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Original Research

Occurrence of Coliforms in Microplastic Associated Biofilm in Estuarine Ecosystem

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Abstract

Coliforms and microplastic pollution are major issues prevailing in the estuarine ecosystem. At present, the Adyar river in Chennai, Tamil Nadu is under threat due to sewage disposal and solid waste dumping. In the present investigation surface water samples were collected from five sampling locations and analyzed for coliforms, microplastics (MPs), and the development of biofilm. The maximum coliform population of 75±6-228±12 MPN/100mL was observed during the pre-monsoon period. The MP content was in the range of 90-230 particles /L, 70-210 particles/L, and 90-190 particles/L for pre-monsoon, post-monsoon, and summer seasons respectively. The development of black crystalline colonies on Congo Red Agar (CRA) medium explained the occurrence of biofilm in the study area. Further, biofilm formation was higher during summer when compared with that of pre-monsoon and post-monsoon seasons. The plastic particles collected from the sampling locations were having rough surfaces. More bacterial colonies were observed on polyethylene (PE) MPs when compared with that of polyamide (PA) and Nylon under laboratory conditions.

Keywords: biofilm, coliforms, estuary, microplastics

Introduction

Coliforms are considered as indicators of feacal contamination and biological deterioration of water [1, 2]. Coliforms are members of the Enterobacteriaceae indicating feacal contamination of water. Among the coliforms *Escherichia coli* are common inhabitants of the intestinal tract of warm-blooded animals including humans. The coliforms not related to feacal origin namely *Citrobacter* sp., *Enterobacter* sp., and *Klebsiella* sp., are also found in soil, vegetation, and surface water [3]. Distribution of feacal coliforms has been reported in rivers [4, 5], lagoons [6], estuaries [7, 8]. Among the different ecosystems, estuaries are considered as important resources, as estuaries are providing food and habitat for fish and shellfish, food for human consumption, areas for tourism and recreation and host of ecosystem services [9-11]. Currently, estuaries are polluted with human and animal release which contains a large amount of pathogenic microorganisms [12, 13], and plastics [14]. MPs distributed in aquatic environments act as vectors to transport pathogens and other harmful pollutants [15]. Furthermore, MPs are providing ecological niche for microorganisms in aquatic systems [15]. Globally few researches have been done on the distribution of MPs in estuaries [16, 17]. In addition, estuaries facilitate the transport of MPs from land to ocean [18]. Furthermore,

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the retention of MPs in estuaries is facilitated by filtering by vegetation and subsequent flocculation [19] makes the estuaries act as sink for MPs [20]. Many studies proved the harmful effects of MPs on biological species including phytoplankton, zooplankton, plants, and animals in aquatic ecosystem [21-24]. In the aquatic systems, microorganisms colonize in cracks and grooves developed on MPs by photo oxidation [24]. The migration ability and large surface area facilitate MPs to absorb pollutants and microorganisms, which serve as long- term migration carriers in the aquatic ecosystems [25-28]. Recently, it has been investigated that microorganisms colonize on MPs by the formation of biofilm [29]. Further, it has been studied that development of microbial community on MPs depends on habitat and season [24]. In addition, the microbial community in the MP associated biofilm could decrease the hydrophobicity and change the functional group of polymers [29]. Furthermore, the bacterial community structure varies depending upon polymer type [30] and *Vibrio* sp. was detected in biofilm formed on MPs. Because, MPs are moving from estuary to marine environment, the surface of MPs may act as a floating substrate for colonization and transportation of microorganisms [31]. In addition, biofilms act as reservoir for bacterial pathogens in polluted water [32, 33]. Although the colonization characteristics of bacterial communities on MPs have been investigated in aquatic environments, the studies conducted to investigate the occurrence MP associated coliforms in estuarine ecosystem is limited. Since, coliforms are considered an important group of bacterial species, more studies are warranted.

Adyar River is an important waterway rises in the Chembarambakkam tank, flows through the city of Chennai, Tamil Nadu, India for a distance of 14 km, before it joins the Bay of Bengal. Adyar estuary starts near the Chettinad palace. At present, the estuary is highly polluted with effluent and domestic waste [34]. It is evident from previous studies that Adyar estuary is highly polluted with MPs of different polymers namely polyethylene (PE), polypropylenre(PP), polystyrene (PS), and polylactic acid (PLA) [35]. Dumping of tons of plastic waste near river mouth contributes the MP pollution in the Adyar estuary. Furthermore, the discharge of untreated sewage and open defecation on the banks of estuary are the major source of bacterial contamination including coliforms in the estuary [36]. No studies were conducted to investigate the occurrence of coliforms in biofilms developed on MPs under estuarine ecosystem. Hence, the present study was conducted to (a) examine the effect of different polymers on the occurrence of coliforms on biofilm (b) explore the surface morphology of polymers after biofilm formation.

