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Studies of detailed Biofilm characterization on fly ash concrete in comparison with normal and superplasticizer concrete in seawater environments

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In cooling water systems, many concrete structures in the form of tanks, pillars and reservoirs that come in contact with aggressive seawater are being deteriorated by chemical and biological factors. The nuclear industry has decided to partially replace the Portland cement with appropriate pozzolans such as fly ash, which could densify the matrix and make the concrete impermeable. Three types of concrete mixes, viz., normal concrete (NC), concrete with fly ash and superplasticizer (FA) and concrete with only superplasticizer (SP) were fabricated for short- and long-term exposure studies and for screening out the better concrete in seawater environments. Biofilm characterization studies and microscopic studies showed excellent performance of FA concrete compared to the other two. Laboratory exposure studies in pure cultures of *Thiobacillus thiooxidans* and *Fusarium oxysporum* were demonstrated for the inhibition of microbial growth on fly ash. Epifluorescence and scanning electron microscopic studies supported the better performance of the FA specimen. Thus, the present study clearly showed that FA concrete is less prone to biofilm formation and biodeterioration.

Keywords: fly ash concrete; sea water; biofilm; biodeterioration; *Thiobacillus*

Introduction

Sulphate attack, chloride attack, carbonation and biodeterioration are some of the phenomena that may cause gradual but severe damages to concrete structures.[1] Biodeterioration takes place under biofilms predominated by acid-producing microbes.[2] Concrete mixture of calcium oxides, hydroxides and carbonates with quartz minerals and water is typically alkaline and has a pH in the range of 11–13.[3] However, the concrete does not remain basic throughout its whole life. The atmospheric CO_2 is capable of reducing the concrete pH to 9.5.[4] Once the pH is lowered, some heterotrophic bacteria and fungi can grow on the surface and reduce the pH. The acid-producing sulphur oxidizing bacteria oxidize sulphur to sulphuric acid, which further reduces the pH of the surface to 3–4.[5–7] The reaction of the biogenic sulphuric acid with cementitious material of the concrete leads to the damage of the concrete structures.[8,9] The corroding layer consists of gypsum and moisture.[10] The formation of ettringite during the acid reaction causes volume expansion leading to internal cracking of concrete structures.[11] This will provide further sites for the penetration of acid into the concrete.[12] Apart from sulphur oxidizing bacteria, nitrifying bacteria such as *Nitrosomonas* sp., *Nitrococcus* sp. also produce inorganic acid. Heterotrophic bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Altermonas* sp. etc., involve in the formation of the biofilm.[13] Sulphate-reducing bacteria is also involved in this process by providing the sulphur source for sulphur oxidizing bacteria.

Several concrete structures in the cooling water system of nuclear power plants are exposed to seawater either directly or indirectly. Concrete undergoes several reactions concurrently when exposed to such aggressive environments like seawater.[14] The surface of concrete is the first line of defense against the marine environment protecting the reinforcements inside.[15] With an impermeable concrete skin, the chemical and biological attack from seawater could be limited.[16] In addition, recommendation like partial replacement of Portland cement by appropriate pozzolans like fly ash is coming up to densify the matrix and to make the concrete impermeable.[17] Nuclear power plants with the new mandate of designing future power plants for 60-100 years are also looking for fly ash modified concrete to maintain the integrity for a long time.[18] Detailed literature review on processing, characterization and properties of fly ash concrete by Mustafa Al Bakri et al. [19] states superior performance of this concrete in seawater and sulphuric acid environments. However, this state of art review clearly shows that until now no systematic and detailed biofilm characterization studies on fly ash concrete

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is carried out. Thus, the present study aims for a detailed investigation on concrete biofilm characterization of three different types of concrete specimens such as normal concrete (NC), concrete with fly ash and superplasticizer (FA) and concrete with only superplasticizer (SP) with molecular identification and phylogenetic analysis to understand the resistance to biofilm formation and biodeterioration by FA concrete.

Materials and methods

Preparation of specimens

Three different types of concrete mix proportions, viz., (a) NC (M 30), (b) Fly ash with SP concrete (FA) (M 35) and (c) superplasticizer (SP) admixture concrete (M 30) were prepared as cube, prisms with reinforcements and cylindrical specimens (only mortar) to conduct a one-year long exposure studies in seawater. The ingredients of the mixtures in all the three types of specimens and the percentage of fly ash in concretes are as follows [20]:

Preparation of cube and prism concrete specimens

The cube specimens of 150 × 150 × 150 mm and prism specimens of 750 × 100 × 100 mm of dimensions were cast with all three mix proportions (NC, FA and SP) for carrying out the biofilm characterization studies.

Preparation of cylindrical mortar specimens

Two different sizes of mortar mixes cylindrical specimens of 90 mm diameter ×15 mm thickness and 35 mm diameter ×10 mm thickness of three types (NC, FA and SP) were cast in the petriplates and cured for 28 days in normal water in the concrete laboratory of the Indira Gandhi Centre for Atomic Research, Kalpakkam. The pH degradation studies, calcium leaching and weight loss of the specimen was tested by the bigger size of the cylindrical mortar specimen and for microscopic and pure culture exposure studies was done by smaller cylindrical mortar specimens.

