolymer matrix in the biofilm would make it function as an ion-exchange matrix enabling the concentration of nutrients.[28] The polymers, being amphoteric and polyelectrolyte in nature, can bind strongly to anionic nutrients like silicate, phosphate and nitrate.[29] It is possible that biofilm inhabitants benefit from specific interactions based on the provision of growth requirements, e.g. filamentous microorganisms which are observed in the biofilm may transport nutrients from the bulk liquid into the biofilm interior and replenish their supply as reported.[20]

It is known that in the later phases of biofilm development in natural waters, protozoans colonise the biofilm.[20] Ciliate protozoans play a critical role as bacterial grazers. The protozoa select their prey primarily based on physical characteristics such as size and shape.[30] By grazing on bacteria trapped in the biofilm the protozoans influence biofilm growth dynamics. They may even influence mass transfer to deeper layers of the biofilm, by creating turbulence through their motion within the biofilm.[20, 30] The protozoans graze on the bacterial population and are important participants in the maintenance of a stable climax biofilm community. During the course of this study, diatoms succeeded protozoan appearance in the aphotic biofilm and it is possible that the grazing of bacteria in the aphotic biofilm by the protozoans might have denuded the bacterial films from the substratum and paved the way for colonization by diatoms. The grazers release bacteria-bound nutrients, which become available for the diatoms, which in turn help in their proliferation. Thus the presence of protozoa in the biofilm may be very important in documenting the expression of metabolic potential of biofilm bacteria.

The initial phase of growth of microorganisms in the biofilm formation depends on the chemistry of the ambient environment, primary settlers, as well as adsorbed molecules at the interface. These parameters may characterize the optimum ecological niche for the further development of the biofilm. During the course of biofilm formation and progression, the microflora affect the environmental chemistry and provide conditions for favorable settlement of

other species. It was observed that the phosphate concentration in the aphotic film increased sharply after 72 h, which was also the time when protozoans were detected in the film in large numbers. Simultaneously, there was also a progressive reduction in the bacterial count, which may be due to protozoan grazing. Subsequent reduction in phosphate levels is probably due to the uptake by heterotrophic diatoms and increased bacterial colonization which was observed after 96 h of biofilm growth. According to Berner[31] phosphate in the biofilm may adsorb, diffuse or complex with inorganic and organic cations and be precipitated in the biofilm. This indicates that although the phosphate accumulation was significant in the initial phase, bacterial growth might have utilized it. It is reported that bacteria remove phosphate and retain it in their biomass making it unavailable to algae.[32] However, a similar trend was not discernible in the case of nitrate. This variation could be the result of the presence of denitrifying bacteria-reducing nitrate and their subsequent grazing by protozoans. It is probable that the observed reduction in the hexose sugar levels in the aphotic biofilm could be attributed to the uptake by heterotrophic diatoms such as *Nitzschia* and *Navicula* species, which were observed in plenty on the panels. Direct encroachment by motile diatoms on the substratum adjacent to denuded areas is possible as most of the heterotrophic diatoms are pennate.[24]

The ability of diatoms to grow on organic substances in the dark is of great advantage over an obligatory photoautotrophic mode of existence in those aquatic environments where cells are subjected to almost complete darkness. Heterotrophic capabilities are fairly widespread in pennate diatoms, which are epiphytic and inhabit environments rich in dissolved organic matter and high silicate: phosphate ratio.[24] Among the pennate diatoms, members of the genus *Nitzschia* sp. and *Amphora* sp. show pronounced heterotrophic property. They also occur in habitats strongly favoring heterotrophic modes of growth. In order to utilize the organic carbon sources, *Nitzschia* sp. attach themselves to suitable substratum and elaborate polymers for adhesion and in immobilizing the hydrolytic enzymes which degrade the complex organic sources for assimilation. Diatom cells are capable of survival in the dark for weeks or even months.[24]

4.2. *Estuarine biofilm*

The nutrient concentrations observed in the estuarine biofilm (Table IIB) were more than those observed in the freshwater and marine environment. Phosphate was accumulated to very high levels. The POC in the biofilm was relatively more than the other biochemical parameters assayed and showed a linear increase with time. Previously published reports[33, 34] say that elevated cellular activity in the initial stages of biofilm growth might be due to cells associated with particulate matter. Thus it can be said that biofilm acts as a trap to accumulate the particulate matter from the surrounding waters. However, this needs further confirmation as to how biofilm influences the rapid accumulation of particulate matter.