Materials and Methods

Study Area and Sample Collection

About 10 L of Surface water samples were collected from different locations of Adyar estuary namely near Kotturpuram bridge, Greenways railway bridge, ThiruViKa bridge, Broken bridge, and Mouth of Adyar estuary. The study area map showing different sampling locations is given in Fig. 1.

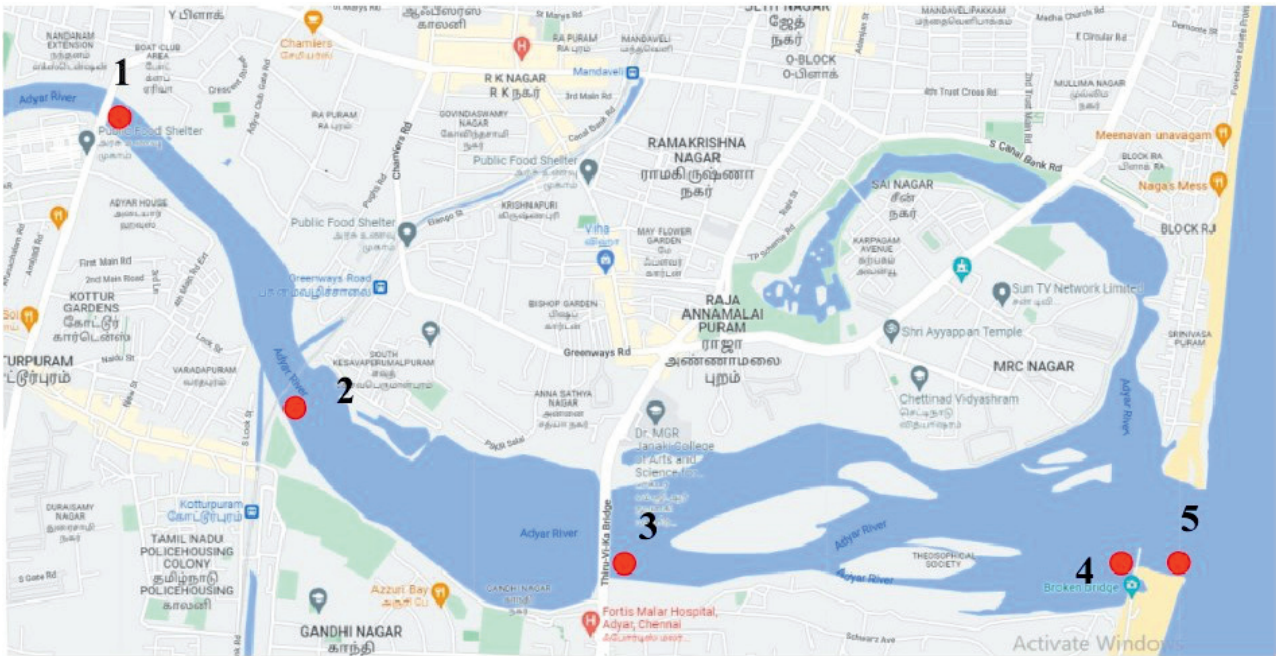

Fig. 1. Study area map showing different sampling locations (● indicates sampling locations) ((1) Kotturpuram bridge (2) Thiru Vi Ka bridge (3) Greenways railway bridge (4) Broken bridge (5) Mouth of Adyar estuary).

The samples were collected during pre-monsoon (September 2021), post monsoon (January 2022), and summer (March 2022) seasons. Samples were collected using metal bucket (10 L capacity). The collected samples were immediately transferred to glass bottles (Borosilicate) of one litre capacity. Appropriate space (1.0% of the container volume) was left for aeration and mixing. The water temperature was determined using digital thermometer (Hanna Instruments, USA), pH was measured using a portable digital pH meter (pHep+; Hanna Instruments, USA), and conductivity was determined by a conductivity meter (PRIMO5; Hanna Instruments, USA), from which salinity was calculated by multiplying with the factor 0.64. The sample containing glass bottles were immediately closed with lid to prevent onsite contamination. The samples were stored in 2.5% formalin solution [37], and the glass bottles with samples were transported to laboratory under iced condition for further study. The water samples collected from estuary was analyzed for phosphate, and BOD [38].