Exposure studies in sea water

Exposure studies of cube and prism concrete specimens in seawater

All concrete specimens were demolded after 24 h of casting and were cured for 28 days in a laboratory atmosphere. After removing from the curing tank, the specimens were immediately exposed to sea water at the Nuclear Desalination Demonstration Project pump house sump at Kalpakkam and specimens were withdrawn after 60, 190 and 360 days.

Exposure studies of cylindrical mortar specimens in seawater

All the three types of cylindrical mortar specimens prepared as NC, FA and SP were exposed to the Biofouling Test

Ingredients:

Cement	(C)	O.P.C./Penna/43 grade
Water	(W)	Potable
Sand	(FA)	River bed (Palar)
Coarse Agg.	(CA)	Hard Blue Granite Rock Aggregate (Machine Crushed)
Fly Ash		Siliceous type IS 3812(Part–I)–2003
SP		Superplasticizer SP-430 (Fosroc Chemicals Ltd., Bangalore, India)

Concrete-Mix N/30/20/60: NC

Ingredients	Cement	Water	Fine agg.	Coarse agg.
In Kg/m^3	(C)	(W)	(FA)	(CA)
Concrete	400	180	684	1168
Mix Preparation	C÷C :	W÷C :	FA÷C:	CA÷C
By Weight	1	0.45	1.71	2.92

Concrete-Mix N/35/20: Concrete with FA (SP)

Ingredients	Cement	Water	Fly Ash	Binder	Sand	Coarse agg.	SP
In Kg/m^3	(C)	(W)	(FA)	(B)	(FA)	(CA)	
Concrete	225	143	150	375	549	1130	6.75
Mix preparation	C÷C :	W÷C:	FA	Total B	FA÷C :	CA÷C	B ÷SP
By weight	0.6	0.38	0.4	1	1.46	3.01	1.8%

Concrete-Mix N/30/20: Concrete with SP

Ingredients	Cement	Water	Sand	Coarse agg.	SP
In Kg/m3	(C)	(W)	(FA)	(CA)	
Concrete	350	154	845	1101	4.2
Mix preparation	C÷C :	W÷C :	FA÷C :	CA÷C	C÷SP
By weight	1	0.44	2.41	3.15	0.9%

Loop (BFTL) seawater sump and withdrawn after 60, 190 and 360 days.

Post exposure studies

Biofilm characterization studies of cube and prism concrete specimens

The biofilm characterization of cube and prism concrete specimens of all the three types (NC, FA and SP) was carried out. The most important parameter of biofilm characterization is the bacterial density. The total bacterial density of aerobic heterotrophs is evaluated as the Total Viable Count (TVC) of aerobic bacteria by culture techniques using seawater agar (Hi Media-M592). The density of different types of microbes like slime formers (*Pseudomonas* sp.), Manganese-oxidizing bacteria, algae, fungi and anaerobic sulphate-reducing bacteria in the biofilm was estimated by culturing in *Pseudomonas* Agar (PSA) (Hi Media-MM119), Manganous Agar (MnA) (Hi Media-M771), Cyanophycean Agar (CA) (Hi Media-M699), Czapek Dox Agar (CDA) (Hi Media-M1170) and modified Postgate medium,[21] respectively. The concrete specimens were collected from sea water and gently washed to get rid of loosely adhering cells. Using a sterile brush, the biofilm on the three sets of specimens both cube and prism were dispersed into 70 ml of sterile phosphate buffer (0.0425 g KH_2PO_4, 0.19 g $MgCl_2$ per litre). Serial dilutions of the bacterial cell suspension were prepared and 0.1 ml of each dilution was plated onto respective media. The plates were incubated for 24–48 h at 32°C and the bacterial density was estimated according to APHA standards.[22] Statistical analysis of the data was carried out using MYSTAT Software. Three replicates of three sets were analysed for each experimental condition. Student's *t*-test was performed to assess significance in the difference between bacterial counts on NC, FA and SP.

The biochemical constituents of biofilm like protein, carbohydrate and chlorophyll were also estimated, which provides the quantification of organic materials in different concrete biofilm. Protein concentration in the biofilm was analysed by Lowry's methods.[23] Carbohydrate content was measured using the Anthrone method.[24] Chlorophyll estimation was evaluated by the acetone extraction method.[25]

Total dissolved solids (TDS) were estimated by the evaporation technique for the combined content of all inorganic and organic substances in the biofilm sample. Total suspended solids (TSS) in the biofilm were also evaluated by the filtration technique.

