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Effects of Nano-coating on biofilm forming bacteria on different boat building materials using 16sr DNA

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Abstract
The present study was aimed to see the effect of nano coating on biofilm formation in different boat building materials. Test panels (15cm h x 8 cm b and 12 mm thickness) made up of selected boat building materials (Steel, Wood, FRP) were tied in an iron frame and hung at a depth of 1.5 m inside the Tuticorin New Port area. The synthesized copper nano powder was mixed with enamel paint and spray coating was done on different boat building material test panels under room temperature. Periodical sampling was done to study the biofilm forming bacteria. Total counts of viable bacteria in untreated controls increased with time. From the biofilm forming bacteria, morphologically dissimilar colonies were randomly selected, isolated and maintained in Zobell Marine slants. A total of four isolates were selected from the test panels (H_1, A_2, A_3, and A4). The selected four isolates were amplified using the primer specific for mt 16S rDNA gene. The first isolate was identified as *Pseudomonas aeruginosa* (H_1) based on 99% similarity. The third isolate was *Vibrio alginolyticus* (A_3). These two species were present in all type of panels. The second isolate was identified as *Ferrimonas futtsuensis* (A_2), which was present in steel control and nano coated panels. The fourth isolate was *Vibrio coralliilyticus* (A_4) which was present in FRP panels and wooden panels. The phylogenetic analysis was preferred with the sequence of four isolates taken in this study and twelve biofouling bacterial sequences already reported by the authors in the previous works.

Keywords: Nanocoating, Boat building materials, Biofilm bacteria, PCR

1. Introduction

Fouling is the undesired deposition of materials on surfaces [12]. These surfaces typically include ships hulls, propeller, fishing nets, anchor, water pipelines etc. Biofouling is the deposition and growth of micro and macro organisms on submerged surfaces. The first step in marine biofouling is the formation of a conditioning film composed of organic material. The second step after formation of the conditioning film is the adhesion and development of biofilm by bacteria and microalgae [1]. There are reports of an increase of up to 18% in fuel consumption caused by microfouling alone[5]. The covering of microorganisms on the substrate is called primary film.

To avoid economic losses, as well as an accelerated deterioration of the artificial structures in contact with seawater, different types of antifouling protections have been used over time. Antifouling is the process of controlling fouling of a surface. Commercial anti-fouling techniques include mechanical cleaning, biocides, toxic coatings etc. Marine biofouling, broad spectrum metal biocides, such as tributyltin (TBT) and all the organic compounds of tin are extremely toxic to non-target organisms [2, 20]. Thus, in 1982, France prohibited the use of TBT in ships with a length less than 25 meters followed by Japan, United Kingdom, United States of America, Norway, Australia and New Zealand etc.

Despite the restrictions imposed on the use of oregano-metallic compounds, copper compounds have re-emerged as a main active ingredient of antifouling coatings in recent years. The copper based mixture works well for short term and serves as an ideal antifouling agent at least for three years after application [7]. Copper based antifouling formulations affect the fouling organisms too. However, their non-target effect is not as much as organo-metallic compounds. When copper is used in nano level, the impacts on the environment is much lesser. The results of scientific studies have revealed that the nanocoating prevent biofilm formation, bacterial adhesion besides the attachment of macro foulers [24].

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The Global ban on TBT as an antifouling biocide has led to the domination by copper-based antifouling technology and it has been estimated that about 15,000 tons of copper are being added to the marine environment every year [9].

In recent years, the application of nanotechnology has revolutionized many areas of material science, agriculture, fisheries, engineering, and medicine. The nanotechnology has a promising future in controlling the biofouling in maritime industries including shipping. Nano coating of the metals having antifouling properties showed positive results in different parts of the world. Nano coating controls many surfaces and bulk properties that are relevant to an antifouling, 'non-stick' surface, such as surface energy, change, conductivity, porosity, roughness, wettability, friction, modulus, physical and chemical reactivity, and compatibility with organisms. Advanced Nano structured Surfaces for the Control of Biofouling (AMBIO); a European Union research project is investigating how to prevent the build-up of organisms on surfaces under marine conditions to avoid biofouling. The project aims to use Nano structuring to significantly reduce the adhesion of organisms to surfaces in aquatic environments, and thus control the fouling process without the use of toxic biocide organotin compounds that prevent fouling by killing organisms [3].