Changes in biofilm community composition with time could be due to adaptation of microorganism to the substratum microenvironment or to the changes in cell adhesiveness.[35] There is always a high degree of diversity among organisms that successfully colonize *in-situ* environments.[36] Viable counts of the estuarine biofilm bacteria showed an increasing trend up to 72 h. Generally, heterotrophic bacteria contribute to 15–50% of primary production and dominate the biomass in the pelagic zone of the open waters. This is because heterotrophs form the ma-

jor component of planktonic microflora. Thus, heterotrophic bacteria are likely to have a substantial impact on carbon and nitrogen cycle and large fraction of ammonia assimilation in marine waters could be attributed to heterotrophic bacteria. A decrease in CAHB was noted after the 4th day (96 h), later on by the end of 5th day (120 h) there was a significant increase. The decrease observed on the 4th day might be due to sloughing of biofilm and also due to grazing by bacteriovores, which included significant protozoan population. Species such as *Vorticella* and *Obelia* sp. were observed after 48 h of biofilm growth. However, there was no decrease in SRB population which could be due to their habitat specificity (anaerobic niche), which make them the bottom microflora in the biofilm and also protect them from grazers. Hence, the biofilm community could have reached its peak metabolic activity by 72 h. Thereafter, it could have led to the enticement of grazers (bacteriovores) which feed on the microflora and induce changes in the architecture of the biofilm.

Bacterial metabolism to a large extent is influenced by local physico-chemical factors.[33] Nitrogen fixation and denitrification are invariably dependent on the availability of utilizable organic carbon which is required as an energy and reductant source for nitrogen fixers and denitrifiers. About half the bacteria found in the sea are capable of reducing nitrate in waters enriched with organic matter and nitrate. Denitrification is a major sink for nitrogen which is important because the coastal marine ecosystems are frequently nitrogen-limited.[37] The two important mechanisms of biological reduction of nitrate are assimilatory and dissimilatory ni-

Table IV
Water quality data of the Edaiyur estuary

Parameter	Unit	Maximum	Minimum	Mean*	SD
Temperature	°C	32.3	29.8	31.1	0.93
pH	pH units	8.3	8	8.2	0.09
Turbidity	NTU	7	3.4	5	1.31
Seston	mg l^{-1}	1.32	0.8	1.3	0.15
Salinity	ppt	34.4	32.7	33.6	0.5
Dissolved oxygen	mg l^{-1}	7.2	5.8	6.7	0.47
Nitrate	µg at N l^{-1}	45	23 7	39	3.8
Nitrite	µg at N l^{-1}	2.2	1.4	1.7	0.23
Ammonia	µg at N l^{-1}	1	0.4	0.8	0.19
Silicate	µg at Si l^{-1}	185	90	146	33
Phosphate	µg at P l^{-1}	44	13	24	9
Chlorophyll a	mg m^3	6.2	3.3	5	0.92
POC	µg C l^{-1}	4.6	2.5	3.7	0.67
Total protein	mg l^{-1}	4 8	2.5	3.6	0.79
Total hexose sugar	mg l^{-1}	3.6	1.6	2.2	0.6
Total lipid	mg l^{-1}	1.8	0.6	0.9	0.2
Diatom count	cells ml^{-1}	8×10^3	4×10^3	5.6×10^3	141
SRB	cfu ml^{-1}	840	118	693	41
NRB	cfu ml^{-1}	7×10^4	3×10^3	4.3×10^4	1120
CAHB	cfu ml^{-1}	3×10^5	3×10^4	9.3×10^4	8×10^3

*Mean for 5 days.