Analysis of Coliforms

Initially M Endo agar medium (Himedia Ltd.,) [39] with the following composition was prepared by suspending the ingredients namely, agar - 15 g, casein hydrolysate - 3.7 g, di-potassium hydrogen phosphate - 3.3 g, lactose - 9.4 g, pararosanilin (fuchsin) - 0.8 g, peptone from meat - 3.7 g, potassium hydrogen phosphate - 1.0 g, sodium chloride - 3.7 g, sodium deoxycholate - 0.1 g, sodium lauryl sulfate - 0.05 g, sodium sulfite - 1.6 g, tryptose, -7.5 g, yeast extract - 1.2 g in 1000 mL distilled water containing 20 mL of 95% ethanol. The final pHof the medium was maintained at 7.2±0.2 using 0.1N HCl. After thorough mixing, the medium was heated with frequent agitation and brought to boil to completely dissolve the powder; removed from heat immediately. After cooling (45 to 50ºC) 4 to 6 mL of the medium was poured in to Petri plates (Borosilicate (50 mm)) under aseptic condition. Subsequently, the collected surface water samples were diluted with sterile distilled water and 25 mL was passed through nitrocellulose membrane filter (pore size 0.45µm, Millipore Corp.Bedford, Mass) fixed in a membrane filter assembly which was fitted with a vacuum pump (LAPRO@S.SFiltration Assembly, India). Afterwards, the filter paper was removed from the filtration assembly using sterile forceps under aseptic condition, and placed on the surface of the medium. The M Endo Agar plates were incubated at 35±0.5°C for 24 hours in an incubator (BSSCO-BSEX -1412, India). After 24 hours, the petri plates were examined and the colonies developed were counted.

Analysis of Microplastics

The water samples (1.0 L) collected from different locations of the Adyar Estuary were processed for examination of MPs [40]. In order to digest organic matter, one litre of each sample was added with 5-15 mL of 1:1 mixture of 10M KOH and H_2O_2(30%). After 3 to 4 days of agitation in a rotary shaker (REMI RS - 36 BL, India), the samples were neutralized with formic acid. The separation of MP particles was achieved by adding 3.61g potassium formate powder/ mL sample (density 1.62 g/mL) using a separating funnel. The uppermost layer of the water phase was filtered using a vacuum filter assembly using glass fiber filter paper (pore size: 2.0 µm, Millipore, UK). Afterwards, the filter paper was removed from the filter assembly, air dried (40°C) and stored in petri plates under closed condition [41]. The plastic particles were carefully transferred to a glass slide using fine-tip tweezers and observed under a stereo microscope (Accu-Scope; Thermo Scientific Inc.) and counted. The examination of the surface morphology of the selected MPs was done by Scanning Electron Microscope (SEM) (Carl Zeiss MA15/EVO 18, resolution of 3.0 nm with a 30-kV SE detector). The MPs samples were fixed on gold SEM stubs using double-sided adhesive carbon tabs [42]. The photographs of the samples were taken using EmCrafts Virtuoso 1.1 operated at 20 kV and at a working distance of 15 nm [43].

Detection of Biofilm

About 100 mL of water samples collected from different locations of Adyar Estuary was subjected to vacuum filtration. The filter paper along with MP was transferred to Perti plates containing Congo Red Agar(CRA) medium [44]. The CRA medium was prepared by supplementing sucrose (5% w/v) and Congo red (Sigma Aldrich (0.08% w/v)) to Brain Heart Infusion Agar(BHIA) (Himedia) developed by Rosenow [45]. Afterwards, the Petri plates were incubated at 35°C for 24-48 hours under aerobic condition. Appropriate controls were maintained without addition of samples. The experiment was performed in triplicates.

Development of Biofilm on MPs Derived from Different Polymers under Laboratory Condition

Preparation of Microplastics

The locally available plastic materials namely plastic carry bag (polyethylene), rope (nylon), and fabric (polyamide) were collected, washed thoroughly, dried, and cut in to small pieces (5mm x 5 mm). Again washed with distilled water, disinfected with ethanol (70%), air dried and kept for further study.

Development and Detection of Biofilm

The experiment was conducted in 250 mL glass beakers (Borosilicate). Before the start of the experiment, the beakers were washed thoroughly,

rinsed with sterile distilled water, and subsequently with sterile estuary water. About 100 mL of estuary water was mixed with 15 numbers (each) of uniformly sized plastic particles (5 mm) and kept under static conditions for 30 days. Flasks were incubated at room temperature without any nutrient additions. Afterwards, the MPs were separated by vacuum filtration. The filter paper (0.45µm) along with MP was transferred to Perti plates containing Congo Red Agar (CRA) medium [44] and the Petri plates were incubated at 35°C for 24-48 hours under aerobic condition. The Petri plates were examined for the development of colonies. The MPs extracted were subjected to SEM analysis to examine the bacterial colonies grown in biofilm developed on different types of MPs, and morphological changes occurred on MPs surface due to biofilm formation.