Approximately 500 different nano structured coatings were prepared, tested in laboratory scale and evaluated for their antifouling effect [24]. Few attempts have been made in the application of nano coating to control biofouling in immersed marine structures and pipelines in India [27] studied the effect of copper nano films on bacteria at Indira Gandhi Centre for Atomic Research, Kalpakkam. With this background, an attempt was made to study the effect of copper nano-coating on different panels of boat building materials against the microfoulers.

2. Materials and Methods
Geographical description of the study area
The study was carried in Tuticorin, a Port City located between latitude 8°15' to 9°0' N and longitude 77°50' to78°15' E bordering Gulf of Mannar, which is known for its marine biodiversity. The jetty of Central Electro Chemical Research Institute (CECRI), Regional Research Centre, located within the Tuticorin New Port area was chosen for testing the panels. This site has no chance for the mix of freshwater to dilute the seawater so that salinity is reasonably stable except during the monsoon season.

2.1 Preparation of test panels
The test panels were made up of *Aini* wood (*Artocarpus hirsutus)*, Fiber Reinforced Plastic (FRP) and mild steel of width size 15 cm height, 8 cm breadth and 12mm thickness. The weight of each panel of wood, FRP and steel were 200gm, 240gm, and 1kg, respectively. These test panels were mounted with the help of 4mm polypropylene rope in an iron frame having a dimension of 106.5cm length and 106.5cm width. Ten panels were mounted in each frame; i.e. five control panels and five coated panels. With the help of a loop provided on the top of the frame, each frame was tied with a 12mm polypropylene rope and suspended in the CECRI jetty, inside the Thoothukudi harbor area. All the frames were suspended from the platform at a depth of 1.5m.

2.2 Sampling schedule
After immersion in seawater, the study panels were sampled periodically for microbial analysis. For microbial analysis, the samples were drawn after 24h of immersion and subsequently after the 1st week, 2nd week, 3rd week and a 4th week from the date of immersion.

2.3 Nano-coating
The nano-coating was done for wood, steel and FRP panels by spray method. The synthesized copper nano powder was mixed at the rate of 0.4μg/ L with enamel paint using magnetic stirrer at 60^0C for 6 h. Then, the paint was spray coated at 2-4 bar / 30 psi pressure and dried for 24 h at room temperature.

2.4 Antifouling coating
For comparative studies, all three-selected boat-building materials were painted with commercially available antifouling paint (Brand name: NOAH Marine Paints, Cochin).

2.5 Isolation of biofilm forming bacteria from the test Panels
For the enumeration of biofilm forming bacteria, a template of size 5 x 3 cm^2 was placed on each test panel and the bacterial film was scrapped using a sterile scalpel and placed in a test tube containing 10 ml of sterile saline. Serial dilutions were done with the same diluent. Zobell Marine Agar (HiMedia) was used for the enumeration of biofilm forming bacteria. Appropriate dilution (0.1ml) was inoculated on the sterile medium and spread plated uniformly with sterile glass spreaders. The plates were left at the room temperature for 30 min.until the sample was completely absorbed by the medium. The Petri dishes were then inverted, stacked in lots and incubated at a temperature of 37˚C for 24 h. The colonies were counted as biofilm forming bacteria and expressed as CFU/Cm2. For purification of the selected colonies, the frequently occurring colonies were streaked on Zobell agar medium, and the single isolated colonies were picked up and maintained in Zobell nutrient slants and held in a refrigerator [17].

Plate 1: Spraying of test panels with copper nano powder mixed paint

2.6 DNA Extraction
From the purified colonies, genomic DNA was extracted using HiMedia DNA extraction Kit. Approximately 1.5ml of overnight broth culture was taken in a 2 ml microfuge tube and 180 μl lysis buffers, 20 μl of proteinase K were added. After homogenization, the tubes were incubated at 55°C for 30 min in a water bath. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the lysed

tissue cell in the tube. The contents were mixed gently and centrifuged at 9200 rpm for 10 min. The top of the aqueous layer was then transferred to a new 1.5 ml microfuge tube. The DNA was precipitated by the addition of equal volume of isopropanol and 0.2ml volume of 10M ammonium acetate and by inverting the tube several times. The tube was centrifuged at 13200 rpm for 10 min. The supernatant was removed by pouring out gently. The pellet was then washed in 500 μl of chilled 70% ethanol, air-dried and resuspended in 100 μl sterile water.