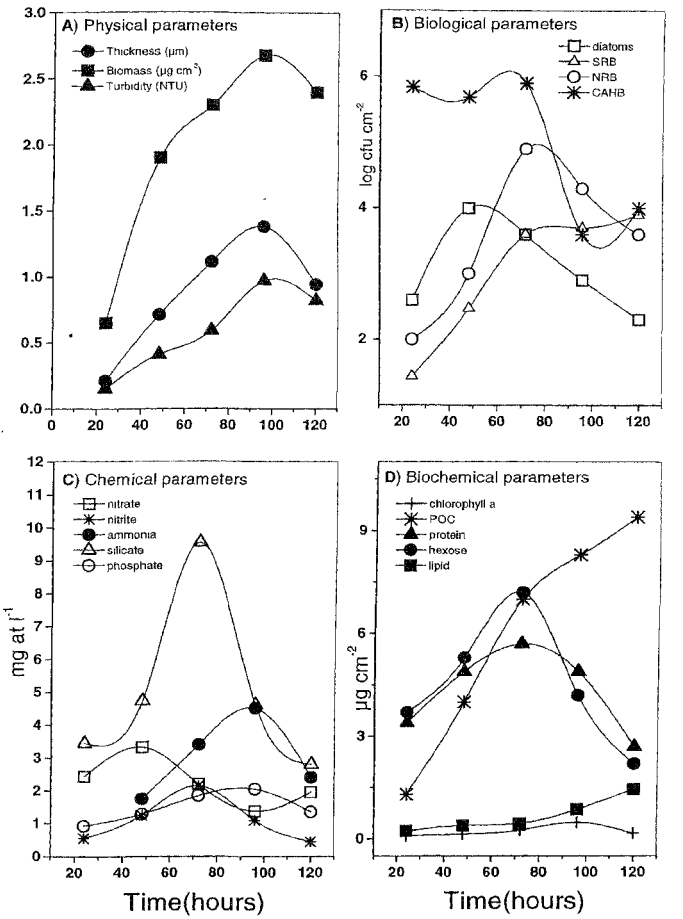

FIG. 3. Multiple chart showing various analyses of estuarine biofilm, A) physical parameters, B) biological parameters, C) chemical parameters and D) biochemical parameters.

trate reduction to ammonia.[38] In assimilatory nitrate reduction, nitrate is taken up and converted to nitrite and then to ammonia. The reduction process is driven by nitrate and nitrite reductases which are not oxygen-sensitive. *Achromobacter, Bacillus, Corynebacterium, Micrococcus denitrificans, Pseudomonas, Serratia* and *Vibrio* species are examples of extremely active nitrate reducers.[39] The denitrifying bacteria observed during the course of this study were *Alcaligens* sp., *Bacillus* sp., *Micrococcus denitrificans* and *Pseudomonas* sp.

Biofilms offer both a potential energy source and reduced microenvironment (subsurface layers of microzones) for denitrification. The process is strictly anaerobic and is regulated by i) the presence of energy sources, ii) absence of oxygen and iii) availability of nitrate, which serves as an electron acceptor. Active microbial metabolism of the biofilm microzones can potentially reduce oxygen in the subsurface layers, while proximity to the adjacent aerobic waters is essential for nitrate supplies.[20] Nitrate reducers are facultative anaerobes and the persistence of denitrification capacity under aerobic conditions is a rule rather than an exception.[39] The concentration of nitrate in the milieu also evokes certain responses in the microorganisms. Diffusion of nitrate from the overlying water is the only nitrate source for denitrification. The regions with greater concentration exhibit faster rates of nitrate reduction.[40] The reduction of nitrate and nitrite to ammonia is an endothermic reaction and therefore is thermodynamically possible only when required energy is forthcoming from an accompanying exothermic reaction. However, on the basis of thermodynamic considerations alone, the coupling through an electron transport chain of organic compounds to the reduction of nitrate to ammonia would also be expected to generate ATP. Thus, dissimilatory reduction of nitrate to ammonia is in fact nitrate respiration followed by passive reduction or possibly detoxification of nitrite to yield ammonia.[41] Many prokaryotes, in addition to filamentous fungi, yeasts, algae, and higher plants, are capable of reducing nitrate to ammonia for biosynthetic purposes.[42] Nitrogen and nitrous oxide are the major products of denitrification at positive redox potentials found in surface layers of biofilms, while the relative significance of nitrate reduction to ammonia increases at negative redox potentials.[43] Since nitrate serves as an electron acceptor the growth of denitrifiers depends on nitrate concentration [nitrate concentration in the biofilm was 540 (mean of 5 day biofilm) times more when compared to bulk water]. Denitrifying bacteria require an electron donor to carry out denitrification process, which is served by the organic matter.[37] The Edaiyur estuarine water is rich in organic matter (up to 2.3 mg l^{-1} of biofilm, calculated from particulate organic matter and hexose sugar content).

