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Research Article

Enumeration of aerobic heterotrophic bacteria in early-stage of biofilm formed on different plastic types

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Abstract

Microplastic pollution is a global concern. In aquatic environment, microplastics act as a new substrate that increases the adhesion of the biofilms. Extracellular polymeric substance (EPS) in the matrix of the biofilm can interact with metal ions and inorganic/organic contaminants. Therefore, microplastics with biofilm can pose serious threats to human and environmental health. In this study, the total number of aerobic heterotrophic bacteria (AHB) in early-stage of biofilm formed on different plastic types commonly used in daily life, namely polyethylene (PE), polypropylene (PP) and polystyrene (PS) was investigated in a water recirculating test system. The Golden Horn Estuary's water was used as a system water. The test system with plastic coupons was operated continuously for 504 h and natural biofilm formation was ensured on the plastic surfaces. The physicochemical properties of system water were measured and bacterial count was performed at each sampling time. Bacterial attachment was observed on all plastic surfaces even within the first hours. Also, macroscopic images proved that the biofilm layer formed and flourished on all plastic surfaces over time. After 504 h, the levels of AHB on the plastic coupons were detected as 1.4×10^5, 1.2×10^5 and 1.8×10^5 cells cm^{-2} for PE, PP and PS, respectively. To investigate the number of bacterial cells in early-stage of biofilm formation on plastic surfaces will create an important basis for future research in terms of evaluating the effects of microplastics on the environment and human health.

Keywords: Aerobic heterotrophic bacteria (AHB), Biofilm, Polyethylene, Polystyrene, Polypropylene

Introduction

Plastic pollution is one of the most serious problems of the century (Thompson et al. 2009). Although plastic production is quite high in the world, there is not enough adequate infrastructures to manage waste or recycle. Plastic waste causes environmental pollution due to their uncontrolled release into nature. These wastes can be transported over vast distance by wind, rain or rivers. During this period, plastics break down into small pieces and turn into a structure called "microplastic". According to the US National Oceanic and Atmospheric Administration (NOAA) microplastics are plastics that are smaller than 5 mm and have an irregular shape and size (Ruz et al. 2012). Microplastics can enter our daily lives with products such as drinking water, table salt, soap, shampoo, mascara, toothpaste, and clothes etc., and have a negative impact on the life of all living things in nature (Yuan et al. 2022). They pose a great danger to the environment and human health due to the fact that they act as reservoirs for microorganisms and pollutants.

Micro or macro-organisms tend to attach to the surfaces to enhance their survival. They can attach more readily to non-polar surfaces (Teflon and other plastics) than to hydrophilic materials (glass or metals) (Donlan, 2002). Hydroids, fungi and pathogenic bacteria can also adhere to the microplastics surface and live for a long time and be easily transported in the marine environment (Carpenter and Smith, 1972; Rummel et al. 2017). Biofilm formation is a complex and dynamic process (Unsal et al. 2019). In the first step of bacterial attachment, organic and inorganic molecules in the bulk are carried toward the surface. These molecules at the solid-liquid interface on surfaces form a conditioning film layer that facilitates the bacterial attachment (Palmer et al. 2007). After the bacterial attachment, microorganisms flourish and form biofilms. Extracellular polymeric substances (EPSs) are biopolymers, synthesized by microorganisms, play an important role in the stage of formation of biofilm.

The Golden Horn Estuary, which is Istanbul's natural harbor that the terrestrial and anthropogenic inputs cannot be completely prevented, was selected as a sampling area. Metal pollution (Balkıs et al. 2011), toxic diatoms (Tas et al. 2016) also, pharmaceutical compounds were detected in high amounts in this area (Korkmaz et al. 2020, 2022). Therefore, the use of the estuary water is a very important source to form the natural biofilms.

EPSs can interact with inorganic-organic pollutants, and metal ions (Bonnineau et al. 2021). Also, they may affect the dynamics and the interactions between biofilms and microplastics. Microplastics with biofilm can increase the environmental and human health risks. Therefore, it is significantly essential to investigate biofilm formed on microplastics. In this work, the total number of aerobic heterotrophic bacteria (AHB) during the early-stage of biofilm formed on different plastic surfaces was investigated. Three different types of

plastic (polyethylene, polystyrene and polypropylene), which have the highest rate of waste in nature, were placed in a water recirculating test system. The system was operated continuously for 504 h and natural biofilm formation was provided.

Materials and Methods

Sampling area

Water samples were taken from the Golden Horn Estuary (approximately from 0-50 cm depth), Kasımpasa region under aseptic conditions. Water samples were taken weekly. All the samples were transported to the laboratory within 1 h. The sampling point is shown in Figure 1.