2.7 Polymerase Chain Reaction
The mt16S rDNA region was amplified by PCR from the isolated genomic DNA using the universal primers. Primers used for PCR analysis were,
Forward: 5'-AGAGTTTGATCMTGG-3',
Reverse: 5'-ACCTTGTTACGACTT-3'
The amplification was done with 25 μl of reaction mixture containing 2.5μl buffer 0.25μl dNTP, 19 μl of molecular grade water, 0.25μl of Taq DNA polymerase, 1μl of each forward and reverse primers and 1μl of template DNA. The PCR protocol comprised of initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 45 sec and a final extension at 72°C for 10 min. The number of cycles was 35. A single discrete band at 1200bP was observed on agarose gel 2%.

2.8 Agarose gel electrophoresis
After the completion of PCR, 4 μl of PCR product was taken and mixed with 1 μl of 6X loading buffer and subjected to electrophoresis on a 2% agarose gel containing ethidium bromide at a concentration of 0.5 μg/ml in TAE (1X) buffer. The gel was analyzed under UV transilluminator and photographed using gel documentation system. Amplified PCR product was purified using column purification as per manufacturer's guidelines and further used for the sequencing reaction.

2.9 DNA sequencing of purified 16S rDNA fragment
After the amplification, the amplified gene products were purified and sequenced by Unibiosys Lab, Cochin, India. Phylogenetic analysis was done using the MEGA software.

3. Results
3.1 Effect of antifouling coating on biofilm forming bacteria associated with different boat building materials
The study was performed with different kinds of boat-built material panels for the duration of four weeks and the results are given in Table 1, 2 and 3. The graphical representations of the results on the effect of antifouling coating on biofilm forming bacteria are depicted in the Figures 1, 2 and 3.

3.2 Effect of antifouling coating on biofilm forming bacteria on various wooden panels
In wooden panels, the biofilm forming bacteria were 4.26, 4.30 and 4.65 log CFU/cm^{2}in control, antifouling painted and nano coated panels, respectively. The counts increased with the duration and were recorded as 6.47, 5.65 and 4.65 log CFU/cm^{2}for control, antifouling painted and nano coated panels respectively after the 4th week. Significant differences were observed in the counts of various wooden test panels except for the 1st week. Compared to control, the effect of antifouling was higher in nano coated wooden panel followed by antifouling painted panel after the 4th week (Table 1).

Table 1: Effect of antifouling coating on microbes in wooden panels

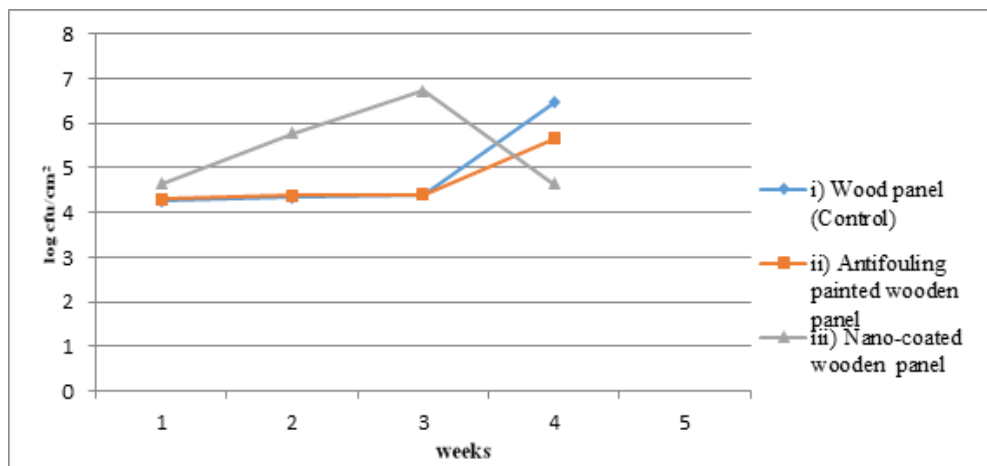| panel type	In weeks (log CFU/cm^2)			
	1st	2nd	3rd	4th
i) Wood Control	4.26	4.34	4.41	6.47
ii) Antifouling painted	4.30	4.39	4.41	5.65
iii) Nano-coated	4.65	5.78	6.74	4.65

Fig 1: Effect of antifouling coating on microbes in wooden panels

3.3 Effect of antifouling coating on biofilm forming bacteria on various steel panels
In steel panels, the biofilm forming bacteria present in control, antifouling painted and nano coated were 4.3, 4.64 and 4.22, log CFU/cm^2, respectively. The counts increased with the duration and were recorded as 4.66, 5.7 and 4.98 log CFU/cm^2, respectively after the 4th week. No significant differences were observed in the counts of the test panels expect on the 4th week (Table 2).