Results and Discussion

Characteristics of Surface Water Samples Collected from Adyar Estuary

The estuaries are the partly opened ecosystems supporting plenty of flora and fauna [46]. The Adyar riverine ecosystem is unique because, it has a creek and estuary. The estuary is polluted due to urban developmental activities [47, 48]. The surface water samples collected from different sampling locations of Adyar estuary was analyzed for temperature, pH, and salinity and the results are illustrated in Fig. 2. The results revealed that, temperature of the surface water samples collected during pre-monsoon, post-monsoon, and summer period was in the range of 25.4±0.2°C-26.9± 0.4°C, 27.9±0.2°C-29.0±0.4°C, and

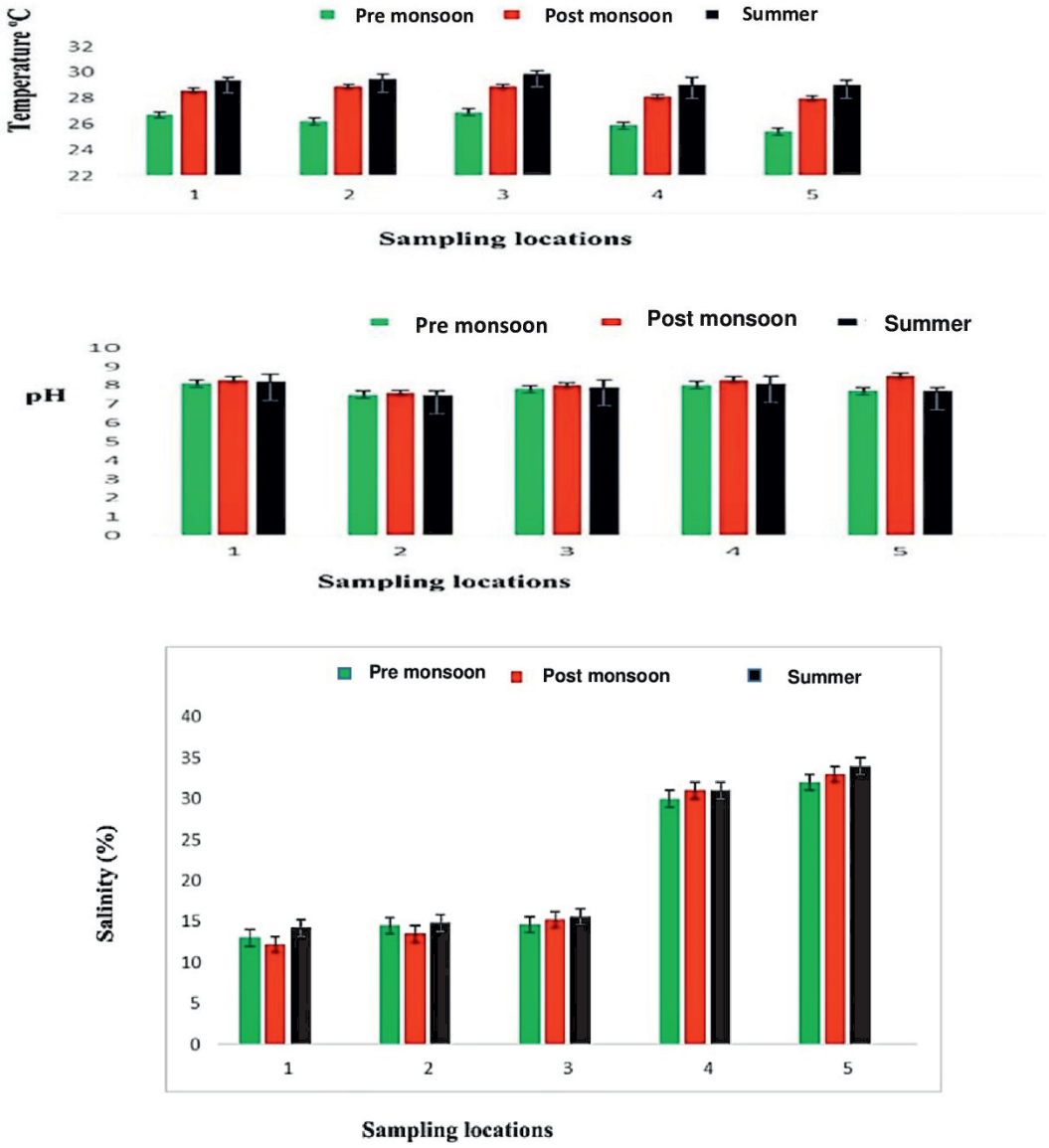

Fig. 2. Graph showing temperature, pH, and salinity of Adyar Estuary at different sampling locations ((1) Kotturpuram bridge (2) Greenways Railway bridge (3) Thiru Vi ka bridge (4) Broken bridge (5) Mouth of Adyar Estuary) Error Bars represent ± Standard Error (N = 5).

28.9±0.2°C-29.9±0.2°C respectively. In the present study, the pattern of temperature variation was same in all the seasons. However, when compared with pre-monsoon and post-monsoon seasons, there was an increase in temperature during summer. In general, the temperature is influenced by solar radiation, evaporation, inflow of fresh water and flow from adjoining coastal waters. The higher temperature during summer might be due to the intensity of solar radiation, and high evaporation rate. The results of the present study are in agreement with the findings of previous researchers [49, 50]. Likewise, the pH of the samples was from 7.5±0.2-8.1±0.8, 7.6±0.4-8.5±0.2, and 7.5±0.4-8.2±0.4 during pre-monsoon, post-monsoon, and summer respectively. The salinity of the samples recorded was 13%±2.2-32%±2.2, 12.2%±2.4-33.2%±2.4 and 14.2%±2.2-34.0%±3.2 for pre-monsoon, post-monsoon, and summer respectively. The alkaline nature of the water is indicating the dominance of marine water inflow [46]. The findings of present study are in concordance with the results of [51, 52]. Further, previous studies suggested that estuaries were driven by the magnitude of river discharges, resulting in variations in physical, chemical, and biological characteristics [53].

The BOD was between 4.1±0.4-10.1±1.2 mg/L, 3.0±0.4-9.2±1.0 mg/L, and 2.8±0.4-9.6±1.4 mg/L respectively for pre-monsoon, post-monsoon, and summer seasons (Table 1). From the results, it is known that in all the seasons, the BOD was higher at riverine component when compared with that of the mouth of the estuary. It has been suggested that the discharge of effluent, and dumping of solid waste, might increase the BOD of the riverine component [54]. Further, the addition of organic matter into the river from feacal matter by the surrounding urban area, and human settlement might be the reason for high BOD [55].