Ammonia produced as part of the denitrification process is often concentrated near the surfaces and nitrifiers are typically active at surficial interfaces where ammonia diffusion overlaps with oxic conditions. When ammonia diffuses to the surface, it gets oxidized by autotrophic nitrifying bacteria to nitrite and finally to nitrate as in other aerobic systems.[44] Although it was reported that heterotrophic bacteria utilize ammonium for growth, in the present study these were not assayed after 120 h. Denitrification process was observed in the biofilm formed in the Edaiyur estuarine water (Fig. 4). Seasonal cycles of nitrification and denitrification have been described in sediments.[45] Evidences suggest that in many coastal environments seasonal trends of denitrification are determined by nitrate availability. Therefore, coherent temporal trends of nitrification and denitrification would be expected in most coastal regions (e.g. sediments). The depression in nitrification and the consequent increase in denitrification is explained by seasonal fluctuations in oxygen penetration into the sediments.[45] These observations could be

compared in the same manner to the phenomenon observed in the biofilm formed in the Edaiyur estuarine water. The biofilm initially showed reduction of nitrate to ammonia and by the end of 120 h showed nitrification process which was evident by an increase in nitrate concentration as a result of oxidation of ammonia to nitrate. Tiedje[46] reported that the activity of enzymes involved in nitrate reduction is partly regulated by extracellular concentration of ammonia and nitrate. Losada *et al.*[47] have reported that nitrate reductase can be inactivated by a brief incubation of cells with ammonia. This indicates that when ammonia level reaches a critical concentration it can act as a repressor of the nitrate reductases.

This study has shown that nitrate reduction and ammonia oxidation happen in a five-day-old (120 h) biofilm. The processes could have happened due to diffusion of ammonia from the anaerobic zone to the oxygen-rich surficial regions of the biofilm. Microbial oxidation of ammonia in the aerobic zone results in nitrate formation and diffusion of nitrate back to the anaerobic zone. This mass transport within biofilm is enhanced by convective flow of the bulk fluid through the water channels that anastomose throughout the biofilm.[20, 26] Hence this natural process might facilitate biofilm metabolism. If that is the case, the ammonia produced is no longer transformed to nitrogen but could be recycled back to the overlying water for primary

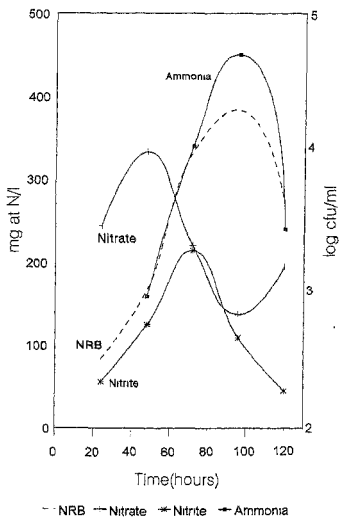

FIG. 4. Denitrification process observed in an estuarine biofilm.