Table 2: Effect of antifouling coating on microbes in steel panels

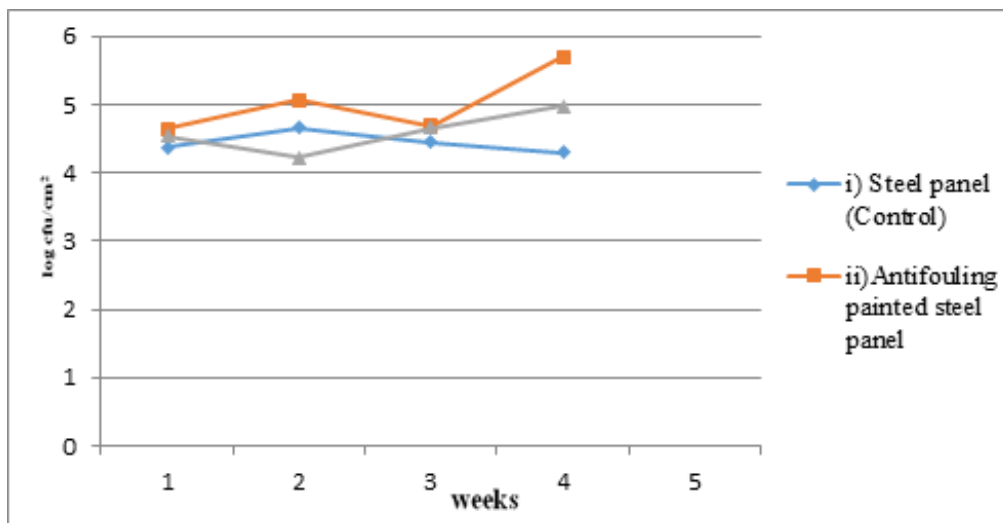| Panel type	In weeks (log CFU/cm^2)			
	1st	2nd	3rd	4th
i) Steel Control	4.37	4.66	4.44	4.3
ii)Antifouling painted	4.64	5.07	4.69	5.7
iii) Nanocoated	4.54	4.22	4.66	4.98

Fig 2: Effect of antifouling coating on microbes in steel panels

3.4 Effect of antifouling coating on biofilm forming bacteria on various FRP panels
In FRP panels, the biofilm forming bacteria present in control, antifouling painted and nanocoated panels were 3.56, 5.3 and 5.41 log CFU/ cm^2, respectively. The counts increased with the duration and were recorded as 5.88, 5.99 and 5.59 log CFU/cm^2, respectively after the 4thweek (Table 3).

Table 3: Effect of antifouling coating on microbes in FRP panels

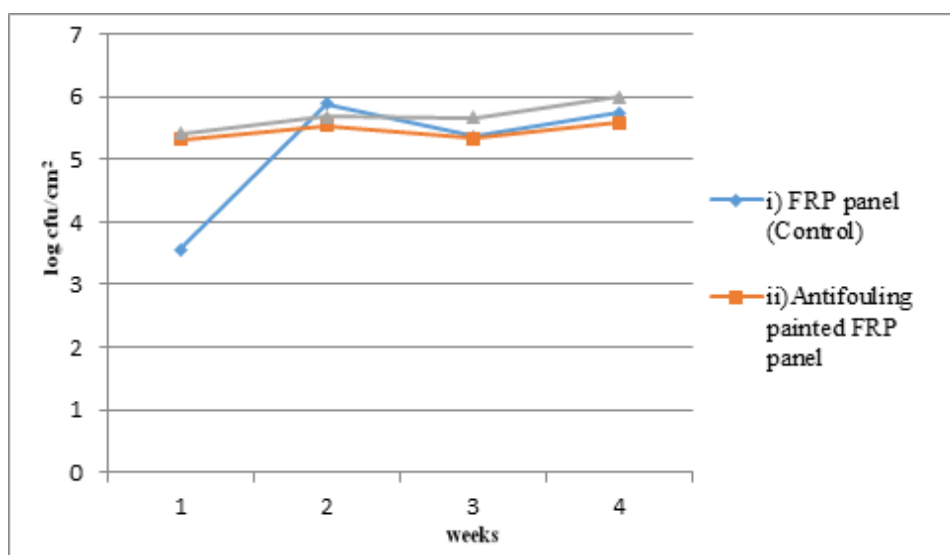| Panel type	In weeks (log CFU/cm^2)			
	1st	2nd	3rd	4th
i) FRP Control	3.56	5.88	5.37	5.75
ii)Antifouling painted	5.32	5.54	5.34	5.59
iii) Nanocoated	5.41	5.68	5.66	5.99