The phosphate content of the samples collected from different sampling locations of Adyar estuary was 0.83±0.2-2.10±0.2mg/L, 0.75±0.4-2.01±0.2 mg/L, and 0.73±0.2-1.97±0.4 mg/L for pre-monsoon, post-monsoon, and summer seasons (Table 1). The phosphate concentration in Adyar estuary is attributed with sewage disposal [54]. However, the less phosphate content at estuary mouth might be due to the limited flow of fresh water, high salinity, and utilization of phosphate by phytoplankton [56, 57].

Coliforms and Biofilm in Adyar Estuary

The surface water samples collected from different sampling locations of Adyar estuary was studied and the results are furnished in Fig. 3. The coliform population was found to be 75±6-228±12 MPN/100 mL, 47±6-197±12 MPN/100 mL, and 36±2-75±6 MPN/100 mL respectively for pre-monsoon, post-monsoon, and summer seasons respectively (Fig. 3). The development of red colonies with a metallic green sheen on M Endo agar medium indicated the occurrence of coliforms in the Adyar estuary (Fig. 4). In general, pathogen levels in surface waters are monitored by measuring the pathogen indicator organisms such as coliforms [58]. Indiscriminate discharge of domestic sewage makes some of the estuaries active hotspots of coliform pollution, deteriorating the entire water quality [59]. It is evident from the present investigation that the coliform population was higher in the riverine component when compared with that of the mouth. The findings of the present study are in agreement with the findings of Soueidan et al [60]. Many studies have reported that freshwater input coupled with storm-water runoff decrease salinity levels, facilitating the survival of coliform bacteria in riverine waters [61].

However, on CRA medium the black crystalline colonies developed were denser during summer for the samples collected from different sampling locations of Adyar estuary when compared with that of pre-monsoon and post-monsoon seasons (Table 2). Further, it has been noted that denser colonies were developed for the samples collected nearby ocean (Broken Bridge, and mouth of the estuary). The findings of present study are in accordance with the findings of Zhang et al. [62] explaining that season and location are

Table 1. BOD and Phosphate content of surface water samples collected from different sampling locations of Adyar Estuary.