Fig. 1. Sampling point (Google Earth, 2022).

Test specimens

Three different polymer types were used (Polyethylene (PE), Polypropylene (PP), Polystyrene (PS)) (Figure 2). All the coupons were prepared as 25x25x10mm dimensions for the experiment. A hole (2.5 mm diameter) was drilled on the edge of the coupons. Before the experiments, the coupons were washed with sterile distilled water then cleaned with acetone and dried in a Pasteur oven. All the coupons were sterilized under ultraviolet light for 4 h.

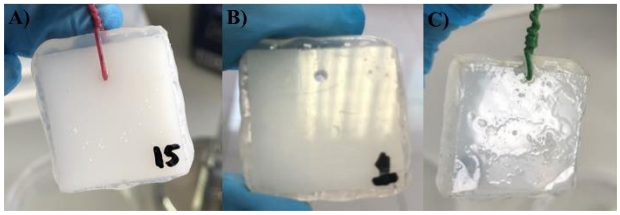

Fig. 2. A) Polyethylene (PE), B) Polypropylene (PP), C) Polystyrene (PS) coupons.

Installation and operation of water recirculating test system

A glass lab-scale (40-L) water recirculating test system was used (Figure 3). The test system was consisted of a pump, the coupon carriers, and coupons. The test system was filled with 20 L Golden Horn water (pH: 7.45, dissolved oxygen (DO): 23,1 mg/L, total suspended solids (TTS): 335 mg/L) and the system was kept at room temperature. The direct entrance of natural light and fresh air were allowed into the system. One liter water was discharged from the recirculating system daily and fresh make-up water was added (2 ± 0.25 L/day). During the experiment period, the evaporation rate was high level. Therefore, more water was added to the system to compensate for water losses by evaporation.

Natural biofilm formation was provided on the coupon surfaces. No extra bacteria or supplements were added to the water. A carrier grid made of steel, was prepared, and placed on the test system to hang the coupons. The coupons were connected to the grid by copper wire. After placing the coupons into the test system, the system was operated for 504 h to observe the early stage of the natural biofilm formation. The coupons were removed from the system after 0, 4, 8, 24, 48, 72, 96, 168, 336 and 504 h.

The physico-chemical parameters such as pH, temperature, total suspended solids (TTS) and dissolved oxygen were measured. Temperature and pH measurements were measured by multiparameter device (YSI Professional Plus model). Dissolved oxygen was measured by Winkler method (Winkler, 1888; APHA, AWWA, WPCP, 1985).

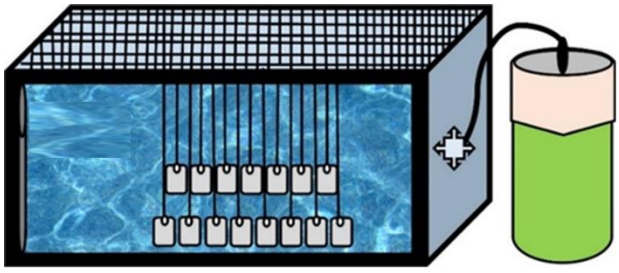

Fig. 3. Water recirculating test system

Enumeration of total aerobic heterotrophic bacteria

Water samples were taken from test system and concentrated by filtration through a sterile 142 mm diameter and 0.22 µm pore size (Sartorius) nylon membrane filter. Filtered samples were re-suspended in 20 ml of sterile Golden Horn Estuary water using Stomacher Lab Blender (Interscience BagMixer) for 2 min. Suspansions were serially diluted 10^{-1} to 10^{-7} and used to enumerate AHB in the test system.

To count AHB in biofilm, all the coupons were rinsed with phosphate buffered saline (PBS) to remove loosely attached planktonic cells. Biofilms on coupons were removed using a sterile applicator. The biofilm constituents were vortexed for 1 min in a 10 mL sterile Golden Horn water. After that the bacterial suspensions were serially diluted to 10^{-7}. R2A agar medium (Merck) was used to enumerate planktonic and sessile bacteria. Plates were incubated at 28 °C for 7 days. All plate counts were performed in triplicate. After the incubation, the colonies were counted by a colony counter (Funke Gerber, Colony Counter) and recorded as colony forming unit (CFU) (Reasoner ve Geldrich, 1985).

Statistical analysis

AHB levels and plastic types comparison was done by one-way ANOVA and Tukey's post-hoc tests. The statistical analysis was performed using IBM SPSS statistics program.