Isolation and Molecular identification and phylogenetic analysis of dominant species of bacteria in the biofilm on the three types of concrete cube specimens (NC, FA and SP) were carried out to understand the diversity of different types of bacteria. The diversity on the different types of concrete will give a picture of biofilm resistance on the modified concrete. The different types of bacteria in the sea water agar plate were isolated as pure cultures and using biochemical tests the genus level identification was done.[26] Then isolation of Genomic deoxyribonucleic acid (DNA) was done by using the Invitrogen Pure kit. Polymerase chain reaction (PCR) was carried out to amplify 16S rRNA gene sequences of the isolates. The PCR products were checked using the Flash gel DNA system (LONZA, Basel, Switzerland) to confirm the amplification of 16S rRNA genes. The PCR products were purified and sequenced using Big Dye Terminator system. The sequences were analysed using bioinformatics tools to construct phylogenetic trees and identify the bacterial isolates to species level.[27]

pH degradation studies using cylindrical mortar specimens

Cylindrical mortar specimens of bigger size were used for pH estimation studies. The surface pH and total pH was carried out by crushing the specimen. A set of three types of cylindrical mortar specimens (NC, FA and SP) exposed to sea water for 60, 190 and 360 days was used for measuring the surface pH by short-range pH papers (HiMedia, HiIndicator pH paper with a pH range 1.0–14.0). Another set of similar specimens of 60 and 360 days were crushed by the weight crusher and powdered. In a beaker, 100 grams of the powdered sample was taken with 400 ml of demineralized water (pH 7) and kept for 24 h and pH was measured by a pH metre (ELICO-LI120).

Microscopic characterization of biofilm on cylindrical motar specimens

The cylindrical mortar specimens of smaller size were used for microscopic studies.

Epifluorescence microscopic study To visualize the biofilms under the epifluorescence microscope, nucleic acid stains like acridine orange (AO) is used. AO or 4, 6 diamino-2-phenylindole (DAPI), is a fluorescent dye used to differentiate between DNA and ribonucleic acid (RNA). The AO bonds with DNA and forms a complex that emits green fluorescence and when it bonds with RNA and forms a complex that emits orange fluorescence.[28] Thus, AO stains all the living active cells with lot of RNA in a biofilm and it will fluorescence orange and lesser active cells with more DNA with green fluorescence. Absence or reduction in fluorescence indicates lesser biofilm formation. Three types of cylindrical mortar specimens (NC, FA and SP) with different seawater exposure conditions with 1, 60, 190 and 360 days biofilm was stained with 0.1% AO solution for 30 min and rinsed with deionized water to remove the excess stain. The stained specimens were observed under an epifluorescent microscope (Nikon Eclipse E600 Epifluorescence Microscope, excitation filter BP 490; Barrier Filter O 515).[29]

Scanning electron microscopic study The cylindrical mortar specimens of all the three types (NC, FA and SP) exposed for 360 days to sea water were cleaned with sterile water. The specimens were fixed with 20% of glutaraldehyde for 24 h and then dehydrated through a series of ethanol-water combination of 20%, 40%, 60%, 80% and 100% and stored in vacuum desiccators. The surface morphological characteristics of the specimens were observed with Carl Zeiss, SUPRA® 55 with GEMINI® Technology with a resolution 1.0 nm at 15 kV. All specimens chosen for scanning electron microscopic (SEM) analysis were coated with gold for electrical conduction.[30]

Laboratory exposure studies

Three types of cylindrical mortar specimens (NC, FA and SP) were exposed to seawater and pure cultures of microbes for accelerated studies. Accelerated studies provide aggressive conditions to study the biodegradation effect in a shorter time.

Laboratory seawater exposure studies on cylindrical mortar specimens for weight loss and calcium leaching

Weight loss and calcium leaching [31,32] were evaluated for all the three types of cylindrical mortar specimens (NC, FA and SP) by exposure in seawater. A total of 10 numbers of pre-weighed specimens of each type was exposed to sea water in three different glass cylindrical jars in static conditions for 30 days of studies. The calcium content in the seawater that indicates the leaching of calcium is estimated by the titration method before and after the exposure of cylindrical mortar specimens.[33] After 30 days, mortar specimens were withdrawn and weighed to estimate the weight loss.

Laboratory pure culture exposure studies on cylindrical mortar specimens

Pure cultures of *Thiobacillus thiooxidans* (MTCC No. – 468) was brought from the Microbial Type Collection Center (MTCC) of the Institute of Microbial Techno logy, Chandigarh. The culture was maintained in Starkey medium ($(NH)_2SO_4 \cdot 0.2$ g, $MgSO_4 \cdot 7H_2O \cdot 0.5$ g, $CaCl_2 \cdot 0.25$ g, $KH_2PO_4 \cdot 3.0$ g, $FeSO_4 \cdot 0.005$ g with 1 g S) as recommended by MTCC. The pure cultures of *Fusarium oxysporum* (MTCC No. – 284) was brought from MTCC of Institute of Microbial Technology, Chandigarh. The culture was maintained in Potato Sucrose Agar (Diced potato 200 g, Sucrose 20 g, agar 20 g, pH 6.5) recommended by MTCC.