Season/ Parameters	Chemical Parameters									
	BOD (mg/L)*					Phosphate (mg/L)*				
	Sampling location									
	1	2	3	4	5	1	2	3	4	5
Pre-monsoon	10.1± (1.2)	6.5 ± (0.8)	4.6± (0.4)	2.9± (0.2)	4.1± (0.4)	1.96± (0.2)	1.66± (0.8)	2.10± (0.2)	1.23± (0.2)	0.83± (0.2)
Post- monsoon	9.2 ± (1.0)	6.6± (1.0)	5.4± (0.8)	3.0± (0.4)	4.2± (0.2)	1.66± (0.4)	1.78± (0.2)	2.01± (0.2)	0.99± (0.2)	0.75± (0.4)
Summer	9.6 ± (1.4)	6.5± (0.6)	5.3± (1.0)	2.8± (0.4)	3.9± (0.8)	1.88± (0.4)	1.69± (0.2)	1.97± (0.4)	1.16± (0.2)	0.73± (0.2)

*Values represent mean of five determinations
Values in parenthesis represent±Standard Error

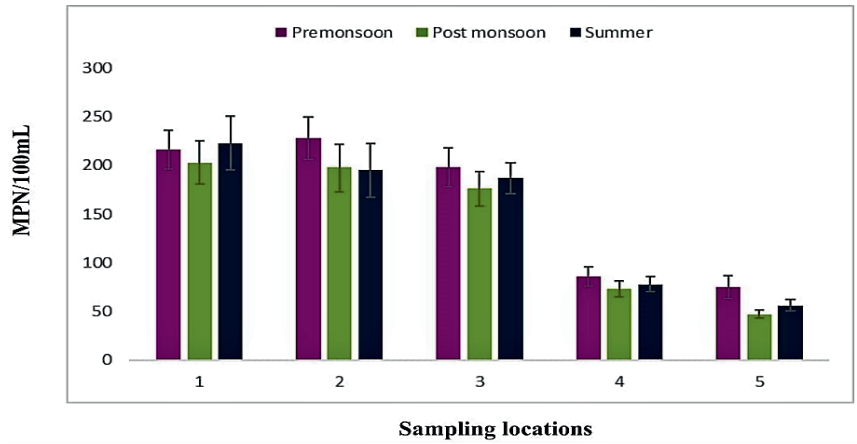

Fig. 3. Most probable number of Coliforms isolated from different sampling locations of Adyar Estuary {(1) Kotturpuram bridge (2) Greenways Railway bridge (3) Thiru Vi Ka bridge (4) Broken bridge (5) Mouth of Adyar Estuary} Error Bars represent ± Standard Error (N = 5).

the key factors determining biofilm formation. Boni et al. [63] suggested the presence of coliforms in biofilms in aquatic environment. Further, salinity is a key factor responsible for biofilm formation. The osmotic shock generated by salinity might have triggered the cells to develop biofilm to protect the cells from adverse environmental conditions [64].

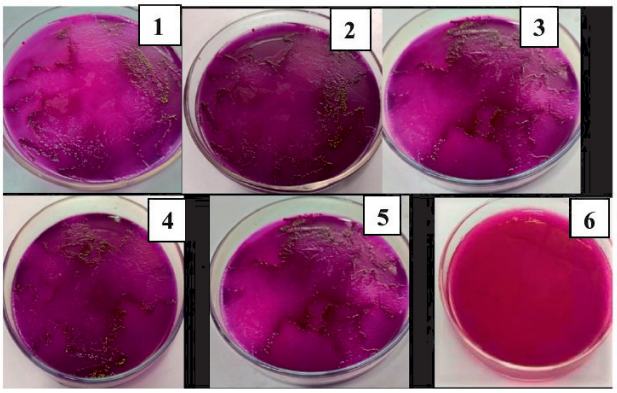

Fig. 4. Growth of *Escherichia coli* with metallic sheen colonies in M-Endo Agar medium isolated from surface water samples collected from different locations of Adyar Estuary {(1) Kotturpuram bridge (2) Greenways Railway bridge (3) Thiru Vi Ka bridge (4) Broken bridge (5) Mouth of Adyar Estuary (6) Control}.