Results

The physicochemical parameters of water samples were determined. Also, the total AHB levels were detected in the test system water and biofilm on plastic surfaces. The chemical parameters of water samples were given Table 1. During the experiment, the average of the temperature values was around 21 ± 1.5°C. The pH values of the test system increased until 48 h then decreased at 96 h and reached the highest value (8.38) at 504 h. The concentrations of DO increased at 4 h then a sharp decrease was observed after 8 h. The lowest value of DO was found as 7.8 mg/L at 96 h. Also, the values of TTS decreased with time and found as 44 mg/L at 504 h.

Table 1. Chemical parameters of test system water over 504 h.

Time (h)	pH	DO (mg/L)	Temperature °C	TTS (mg/L)
0.5	7.45	23.1	21	335
4	7.68	26.6	21.7	64
8	7.72	9.0	22.6	143
24	7.82	8.3	21.9	96
48	7.85	8.0	22.3	103
72	7.79	8.1	22.1	35
96	6.37	7.8	21.8	40
168	7.75	9.7	19.5	32
336	8.37	8.9	21	36
504	8.38	8.3	22.1	44

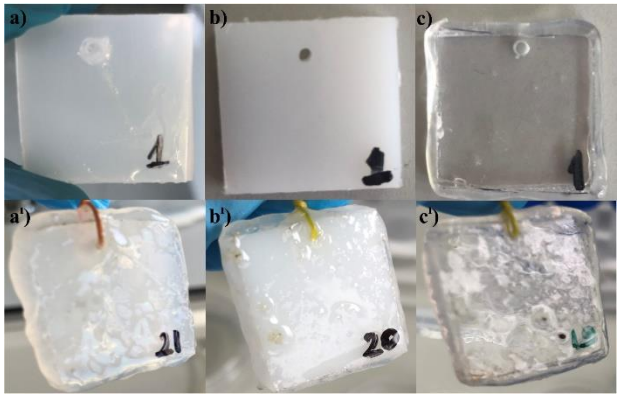

Fig. 4. Macroscopic view of biofilm on plastic coupons after 0 h (a, b, c) and 504 h (a', b', c') of exposure to test system water ((a, a') PE, (b, b') PP, (c, c') PS).

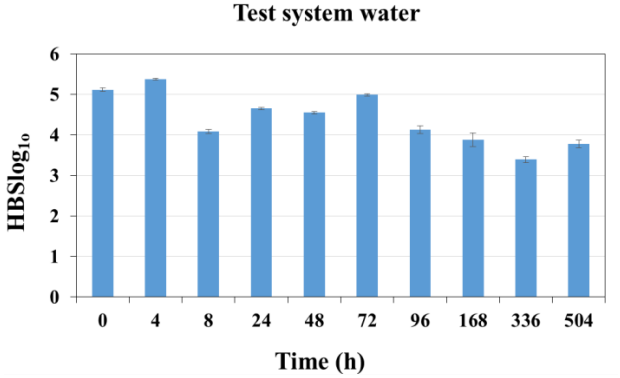

Fig. 5. The levels of total aerobic heterotrophic bacteria in the test system water for 504 h.

These results indicated that the biochemical reactions took place within the test system. Macroscopic images also showed that a biofilm layer formed and flourished on the plastic surfaces over time (Figure 4). After 0 h, the biofilm layer was not visible. However, the biofilm appeared in a yellowish-light brown color and gelatinous after 504 h.

The levels of total aerobic heterotrophic bacteria in the test system water were given in Figure 5. Irregular increases and decreases were detected in the number of bacteria in the test system water during the experiment. The maximum value was determined after 4 h as 2.3×10^5 cfu/ml. After 72 h, the number of bacteria decreased until 336 h and reached the minimum value (2.5×10^3 cfu/ml). However, a slight increase was found after 504 h (Figure 5).

For the first 8 h, the levels of AHB were similar for all plastic types (Figure 6). After 24 h, an increase was detected in the levels of AHB on PE and PP plastic surfaces. The maximum AHB level was found on PE plastic surface at 48 h as 4.2×10^5 cells cm^{-2}. However, after 72 h, the bacterial count decreased and reached its lowest level. At the end of the experiment, the level of AHB on the plastic coupons were detected as 1.4×10^5, 1.2×10^5 and 1.8×10^5 cells cm^{-2} for PE, PP and PS, respectively.