The three types of cylindrical mortar specimens (NC, FA and SP) were exposed to pure cultures of *T. thiooxidans* and *F. oxysporum* in semi-static conditions.

Every week 100 ml of media is replaced to keep the culture growing in semi-static conditions. Initially, the pH of the grown culture will be low and by exposure to alkaline mortar specimens the pH will increase. Subsequently as the mortar specimen supports the growth of the microbe, the pH will again decrease. Thus, the deterioration of specimens was monitored as the pH reduction of the culture and also by estimating the weight loss of the specimens after exposure.

The starkey broth solution was prepared by the combination of potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4 \cdot 7H_2O$), calcium chloride ($CaCl_2$), ferrous sulphate ($FeSO_4$) and aluminium sulphate (Al_2SO_4) in three separate jars, each containing 2 l of the solution in each jar and autoclaved.[34] Elemental sulphur was dusted on to the surface of the starkey broth and the steam sterilized. The initial pH of the starkey broth was checked as 7.0. After cooling the starkey broth solutions, the pure culture of *T. thiooxidans* was inoculated in this solution and kept in static conditions. When the pH was reached after the growth of bacteria to 1–2, the three different types of fresh cylindrical mortar specimens (NC, FA and SP) with pH of approximately 11–12 were exposed to the *T. thiooxidans* cultured solutions. The pH reading of this solution was monitored from the 1st, 7th, 15th, 30th, 45th and 60th day and 240th day. Weight loss of the specimens was also checked after 240 days of exposure.

Czapek Dox Broth (CDB) (Hi Media-M1170) was utilized to prepare fungus growth media.[35] The quantity of the culture was taken as 1.5 l and the initial pH of the broth solution was measured as 7.68. The 1 ml of pure fungal culture of *F. oxysporum* was inoculated into this and grown for 4–5 days to obtain a pure culture of *F. oxysporum*. Then cylindrical mortar specimens (NC, FA and SP) were exposed to a pure fungal culture in the static condition to monitor the pH reduction and weight loss after 45, 60, 90 and 300 days.

Results

Biofilm characterization studies of cube and prism concrete specimens

Figures 1 and 2 show the density of the aerobic and anaerobic bacterial biofilm on three different types of cube and prism concrete specimens (NC, FA and SP). The lowest density of aerobes and anaerobes was in the biofilm on FA concrete specimens. Table 1 gives the density of the different types of microbes like slime formers (*Pseudomonas* sp.), Manganese-oxidizing bacteria, algae and fungi in the biofilm of three types of cube and prism concrete specimens (NC, FA and SP). The density of all the types of microbes was also least in the biofilm on the FA concrete specimens.

The combined content of all inorganic and organic substances in the biofilm sample as TDS and TSS on the three types of cube and prism concrete specimens (NC, FA and SP) which was also least on the FA concrete (Figures

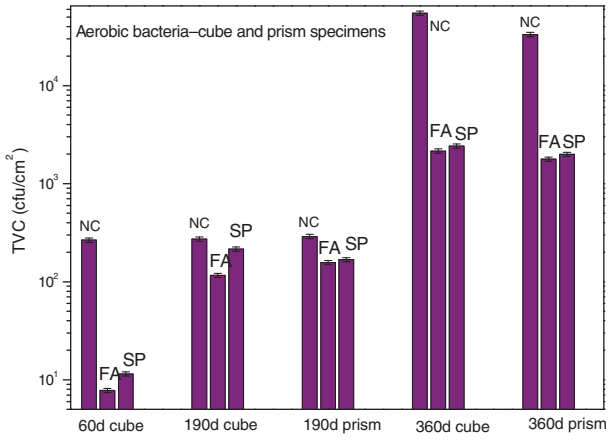

Figure 1. TVC of aerobic heterotrophic bacteria in the biofilms on three types of cube and prism specimens (NC, FA and SP).

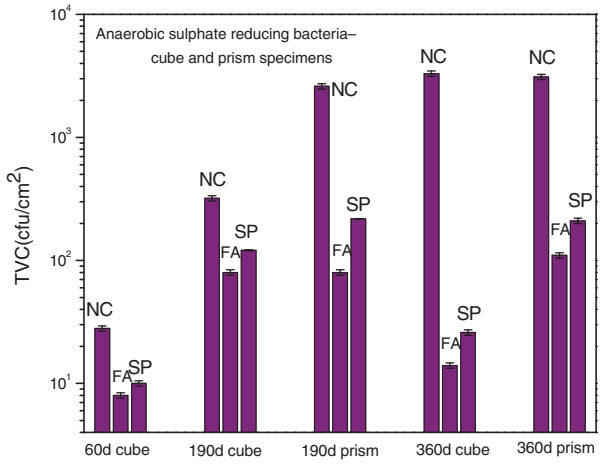

Figure 2. TVCs of anaerobic sulphate-reducing bacteria in the biofilms on three types of cube and prism specimens (NC, FA and SP).