Fig 3: Effect of antifouling coating on microbes in FRP panels

3.5 Isolation of biofilm forming bacteria
From the biofilm forming bacteria, morphologically dissimilar colonies were randomly selected, isolated and maintained in Zobell Marine slants. A total of four isolates were selected from the test panels (H$_1$, A$_2$, A$_3$, and A4). Two of the isolates (H$_1$, A$_3$) were present in all the test panels. Third isolate (A$_2$) was isolated from steel control panel, particularly from the nano coated panel. Fourth isolate (A$_4$) was found in both FRP and wooden test panels.

3.6 Amplification of 16s rDNA from biofilm forming bacteria

The selected four isolates were amplified using the primer specific for mt 16S rDNA gene. The selected primers consistently amplified the gene in all the bacterial species at 1200 bp, which was clearly visualized in the agarose gel electrophoresis (Fig. 4). The first isolate was identified as *Pseudomonas aeruginosa*(H$_1$) based on 99% similarity. The third isolate was *Vibrio alginolyticus*(A$_3$). These two species were present in all type of panels. The second isolate was identified as *Ferrimonas futtsuensis*(A$_2$), which was present in steel control and nano coated panels. The fourth isolate was *Vibrio coralliilyticus* (A$_4$) which was present in FRP panels and wooden panels.
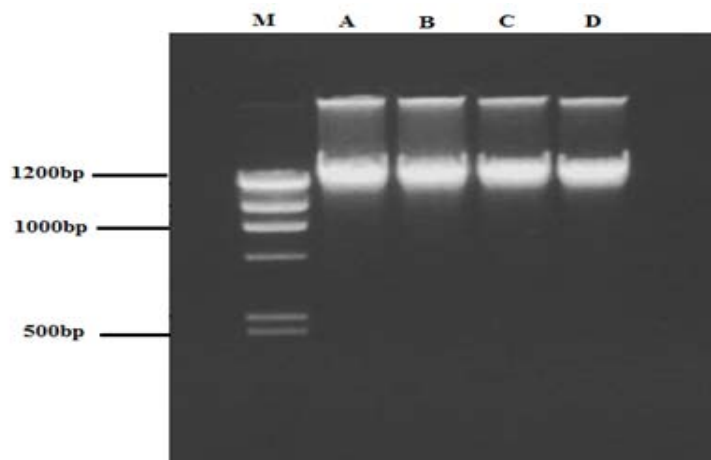
Fig 4: Ethidium bromide stained 2% agarose gel showing results of electrophoretic analysis of amplified 18S rRNA gene eukaryotic specific primer obtained from different bacterial species. Lane M- 1200 bp DNA marker; Lane A- *Pseudomonas aeruginosa*; Lane B – *Ferrimonas futtsuensis*; Lane C-*Vibrio alginolyticus* Lane D – *Vibrio coralliilyticus*

3.7 Sequencing of the amplified 16S rDNA gene region

The amplified 16S rDNA fragments were given for sequencing and the results are presented in Table 6. All the four species, viz. *Pseudomonas aeruginosa, Ferrimonas futtsuensis*, *Vibrio alginolyticus, Vibrio coralliilyticus* showed more than 99% homogeneity based on the entries available in the NCBI databases.

Table 4: Details of BLAST analysis, the percentage of similarity and NCBI accession numbers of marine biofilm forming bacteria isolated from different kinds of test panels.