production or oxidized to nitrate and diffuse back to the bottom layers to further support biofilm production. This sequence constitutes a positive feedback loop that allows biofilm metabolism to catalyze itself once initiated. In fact, biofilms seem like an ideal location for coupled rates of nitrification and denitrification which could be analogous to the one observed by Lindau and Delaune[48] in rhizosphere of marsh grasses. This is a unique observation in biofilms although the processes were dealt with elsewhere in detail with respect to sediments.[49]

5. Conclusions

1. The results of a study of various physical, chemical and biological parameters, analyzed in naturally lit (photic) as well as dark (aphotic) environments in a freshwater system at 24-h intervals up to 120 h, showed a major influence of light on many of the biofilm parameters studied. Biofilm thickness increased from 52 to 128 μm in the photic environment and from 17 to 30 μm in the aphotic biofilm. Biofilm biomass, chlorophyll and other biochemical constituents were higher under illuminated conditions. Diatom counts, as could be expected, were higher on the photic panels. Protozoan grazing of biofilm bacteria was observed after 72 h in both the sets. Nutrients were found to be enriched in the biofilm *vis-a-vis* ambient water. Concentration factors for the nutrients were consistently higher in the aphotic biofilm when compared to those in photic conditions. The conclusions arrived at from the study are; photic biofilms are invariably rich in diversity and bulk, with biotic components consisting of bacteria, fungi, diatoms, cyanobacteria and filamentous algae. Aphotic biofilms are predominantly bacterial, with heterotrophic diatoms forming a minor component in the older biofilms. Nutrients are found enriched in the biofilm, with aphotic biofilms showing greater concentration. SRB proliferation in photic biofilm is slower when compared to that in aphotic ones, which could be due to greater oxygen tension in the former.

2. Analysis of the estuarine water showed elevated nutrient (silicate, nitrate and phosphate) levels, heterotrophic bacteria and nitrate reducers. A study of the biofilm parameters showed considerable spatial and temporal variations. Biofilm thickness, turbidity and biomass showed similar trends of temporal distribution. The biofilm nutrient levels were 100–1000 times higher than those in the ambient water. Diatom counts and hexose sugar content of the biofilm showed very good correlation ($r = 0.97$ and $p = 0.0001$). The initial phase of biofilm development was influenced by bacteria and diatom population (up to 72 h), later bacteriovores changed the biofilm architecture. Anaerobic bacteria such as nitrate and sulfate reducers were observed within 24 h of biofilm formation. Denitrification phenomena was observed up to 96 h of biofilm growth; however, by the end of 120 h nitrification process had set in. This was evident by the significant increase in nitrate concentration in the biofilm. Thus, the estuarine biofilm appears to be like an archetype for studying coupled rates of denitrification and nitrification processes.

References

1. ALLISON, D. G. AND GILBERT, P. Bacterial biofilms, *Sci. Prog.*, 1992, **76**, 305–321.

2. LIU, D., LAU, Y. L., CHAU, Y. K. AND PACEPAVICIUS, G. J. Characterization of biofilm development on artificial substratum in natural water, *Wat. Res.*, 1993, **27**, 361–367.

3 VAISANEN, O M., NURMIAHO-LASSILA, E. L , MARMO, S. A. AND SALKINOJA-SALONEN, M. S. — Structure and composition of biological slimes on paper and board machines, *Appl. Environ. Microbiol.*, 1994, **60**, 641–653.

4. CHARACKLIS, W. G. AND COOKSEY, K E. — Biofilms and microbial fouling, *Adv Appl Microbiol.*, 1983, **29**, 93–138.

5 BHOSLE, N. B. *et al* — Biological and biochemical characterization of microfouling on aluminium panels placed in the Arabian Sea, *Proc. Indian Natn. Sci. Acad. B*, 1989, **55**, 51–56.

6 PEDERSEN, K — Biofilm development on stainless steel and PVC surfaces in drinking water, *Wat. Res* , 1990, **24**, 239–243.

7. JONES, S E. AND LOCK, M A — Seasonal determination of extracellular hydrolytic activities in heterotrophic and mixed heterotrophic/autotrophic biofilms from two contrasting rivers, *Hydrobiologia*, 1993, **257**, 1–16.