S-1–2). The quantification of organic materials in the biofilm as the amount of protein,[36] carbohydrate,[23,37] and chlorophyll [38] is given in Table 2. The concentration of protein and carbohydrate was least on the FA concrete. The amount of chlorophyll indicating the total algal content was also least on the FA modified concrete.

A total of 15 bacterial isolates, each 5 from 3 different cube concrete specimens (NC, FA and SP) exposed to sea water with different colony morphology have only been taken for phylogenetic analysis and molecular identification and the number of bacterial isolates taken for the above analysis was not the reflection of the total bacterial density present in the exposed specimens.

The relationship between the sequences of the ribosomal library and the related organisms from the GenBank database were analysed and three neighbour-joining phylogenetic trees were constructed for the group *Firmicutes* (Figures S-3–5) to be analyse. The similarity and species identified with the phylogenetic analysis have been given in Table 3. In all the three phylogenetic trees, most of the isolates belonged to *Bacillus* and *Staphylococcus* genera, exhibiting a high nucleotide sequence similar to the species of *Bacillus* and *Staphylococcus* genera being the database sequences.

The neighbour-joining tree was constructed with the query samples and *Firmicutes* with a boot strap value of 1000. The boot strap percentage greater than 50 were indicated in the nodes of each branch. From the phylogenetic relationship that was obtained, the most prevalent species found in NC specimens is *Bacillus* sp., whereas in the

Table 1. Density of different types of microbes in the biofilm of three types of cube and prism specimens (NC, FA and SP).

No. of days	Type of specimens	PSA (cfu/cm^2)	MnA (cfu/cm^2)	CDA (cfu/cm^2)	CA (cfu/cm^2)
60th Day cube specimens (150 mm×150 mm)	60dN^0	1.01×10^2	0.78×10^2	78	50
	60dF^0	62	6	5	7
	60dS^0	88	35	18	13
190th Day of cube specimens (150 mm×150 mm)	190dN^0	2.43×10^3	94	1.1×10^2	7.3×10^2
	190dF^0	1×10^2	50	6.8×10^1	1.8×10^1
	190dS^0	1.24×10^2	68	9.6×10^1	4.5×10^1
190th Day of prism specimens (750 mm×100 mm)	190dN^0	2.43×10^3	1.1×10^2	1.08×10^3	4.0×10^3
	190dF^0	1.30×10^2	4.8×10^1	2.7×10^1	2.3×10^1
	190dS^0	1.50×10^2	5.08×10^1	8.9×10^1	3.7×10^1
360th Day of cube specimens (150 mm×150 mm)	360dN^0	3.14×10^4	1.82×10^3	1.2×10^3	5.8×10^2
	360dF^0	1.60×10^2	1.29×10^1	5.8×10^1	2.3×10^1
	360dS^0	2.15×10^2	1.86×10^1	8.6×10^1	3.9×10^1
360th Day of prism specimens (750 mm×100 mm)	360dN^0	2.59×10^4	1.21×10^3	11.9×10^3	5.2×10^3
	360dF^0	1.37×10^2	6.2×10^2	3.8×10^1	3.3×10^1
	360dS^0	1.99×10^2	1.11×10^2	9.2×10^1	4.1×10^1

Table 2. Amount of protein, carbohydrate and chlorophyll in the biofilms on the three types of cube and prism concrete specimens (NC, FA and SP).

Days	Types of specimens	Protein ($\mu g/cm^2$)	Concentration ($\mu g/cm^2$) of carbohydrate (glucose)	Chlorophyll concentration ($\mu g/cm^2$)
60th Day cube specimens	NC	2963.33	355.56	240.89
(150 mm × 150 mm)	FA	2163.33	55.56	97.78
	SP	2315.56	108.89	131.67
190th Day cube specimens	NC	3156.67	1551.11	348.89
(150 mm × 150 mm)	FA	2252.22	191.11	135.56
	SP	2518.89	628.89	270
190th Day of prism specimens	NC	1431.56	548.44	254.06
(750 mm × 100 mm)	FA	1128.28	293.91	99.37
	SP	1225.31	371.25	148.12

Table 3. 16S rRNA sequence analysis of bacteria isolated from the three types of concrete specimens (NC, FA and SP).