S. No	Assigned code	Sequence length (bp)	Similarity (%)	BLAST results	NCBI's accession
1.	*H1*	977	99	*Pseudomonas aeruginosa*	JQ659528
2.	*A2*	989	99	*Ferrimonas futtsuensis*	JQ799090
3.	*A3*	1007	99	*Vibrio alginolyticus*	KJ872832
4.	*A4*	994	99	*Vibrio coralliilyticus*	EU372917

3.8 Phylogenetic analysis methods

The phylogenetic analysis was preferred with the sequence of four isolates taken in this study and twelve biofouling bacterial sequences already reported by the authors in the previous works. The results showed two major divergences among the species based on Maximum Likelihood, Neighbour Joining methods and Minimum Evolution methods (Figures 5, 6 and 7). Inner level divergences observed in all the bacterial species except between two *Vibrio spp*. These four-diverged species have closeness measured as less than 0.1 in divergence scale. *Vibrio alginolyticus* and *Vibrio corallilyticus* are found in the same branch whereas *Pseudomonas aeruginosa* and *Ferrimonas futtsuensis* diverge in two distinct inner nodes. Thus, the overall phylogenetic analysis revealed that the *Pseudomonas aeruginosa, Ferrimonas futtsuensis, Vibrio alginolyticus* and *Vibrio corallilyticus* are distantly related with other selected species of bacteria involved in biofouling activities.
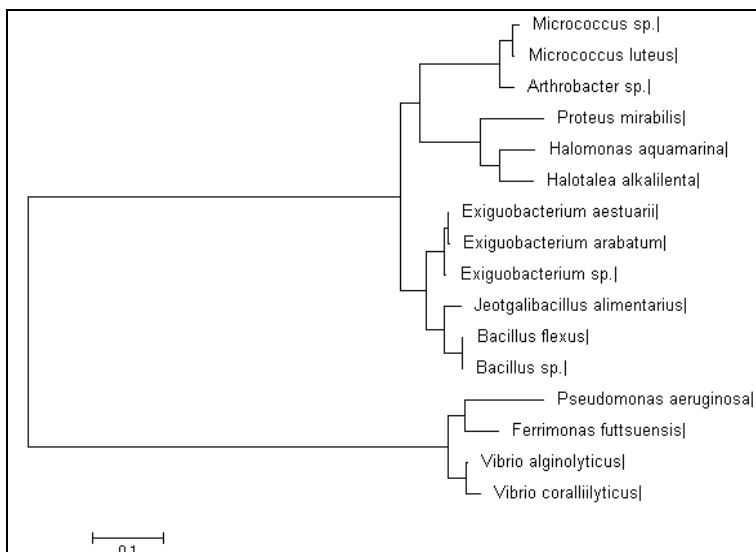

Fig 5: Phylogenic tree constructed using the Maximum Likelihood Method showing the relationships of the rDNA sequences from the marine biofilm bacteria adhered on different kinds of boat building materials
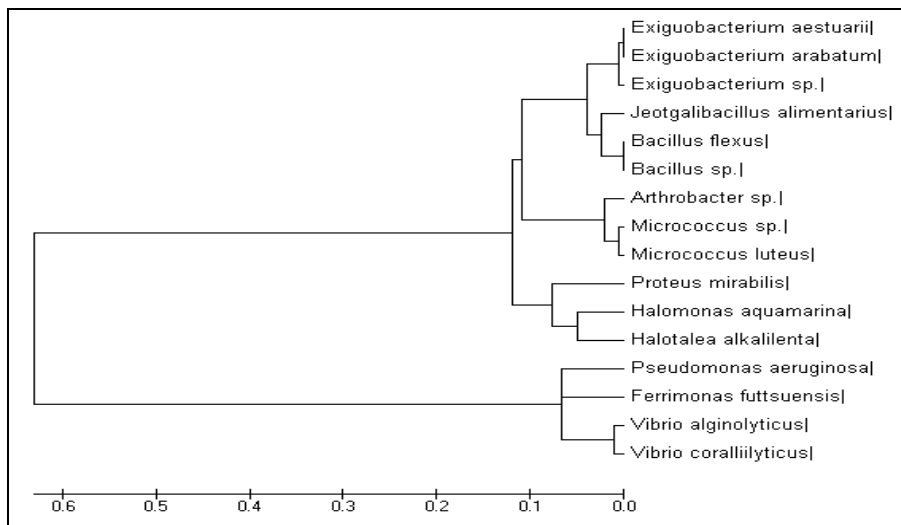
Fig 6: Phylogenic trees constructed using the Neighbour-joining Method of calculating the distance parameter in MEGA software.
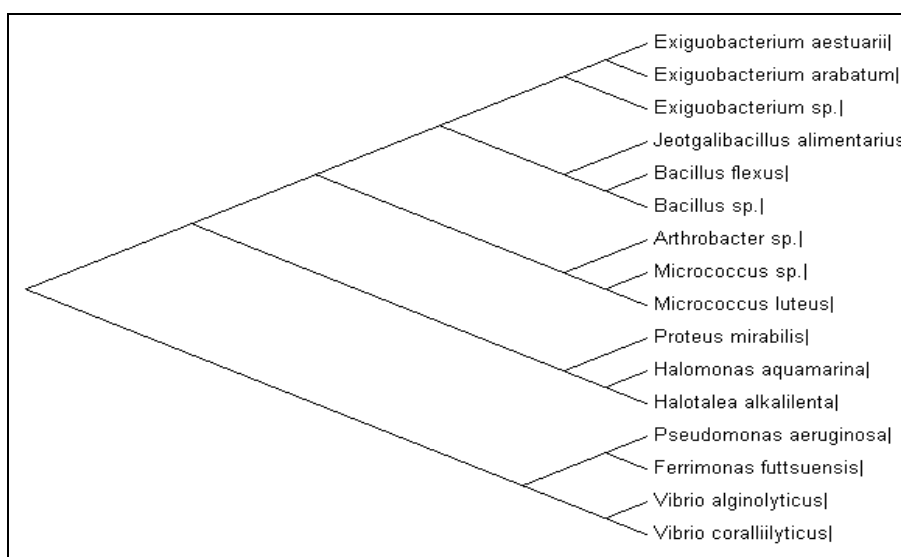