MPs in Adyar Estuary

MPs are of significant concern because of their potential physical impacts and toxic effects on organisms following ingestion. The MPs content of surface water samples collected from different locations of Adyar estuary is presented in Table 3. It is observed that MPs content was in the range of 90-230 particles/L, 70-210 particles/L, and 90-190 particles/L respectively for pre-monsoon, post-monsoon, and summer seasons respectively. In all the cases, sampling location 1(near Kotturpuram Bridge) reported the maximum number of MP particles when compared with that of mouth of estuary. The rapid industrialization coupled with informal settlements along the river banks led to heavy pollution in aquatic systems [65-67]. Further, studies have demonstrated that about 10% of the untreated wastewater is discharged in to the Adyar river and more accumulation of MPs exists in areas where water collects [68, 69]. Hitchcock et al. [70] suggested that MP pollution is more in places where human impact is more and positively related with antecedent rainfall.

Table 2. Growth of black crystalline colonies on CRA medium.

Season/Number of positive plates	Sampling locations				
	1	2	3	4	5
Pre monsoon	+	++	++	++	++
Post monsoon	++	++	+++	+++	+++
Summer	+++	+++	+++	+++	+++

{(1) Kotturpuram bridge (2) Greenways Railway bridge (3) Thiru Vi Ka bridge (4) Broken bridge (5) Mouth of Adyar Estuary} (+ less growth, ++ moderate growth, +++ more growth).

Table 3. Microplastic content in surface water samples of Adyar estuary.

Season/Number of positive plates	Number of MP particles/L				
	Sampling locations				
	1	2	3	4	5
Pre monsoon	230	190	140	130	90
Post monsoon	210	200	160	100	70
Summer	190	210	180	110	90

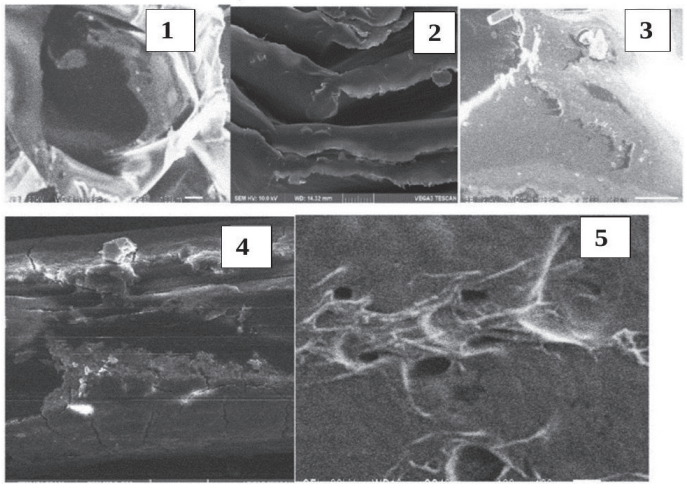

Fig. 5. Electron Micrographs of degraded and weathered MP fragments examined in surface water samples of Adyar Estuary ((1) Kotturpuram bridge (2) Greenways Railway bridge (3) Thiru Vi Ka bridge (4) Broken bridge (5) Mouth of Adyar Estuary).

The SEM images of MP fragments collected from the sampling locations were found with rugged surfaces (Fig. 5). Previous studies suggested that when plastics are exposed to factors such as temperature, radiation, and moisture content, they are broken into small particles [71]. The investigation of our finding is in agreement with the findings of Don et al [72]. The surface of virgin plastics is smooth, and flat, whereas the surface of weathered plastics is rough, and irregular with cracks, fractures, notching, pits, and bumps [72].

MP Associated Biofilm Formation under Laboratory Condition

The locally available plastics representing PE, PA, and Nylon incubated in estuarine water were extracted by vacuum filtration and transferred to Petri plates containing CRA medium. The development of black crystalline colonies confirmed the formation of biofilm (Fig. 6). The biofilm forming strains develop colonies of rough and black color [73]. Further, the biofilm developed on MPs was confirmed by SEM analysis (Fig. 7). Among the different polymer type, more bacterial adhesion was observed on PE, when compared with that of PA, and Nylon suggesting biofilm formation. It is evident from previous studies that microorganisms could be easily colonizing plastics in aquatic environment, and causing physical and chemical changes on plastics including degradation [74, 75]. In addition, bacteria feeding on organic particles within the polymer may create conducive conditions for microorganisms to colonize on different types of plastics leading to the formation of biofilm on plastics [76, 77]. Furthermore, biofilm formation might reduce the hydrophobicity of PE MPs and increase the abundance of hydrophilic C-O and C=O groups on the surface of PE. In addition, on exposure to seawater, MPs absorb organic and inorganic nutrients from seawater and form a conditioning film by the deposition of biomolecules namely glycoproteins, lipids, nucleic acids, ions, polysaccharides and proteins [78]. However, previous studies suggested that, the attachment of microorganisms to MP depends on the particle size and polymer type [79], adsorption property [80], geographical location [81]. But, Duanet al [82], reported that microbial colonization on plastic surfaces did not significantly vary with the type of polymers, but it was by the surface hydrophobicity, morphology, and chemical gradient of the plastisphere. In the present study, coliforms present in the estuary water might have contributed for biofilm formation. Coliforms produce exopolysaccharides, which facilitate attachment or adhesion on substrates [83]. However, thorough study is necessary to explore the bacterial community