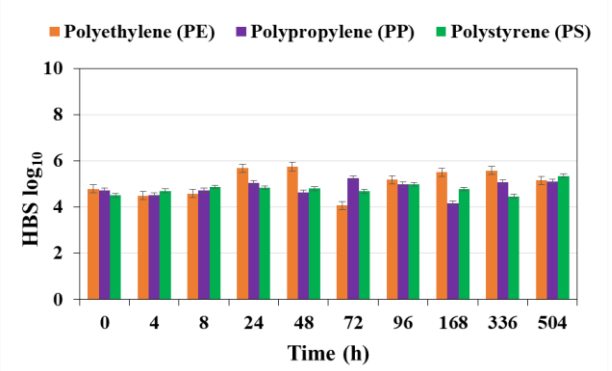

Fig. 6. The levels of total aerobic heterotrophic bacteria in biofilm on PE, PP and PS surfaces for 504 h.

Discussion and Conclusion

Biofilm formation can be described in five steps:

(i) reversible attachment,
(ii) irreversible attachment,
(iii) the production of EPS,
(iv) biofilm maturation and
(v) biofilm dispersal (Muhammed et al. 2020).

The initial attachment is the fundamental part to develop biofilm formation that encourages the other cells to attach to surfaces and maintain their survival. The surface recognition could be fast by bacteria; however, the maturation process may take within weeks (Unsal et al. 2023).

In this study, the bacterial attachment was observed on all plastic coupons exposed to the Golden Horn Estuary's water even within the first hours (Figure 4 and 6). Some researchers also reported that the microorganisms can colonize immediately on floating plastic surfaces (Kaiser et al. 2017; Lobelle and Cunliffe 2011). These colonizing microorganisms are generally bacteria, diatoms, coccoliths and bryozoans (Reisser et al. 2014).

Contrary to the number of AHB in biofilm (sessile cells), the AHB in the water (planktonic cells) reached the maximum value after 4 h ($2.3x10^5$ cfu/ml). Afterwards the fluctuation was observed in the number of planktonic cells (Figure 5). It indicates that the bacteria switch from the water phase to biofilm phase is faster, in particular within the first hours. After the biofilm reached a certain maturity, a decrease was observed in transition of bacteria to water (Figure 6). As the biofilm matures, EPS hold the cells together and provide protection from different stress conditions (Costa et al. 2018; Vu et al. 2009). During the experiment, it was determined that the total number of bacterial cells did not increase regularly over time. Carson et al. (2013) reported also similar results. It shows that the formation and development processes of biofilm have a dynamic structure (Unsal et al. 2019). The maximum level of AHB was detected on PE after 48 h. Also, statistical analysis showed that there is no relationship between AHB levels and plastic types ($p>0.05$). Bacterial attachment depends on many factors such as material surface topography, structure, roughness, surface charge and stiffness (Zheng et al. 2021). All types of plastic have different properties like metals, such as surface properties, crystallinity, particle shape, polymer type and additives compounds (Lambert et al. 2017). Since the morphological structures of these selected polymers will play an important role in biofilm development, each polymer type was also examined in terms of surface morphology and the degree of crystallinity. The degree of crystallinity indicates whether the polymer chains are crystalline, that is, in regular stacking form or tend to be more amorphous. Generally, produced under the same conditions the degree of crystallinity of the polymers from highest to lowest are polyethylene, polypropylene and polystyrene, respectively (Ebewele, 2000; Fried, 2014). This factor also affects the process of bacterial adhesion to plastic surfaces (Almaguer-Flores et al. 2015). McGivney et al. 2020 reported that physicochemical properties of PE, PP, PS were changed during the biofilm formation. Also, they found that the degree of crystallinity of PE was increased after biofilm formation. In this study, the maximum number of AHB was detected on PE. An increase in crystallinity may help biofilm development.

Biofilms act a vector to spread the pathogens (Cholewińska et al. 2022). Also, the formation of biofilms on microplastics in water has high environmental risks for aquatic animals (He et al. 2022). Thus, to investigate the physical, chemical and biological structure of biofilm on plastic surfaces (such as the isolation and identification of bacteria in biofilm and the determination of the EPS content) is an important basis for future research in terms of evaluating the effects of microplastics on the environment and human health.

The following conclusions can be drawn from the obtained data.

- Bacterial attachment was observed on all PE, PP and PS coupons.
- During the early-stage of biofilm formation total number of bacterial cells on plastic surfaces was unstable.
- The maximum AHB level was found on PE plastic surface at 48 h as 4.2×10^5 cells cm^{-2}.
- The bacterial attachment depends on many factors such as material surface topography, structure, the degree of crystallinity, roughness, surface charge and stiffness.

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