Fig 7: Phylogenic trees constructed using the Minimum Evolution Method

4. Discussion
4.1 Effect of antifouling coating on biofilm forming bacteria on various test panels
Deposition of microorganisms on surfaces and biofilm formation is an important bacterial survival strategy. Biofilms occur spontaneously on both inert and living systems, being of concern to a wide range of scientific disciplines. In industry, biofilms can have a detrimental impact because of accumulation at interfaces [6, 8]. Competition for living space is more intense in the marine environment; hence all submerged surfaces in the marine environment are rapidly colonized by bacteria and they form the important component in the development of a fouling community [22]. Biofilms are formed by microbial cells embedded in an exopolymeric matrix. The extracellular matrix is mainly composed of polysaccharides and proteins, although other compounds such as DNA and humic substances [18, 23].

Various workers have described the developing stages of the primary film and ecological succession of complex micro fouling communities in the temperate and subtropical environments [22, 28]. These studies suggest that bacteria and diatoms were the first organisms to appear on various surfaces placed in the aquatic environment. Our observation on the micro fouling material formed on different kinds of materials agrees with the above findings. Bacteria were abundant in the micro-fouling material developed on wooden panels in marine waters. Bacterial numbers were found to be significantly related to various kinds of boat-building materials. (Table- 3, 4 and 5) In our present study, determined the number of bacterial species found in biofilm on various kinds of boat-building materials. These studies have also reported that the cultivatable bacterial numbers on the surfaces quite differed when examining the formation of biofilm up to four weeks duration. When compared to all other materials, wooden control panel harbors many bacteria followed by FRP control and Steel control panel with little variation.

In agreement with the present result, Fletcher and Marshall (1982) [14] and Zacheus (2000) [32] stated that the pattern of colonization of the submerged surfaces by bacteria was influenced by physical and chemical characteristics of the surface. The formation of biofilm was heavily affected due to various factors and one of such factor is the nature of surface materials and the nature of aquatic systems. From the obtained results, confirm that control wooden surfaces support

more microorganisms than FRP and Steel materials. The antifouling nature of paints repels the bacterial attachment than control.

Marszalek *et al*., (1979) [22] was also reported that loss of surface, physical stability or the release of corrosion products which may be toxic at increased concentrations and it makes biologically unfavoured of fouling process. Therefore, there is a difference in the succession of micro biota in stainless steel and FRP panels may be due to differences in materials and nature of the surfaces used. The assessment of bacterial population in the mild steel inferred the slow rate of succession of the bacterial load with respect to the time interval. This may be due to corrosive nature and changes in ionic charges of the substrates. Biofilm of the organism may influence the corrosion of metals. The development of microbial biofilm on the metal surface is also known to be influenced by several factors including temperature and dissolved oxygen of seawater (4, 10, and 15). Little and Wagner (1997) [21] have reported that the rate of initial bacterial colonization in a substratum is dependent, i.e. not all surfaces are colonized at the same rate or to be same the extent. Hence the observed differences in biofilm bacterial load with the different substrate are universal.