8 VAN DER KOOIJ, D. *et al.* — Biofilm formation on surfaces of glass and Teflon exposed to treated water, *Wat. Res.*, 1995, **29**, 655–662.

9 BAKKE, R AND OLSSON, P Q. — Biofilm thickness measurements by light microscopy, *J. Microbiol. Meth.*, 1986, **5**, 1–6

10. PARSON, T. R , MAITA, Y. AND LALLI, C. M — *A manual of chemical and biological methods for seawater analysis*, Pergamon Press, 1992.

11 APHA — *Standard methods for the examination of water and wastewater*, 17th edn, American Public Health Association (APHA), Washington DC, 1989.

12. SHARMA, M. O., BHOSLE, N. AND WAGH, A .B — Methods for removal and estimation of microfouling biomass, *Indian J. Mar. Sci.*, 1990, **19**, 174–176

13. ZOBELL, C. E — *Marine microbiology*, Bishen Singh Mahendrapal Singh, Dehradun, India, 1990.

14. POSTGATE, J. R. (ED) — *The sulfate reducing bacteria*, 2nd edn, Cambridge University Press, 1984.

15. BERGAN, T. — Human and animal pathogenic members of the genus *Pseudomonas*, In *The prokaryotes*, Vol. I, *A Handbook on habitat, isolation and identification of bacteria* (M. P Starr *et al.*, eds), Springer-Verlag, 1986.

16. KRIEG, N. R AND HOLT, J. G. — *Bergey's manual of systematic bacteriology*, Vol. 1, Williams & Wilkins, 1984

17. LOWRY, O. H., ROSE-BROUGH, N. J., FARR, A. L. AND RANDALL, R. J — Protein measurement with folin phenol reagent, *Biol. Chem.*, 1951, **193**, 265–275.

18. DUBIOS, M. *et al.* — Colorimetric method for determination of sugars and related substances, *Anal Chem.*, 1956, **28**, 330–356

19. RAO, T. S. AND HARBOLA, P. C. — Lipid profile of *Clostridium perfringens* Type D, *Int. J. Anim. Sci.*, 1994, **10**, 153–156.

20. CHARACKLIS, W. G. AND MARSHALL, K. C — *Biofilms*, Wiley, 1990.

21. KINNE, O. — *Marine ecology*, Vol. I, Environmental factors, Part I, Wiley, 1972.

22. LOCK, M. A. — Attached microbial communities in river. In *Aquatic microbiology* (Ford, T. E., ed.), Blackwell Scientific, 1993.

23. RAO, T S. AND NAIR, K. V. K. — Microbiologically influenced stress corrosion cracking failure of admiralty brass condenser tubes in a nuclear power plant cooled by freshwater, *Corros. Sci.*, 1998, **40**, 1821–1836.

24. HELLEBUST, J. A AND LEWIN, J. — Heterotrophic nutrition. In *The biology of diatoms, Botanical monographs*, Vol. 13 (Werner, D., ed.) Blackwell Scientific, 1977.

25. DEXTER, S. C, SULLIVAN, J. D., WILLIAMS, J. AND WATSON, S. W. — Influence of substrate wettability on the attachment of marine bacteria to various surfaces, *Appl. Microbiol.*, 1975, **30**, 298–308.

26. COSTERTON, J. W. *et al.* — Microbial biofilms, *A. Rev. Microbiol.*, 1995, **45**, 711–745

27. CHRISTENSEN, P. B., SORENSEN, J. AND REVSBECH, N. P. — Denitrification in nitrate rich streams: Diurnal and seasonal variation related to benthic oxygen metabolism, *Limnol. Oceanogr.*, 1990, **35**, 640–651.

28. FLETCHER, M. — Comparative physiology of attached and free living bacteria. In *Microbial adhesion and aggregation* (Marshall, K. C., ed.), Springer-Verlag, 1984.

29. ROBB, I. D. — Stereo-biochemistry and function of polymers. In *Microbial adhesion and aggregation. Dahlem Conf. on Adhesion*, (Marshall, K. C, ed.), Springer-Verlag, 1984.