Name of the isolates	Taxonomic phylum	Closest relationship in Genbank	Similarity (%)	Microbial group identified
BRNS-N1	Firmicutes	*Bacillus subtilis* (AY553100)	99.4	*B. subtilis*
BRNS-N2		*Staphylococcus pasteuri* (AY553127)	99.6	*S. pasteuri*
BRNS-N3		*Bacillus tequilensis* (JF411295)	99.5	*B. tequilensis*
BRNS-N4		*Bacillus thuringiensis* (JQ621963)	99.6	*B. thuringiensis*
BRNS-N5		*Bacillus cereus* (HQ156459)	98.8	*B. cereus*
BRNS-F1		*B. cereus* (EU915687)	99.1	*B. cereus*
BRNS-F2		*S. pasteuri* (AF532915)	98.8	*S. pasteuri*
BRNS-F3		*B. thuringiensis* (JQ579628)	99.0	*B. thuringiensis*
BRNS-F4		*Staphylococcus epidermidis* (EU373368)	99.4	*S. epidermidis*
BRNS-F5		*Bacillus* sp. (AY189746)	98.6	*Bacillus* sp.
BRNS-S1		*S. pasteuri* (FJ380982)	99.6	*S. pasteuri*
BRNS-S2		*S. pasteuri* (AF532917)	98.6	*S. pasteuri*
BRNS-S3		*Bacillus* sp. (DQ323748)	99.1	*Bacillus* sp.
BRNS-S4		*Bacillus* sp. (GU272363)	97.9	*Bacillus* sp.
BRNS-S5		*S. pasteuri* (HQ908742)	99.2	*S. pasteuri*

FA concrete specimens and SP concrete specimen, the predominant species being *Staphylococcus* sp.

pH degradation studies using cylindrical mortar specimens

Table 4 shows pH degradation on the surface of three cylindrical mortar specimens (NC, FA and SP) exposed to sea water for 60, 190 and 360 days. The control specimens showed a pH greater than 12 and least reduction of pH 7 was exhibited by the concrete. Table 5 shows pH degradation of the three crushed cylindrical mortar specimens for control specimens, 60 and 360 days.

Table 4. pH degradation on the surface of three types of cylindrical mortar specimens (NC, FA and SP) exposed to sea water.

Sample type/days	Control sample	60 days	190 days	360 days
NC	> 12	10	7.3	6.0
FA	> 12	11.5	8.5	7.0
SP	> 12	10.5	7.8	6.5

Table 5. pH degradation of three types of crushed cylindrical mortar specimens (NC, FA and SP) exposed to sea water.

Sample type	Control sample	60 days	360 days
NC	12.33	11.22	9.0
FA	12.38	11.70	9.8
SP	12.48	11.43	9.3

Microscopic characterization of biofilm on cylindrical motar specimens

Epifluorescence microscopic study

Epifluorescence micrographs of the three types of cylindrical mortar specimens (NC, FA and SP) with different seawater exposure conditions with 1, 60, 190 and 360 days biofilm stained with 0.1% AO solution for 30 min and rinsed with deionized water is shown in Figures 3 and 4. There was no biofilm on all the three cylindrical mortar specimens after one day exposure which confirmed with green fluorescence (Figure S-6). The maximum red fluorescence

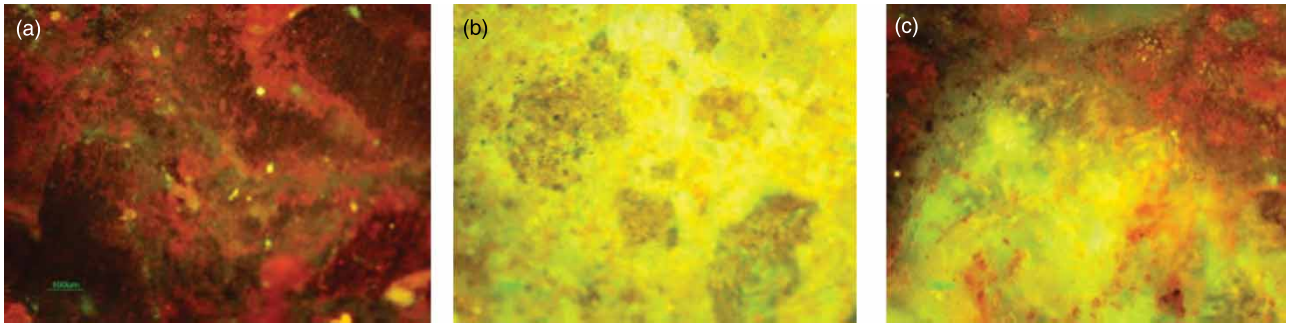

Figure 3. Epifluorescence micrographs of the biofilm on cylindrical mortar specimens of NC (a), FA (b) and SP (c) exposed to seawater for 60 days.