4.2 PCR method for the identification of bacteria using 16S rDNA gene

Murthy *et al.,* 2004 [27] studied the biofilm control using plate heat exchangers of surface seawater from the open ocean for the OTEC power plant. In the microbiological analysis of biofilms revealed that four distinct types of bacterial colonies were present and it is predominant bacteria to isolate, viz. *Vibrio, Flexibacter*, *Pseudomonas and Aeromonas*, the total viable bacteria in untreated controls were observed and tend to amplify counts with the age of the biofilms. There was a highly significant variation (P = 0.0001) between chlorinated and control biofilms. Feng *et al.* (2000) [13] and Yamanaka *et al.* (2005) [31] reported the mechanism of the inhibitory action of silver ions on microorganisms. They concluded that microbes when treated with silver ions, lose their DNA replication ability, expression of ribosomal subunit proteins and other cellular proteins, and inactivation of enzymes essential for ATP production. It has also been hypothesized that Ag+ primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain. However, the mechanism of bactericidal actions of silver nanoparticles is still not well understood.

Jin *et al.* [19] investigated the structure of pioneer communities of marine biofilms developed on three kinds of artificial surfaces (acryl, glass and steel coupons) kept submerged in seawater. The composition of bacterial communities was analyzed by terminal restriction and nucleotide sequencing of 16S rRNA. From this study, indicated some species of γ-*Proteobacteria* were more important as the pioneering population. Dhanasekaran *et al.* (2009) [11] studied the screening of biofouling activity in a marine bacterial isolate from ship hull. In this study, 11 isolates were obtained from three ships from Royapuram harbor, Chennai, Tamil Nadu. Among the 11 isolates only DR4 showed maximum biofouling activity in the micro titer plate assay with a significant optical density of 0.596 and the isolate was similar to *Bacillus* sp. Marine bacteria from the hull of a ship in the form of biofilms or micro fouling were isolated, cultured, and Identified by phylogenetic analysis using 16S rDNA sequences by Inbakandan *et al.* (2013) [17]. Among them, 16

strains of the Firmicutes were dominant (12.5%), CFB group bacteria (6.25%) and Enterobacteria (6.25%).

In our study, the collected bacterial samples were investigated for the isolation, identification, sequencing, characterization bacterial species and their genetic makeup through advanced molecular biological studies including polymerase chain reaction, electrophoresis and sequencing techniques. In this study, help us to predict the mode of binding, genetic materials responsible for biofouling, etc. The polymerase chain reaction (PCR) is one of the most important and widely used technique to quantify the nucleotide samples for further level analysis like isolation and characterization. The isolated and amplified samples were further used for identification of base pairs using sequencing techniques, 16S rDNA were identified, and unread codons present in 3' and 5' prime ends and used for similarity search using an NCBI's tool, BLAST. BLAST help to identify the closely related or similar sequences of bacterial strains present in the Gene bank database. It also helps to identify the genetic makeup of identified bacterial DNA, which responsible for biofouling activities. Bacterial species, such as *Pseudomonas aeruginosa,* [25] *Ferrimonas futtsuensis, Vibrio alginolyticus* and *Vibrio corallilyticus* were identified. From the result, *Pseudomonas sp* was the most dominant bacteria in all samplings and then *Vibrio corallilyticus* was dominant in FRP.

The data presented here on the diversity of biofilm bacterial strains in the experimental panels indicated, that most of the strain identified were gram negative in nature. Among biofilm bacterial strains isolated, the most predominant bacterium recorded in all the experimental panels was *P. aeruginosa* (29 – 35%) and next dominant biofilm bacterial strains registered were *V. alginolyticus* with the percentage occurrence range of 17 – 19% respectively. The other biofilm bacterial strains were recorded in lesser proportion (1 – 10%). From genetic profiling of identified bacterial samples is an imperative research will focus and deliver the unknown information about bacterial species and genetic relationship among the different species. From the knowledge obtained from the sequence, similarity will help up to identify the target site of bacteria for the development more potent antifouling materials in future.

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