30. FENCHEL, T — Suspension feeding in ciliated protozoa: functional response and particle size selection, *Microbial Ecol.*, 1980, **6**, 1–11.

31. BERNER, R. A. — Kinetic models for the early diagenesis of nitrogen, sulfur, phosphorus and silicon in anoxic marine sediments. In *The Sea*, Vol. 5, *Marine chemistry* (Goldberg E. D., ed.), Wiley, 1974.

32. COLE, J. J. AND CARACO, N. F. — The pelagic microbial food web of oligotrophic lakes. In *Aquatic microbiology: An ecological approach* (Ford, T. E., ed.), Blackwell Scientific, 1993.

33. RAO, T. S. — *Studies on biofilm formation in natural aquatic environments in the context of microbial corrosion*, Ph. D. Thesis, Anna University, Chennai, India, 1999.

34. COWAN, M., WARREN, T. M. AND FLETCHER, M. — Mixed species colonisation of solid surfaces in laboratory biofilms, *Biofouling*, 1991, **3**, 23–36.

35. HIRSH, P. — Microcolony formation and consortia. In *Microbial adhesion and aggregation* (Marshall, K. C., ed), Springer-Verlag, 1984.

36. BITTON, G. (ED) — *Wastewater microbiology*, Wiley-Liss, 1994.

37. KELSO, B. H. L., SMITH, R.V., LAUGHLIN, R. J. AND LENNOX, S. D. — Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation, *Appl. Environ. Microbiol.*, 1997, **63**, 4679–4685.

38. PAERL, H. W. — Interaction of nitrogen and carbon cycles in marine environments. In *Aquatic microbiology: An ecological approach*, Blackwell Scientific, 1993.

39. LLOYD, D., BODDY, L. AND DAVIES, K. J. P. — Persistence of bacteria denitrification capacity under anaerobic conditions: the rule rather than the exception, *FEMS Microbial Ecol.*, 1987, **45**, 85–190.

40. KING, D. AND NEDWELL, D.B. — Adaptation of nitrate reducing bacterial communities in estuarine sediments in response to overlying nitrate load, *FEMS Microbial Ecol.*, 1987, **45**, 20–25.

41 JORGENSEN, B. B. AND SORENSEN, J. *Seasonal cycles of oxygen, nitrate and sulfate reduction in estuarine sediments· The significance of a nitrate reduction maximum in the spring*. Marine Ecology Progress Series, Vol. 24, 1985, pp 65–74.

42 TRISKA, F J AND OREMLAND, R. S Denitrification associated with periphyton communities, *Appl Environ. Microbiol.*, 1981, **42**, 745–748.

43. SORENSEN, J Capacity of denitrification and reduction of nitrate to ammonia in a coastal marine sediment, *Appl. Environ Microbiol.*, 1978, **35**, 301–305.

44. PROSSER, J. I. Autotrophic nitrification in bacteria, *Adv. Microbial Physiol* , 1989, **30**, 125–181.

45. TUPAS, L. AND KOIKE, I. Amino acid and ammonium utilisation by heterotrophic marine bacteria grown in enriched seawater, *Limnol. Oceanogr.*, 1990, **35**, 1145–1155

46. TIEDJE, T. J Ecology of nitrification and dissimilatory nitrate reduction to ammonium. In *Biology of anaerobic micro-organisms*, (Zehnder, A. J. B., ed.), Wiley,1988.

47. LOSADA, M. *et al.* Inactivation and repression by ammonium of the nitrate reducing system in *Chlorella*, *Biochem. Biophy. Res. Commun.*, 1970, **38**, 1009–1015.

48. LINDAU, C W. AND DELAUNE, R D. Dinitrogen and nitrous oxide emission and entrapment in *Spartina alterniflora* saltmarsh soils following addition of N-15 labelled ammonium and nitrate, *Estuarine Coastal Shelf Sci* , 1991, **32**, 161–172

49. SEITZINGER, S. Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance, *Limnol. Oceanogr.*, 1988, **22**, 702–724.