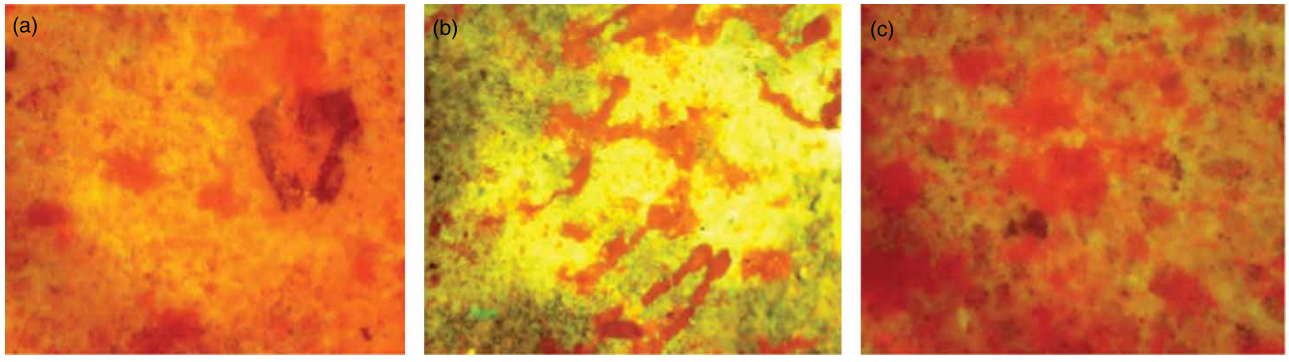

Figure 4. Epifluorescence micrographs of the biofilm on cylindrical mortar specimens of NC (a), FA (b) and SP (c) exposed to seawater for 360 days.

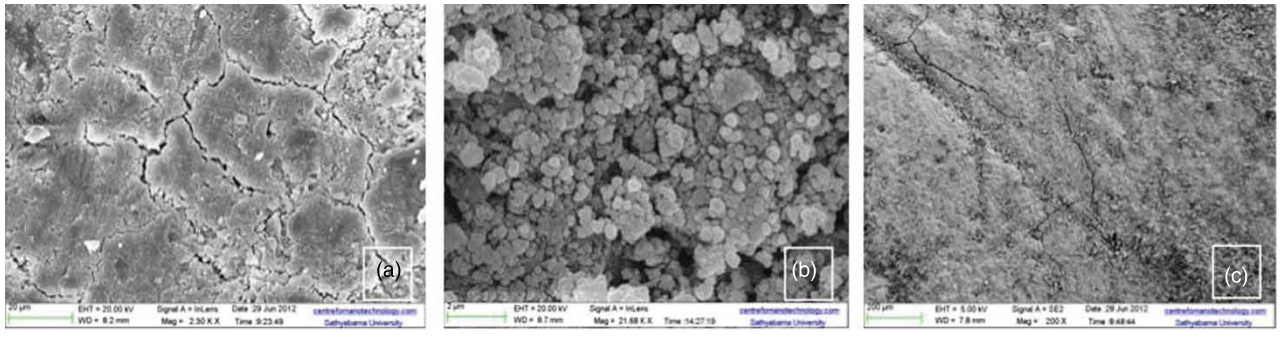

Figure 5. Scanning electron micrographs of cylindrical mortar specimens of NC (a), FA (b), SP (c) exposed to seawater for 360 days.

indicated good biofilm formation on NC specimens. The FA and SP specimens showed very less biofilm formation as compared with the NC specimens.

SEM study

The scanning electron micrographs of all the three cylindrical mortar specimen (NC, FA and SP) without exposing to sea water were taken as reference specimens (Figure S-7). This is compared with SEM micrographs of 360 days seawater exposed specimens of all the three types as shown in Figure 5. The SEM micrographs reveal partly decalcified area and no continuity in the structure formation on NC and SP cylindrical mortar specimens exposed to seawater for 360 days. However, SEM micrographs of FA specimens confirmed more compact structure indicating absence of degradation.

Laboratory exposure studies

Laboratory seawater exposure studies on three types of cylindrical mortar specimens for weight loss and calcium leaching analysis

The percentage weight loss and calcium leaching was observed for the three cylindrical mortar specimens (NC,

FA and SP) exposed to seawater for 30 days in the laboratory (Figures S-8–9). FA specimens showed least weight loss. However, the calcium leaching was highest for the FA specimens.

Laboratory pure culture exposure studies on cylindrical mortar specimens

The cylindrical mortar specimens (NC, FA and SP) were exposed to pure cultures of *T. thiooxidans* and *F. oxysporum* in semi-static conditions and the deterioration of the specimens was monitored as pH reduction of the culture and also by estimating the weight loss of the specimens after exposure.

The pH of the well-grown culture of *T. thiooxidans* was between 2 and 3 and the pH was increased to 11, when the mortar specimens were newly introduced. However, further growth of bacteria was reflected by the reduction of pH and that was highest in the case of NC specimens (pH 6.5) and least with FA specimens (pH 7.5). The weight loss was also least for FA specimens (0.06 g) compared to NC specimens (0.08 g) in cylindrical mortar specimens. A similar trend was seen in fungus culture also. The pH reduction was least for FA specimens (7.7) compared to NC specimens (6.5) (Figure S-10).