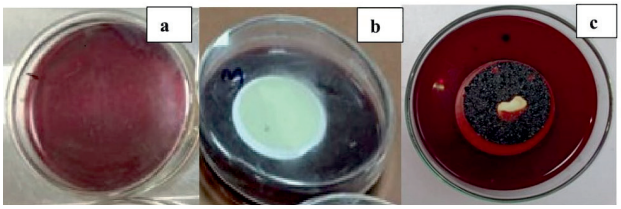

Fig. 6. Photograph showing black crystalline colonies developed on CRA medium indicating biofilm formation on MPs (a) CRA medium immediately after plating (without sample) b) CRA medium with filter paper (without colony development) c) Biofilm (black crystalline colonies) developed on filter paper with MPs in CRA medium.

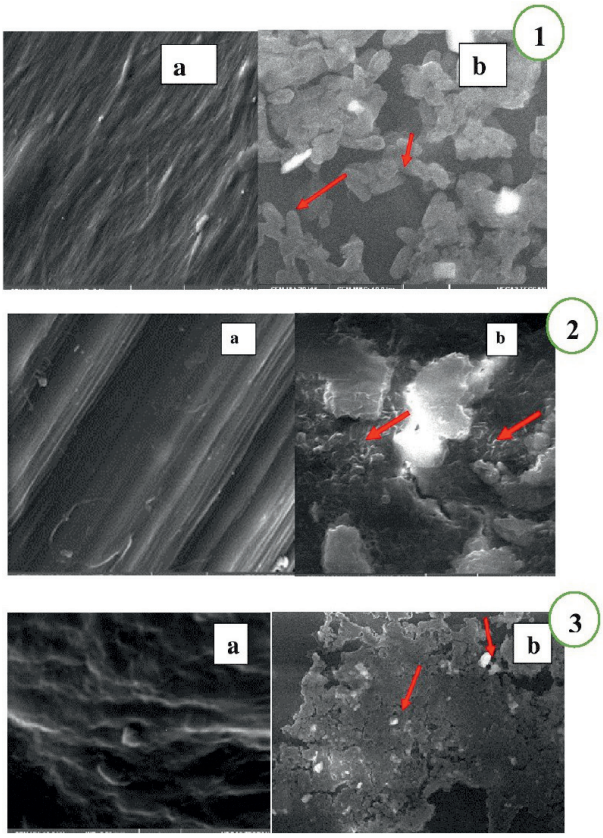

Fig. 7. Biofilm formation on virgin MPs immersed in estuarine water (1) Polyethylene (2) Polyamide (3) Nylon (Red arrows indicate colonization of bacteria on MP). n all the cases (a) Virgin (b) bacterial colonization and weathered and degraded surfaces of MPs.

structure within the biofilm, and composition of various biomolecules secreted by bacteria within the biofilm.

Conclusions

The present study, explored the occurrence of coliforms, MPs, and biofilm in estuarine ecosystem. The coliform population was more at riverine side when compared with that of mouth of estuary irrespective of season. The development of biofilm was more prevalent in samples collected from estuarine mouth irrespective of season suggesting salinity is the key factor for biofilm development. The MPs extracted from estuarine water and visualized under SEM revealed the presence of cracks, pits, and grooves explained the occurrence of either weathering or microbial degradation. *In situ* study conducted to examine the development of biofilm on different polymers insinuated that adhesion was more on surface of PE than that of PA and Nylon. Hence, based on the results of present investigation, it is concluded that MPs can serve as a hub for coliforms in estuaries. Since, MPs can enrich antibiotic resistant bacteria (ARB) and pathogens that are causing adverse effect on human and natural biota, more research is needed. The different abiotic and biotic factors influencing MP based biofilm must be focused. Furthermore, knowledge based on scientific findings must be shared with the public in order to take immediate steps to prevent the entry of plastic and sewage into estuaries.

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Conflict of Interest

The authors declare no conflict of interest.

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