Discussion

Three different types of concrete mix proportions of cube and prism concrete specimens and cylindrical mortar specimens (NC, FA and SP) were prepared to conduct one-year long exposure studies in seawater. These studies aimed for detailed microbiological investigations on fly ash concrete surfaces in comparison with NC and superplasticizer concrete to get a deeper understanding of the probability of biodeterioration of fly ash concrete.[39] The important parameters of biofilm characterization involved total bacterial density of aerobic and anaerobic bacteria and different types of microbes like slime formers, manganese-oxidizing bacteria, fungi and algae. The organic and inorganic constituents of the biofilm were estimated. The molecular identification and phylogenetic analysis of bacteria in the biofilms of the three types of cube specimens (NC, FA and SP) were also carried out.[40,41]

Cube and prism concrete specimens were used for the study of detailed biofilm characterization. The total density of aerobic heterotrophic bacteria was highest on NC (5.48×10^4 cfu/cm^2) and least on fly ash concrete (2.15×10^3 cfu/cm^2) after 360 days of exposure in sea water. The density of *Pseudomonas* bacteria, *Manganese-oxidizing* bacteria, total *fungal density* and *algal density* was also evaluated and found the least density on FA modified concrete specimens. The density of the anaerobic sulphate-reducing bacteria was also less on FA concrete specimens. Thus, the total density of microbes in the biofilm was less significant on the fly ash concrete surface.

The concentration of TDS was measured as 107.4, 56.4, and 86.3 mg/cm^2 for NC, FA and SP cube concrete specimens, respectively, after 360 days exposure in seawater. The three types of prism concrete specimens also showed similar trends. The concentration of TSS was measured as 43.8, 26.4 and 36.0 mg/cm^2 for NC, FA and SP cube concrete specimens, respectively, after 360 days of exposure in seawater. The quantity of protein and carbohydrate indicating organic biomass in the biofilms was also least on FA cube concrete specimens compared to NC. The chlorophyll content of the biofilm indicating total algal density in the biofilms formed on all the three types of concrete specimens was also measured and found that there was a decrease in the chlorophyll content on the FA. Thus, the inorganic and organic content along with total algal density was least in the biofilm formed on the fly ash concrete specimen.

The phylogenetic analysis showed the identification of bacterial isolates up to the species level and the presence of dominant bacterial species isolated from the three different concrete specimens. From the phylogenetic relationship, it is confirmed that the most prevalent species found in NC is *Bacillus* sp., whereas in FA and SP, *Staphylococcus* sp. are the predominant species among the isolates of the two specimens. Between FA and SP, density of *staphylococcus* was less on FA concrete.

The pH reduction on the surface of the three cylindrical mortar specimens (NC, FA and SP) was measured during long-term exposures in seawater. Fly ash mortar specimen surface showed least reduction of pH 7 after 360 days compared to pH 6.0 on normal mortar specimen. The total specimen was crushed and pH measured to get the pH reduction of the whole specimen with long-term exposure in seawater. The pH reduction of the whole specimen was lesser compared to the surface of specimen. However, the reduction was least on Fly ash specimen (pH 10) compared to normal specimen (pH 9.2). Thus among the three types of mortar specimens fly ash mortar specimen showed better resistance to pH reduction during seawater exposures.

The biofilms formed on three types of cylindrical mortar specimens (NC, FA and SP) were visualized by epifluorescence microscopy. Nucleic acid stain (AO) was used for this purpose. This stains all active bacterial cells with high density of RNA as orange-red. Absence or lesser orange-red fluorescence indicates absence or lesser biofilm formation. The epifluorescence micrographs clearly show that by 60 days orange-red fluorescence starts on both NC and SP cylindrical mortar specimen surface. However, the orange-red streaks of biofilm on the FA specimens could be visualized only on the surface after 360 days exposure in the seawater.

The morphological changes on the three types of cylindrical mortar specimens (NC, FA and SP) due to long-term seawater exposures were observed in scanning electron microscope. The SEM micrographs of FA specimens verify the compact structures after 360 days of exposure in

sea water whereas NC and SP surface showed formation of cracks due to long-term exposure.

Weight loss studies by exposing three types of cylindrical mortar specimens (NC, FA and SP) for 30 days in seawater in the laboratory showed 1.0763 g in NC, 0.3929 g in FA and 1.0630 g in SP modified specimens. This confirmed least weight reduction for fly ash specimen. However, this study also showed higher calcium leaching from the fly ash mortar specimens despite least weight loss.

A laboratory accelerated tests was adopted for detecting biodeterioration resistance of fly ash cylindrical mortar specimens in comparison with normal and super plasticizer modified mortar specimens. The chief culprits of biodeterioration like sulphur oxidizing *T. thiooxidans* and acid producing *F. oxysporum* was selected for providing aggressive conditions. Fly ash cylindrical mortar specimens exposed to pure cultures of *T. thiooxidans* and *F. oxysporum* showed least weight loss and pH reduction.

Conclusion

Thus, our detailed microbiological studies on biodeterioration of three type of cube and prism concrete specimens and cylindrical mortar specimens showed excellent resistance of Fly ash concrete to biodeterioration under biofilm forming conditions. However, calcium leaching studies on cylindrical mortar specimens showed higher calcium leaching. It may be a concern for long-term exposure and future studies will be done on nanophase modification of FA concrete to address these problems.

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Supplementary information

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