Biofilm Growth Characteristics at Different Diesel Leakage Concentration

Fang Liu, Wei Yang, HuiYun Zhong, JinJin Lu, and Chaocheng Zhao

Abstract—**Petroleum products leakages result in important effects on the normal operation of circulating cooling water system. However, relatively little research has been done to explore effects of petroleum products leakages on biofilm growth characteristics. In this study, diesel as the experimental subject representing petroleum products, effects of diesel addition on biofilm growth characteristics were investigated. Increase of diesel addition led to biofilm EPS increase with diesel concentration less than 200 mg/L, then EPS content were kept relatively stable with diesel concentration more than 200 mg/L. Protein contents were found at relatively higher levels than polysaccharides in the biofilm with diesel concentration more than 200 mg/L. Expect for 100 mg/L diesel, increasing diesel concentration enhanced biofilm detachment ratio with respect to the control test. Although biofilm wet weight tended to increase with diesel concentration rising from 0 to 1500 mg/L, there was a marked reduction in lipid phosphorus content with increase of diesel concentration from 200 to 1500 mg/L. The reduction indicated that diesel was toxic to microbial population in biofilm.**

Index Terms—**Circulating cooling water, leakage, diesel, biofilm, growth characteristics.**

I. Introduction

In the circulating cooling water system of petroleum refineries in China, petroleum products leakages generally exist. Poor quality of heat exchangers, outdated hermetic sealing technique, aging pipelines, improper operation and other reasons all can lead to petroleum products leakages. In order to reduce the leakage hazards, refineries usually increase pollutants emissions, displace the systems and reduce the concentration index [1]. The above operation modes undoubtedly intensify the contradiction between water consumption increasing and water resources shortage. Further, if sewage from circulating cooling water is discharged into natural water, residues of petroleum products will cause biohazardous effects on human beings as well as other living organisms in the environment. So, petroleum products leakages should be paid enough attention.

Biofilm in general is defined as the accumulation of deposits associated with living organisms growth such as microorganisms (bacteria and algae) and macro-organisms (sponge species, etc.), which are very common, especially in industrial cooling towers [2]. Biofilm can cause equipment damage through corrosion, down time, decrease energy efficiency due to increasing hydraulic pressure (pumping costs), local clogging of cooling towers and increasing heat transfer resistance [3]. According to researcher's result [4], almost half of the defects in cooling water systems were due to problems related to biofilm. Biofilm problems do not arise from microorganisms which have suddenly invaded the system, but are much more likely to be caused by an increase in nutrient concentration or an absence of inhibiting factors. Wijeyekoon et al. manifested that there was obvious difference on structure, porosity and distribution of biofilms formed under different organic carbon concentrations [5]. Shafahi et al. found that organic carbon concentrations affected biofilms' thickness and pore structure directly [6].

Biofilm is composed of microbial cells and extracellular polysaccharide structures (EPS). EPS can enhance biofilm resistance to environmental stress and antimicrobial agents [7]. The production of EPS is known to be affected by nutrients status of the growth medium and the availability of carbon. For these reasons, the biofilm which grows at different nutrient levels in the circulating cooling water system will have the different EPS production and structure. There are contradictory reports in the literature about EPS composition especially with the ratio of protein to polysaccharide (PR/PS). Some researchers indicated that certain EPS from wastewater biofilms had a higher concentration of proteins than polysaccharides and some others showed that polysaccharides were found to be dominant in the biofilm [8], [9].

A better understanding of biofilm behavior is particularly important due to many serious problems associated with biofilm presence. Once developed, the biofilm was harder to be removed completely [10], [11]. Mechanical forces is a parameter often involved in biofilm removal, since the application of sole chemical agents tended to leave the biofilm intact when no mechanical treatment was implemented in the control process [12]. An important biofilm feature involved in the recalcitrance to current biofilm control procedures is the mechanical stability. If petroleum products leak into the circulating cooling water, leakages will affect the biofilm mechanical stability by influencing biofilm structure. This problem is worth studying for ensuring normal operation of circulating cooling water system.

Understanding the contribution of petroleum products leakage to biofilm development are important requirements for cooling water system management strategies. However, little experimental research has been done about the effects of petroleum products leakage on controlling biofilm formation

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and development in the circulating cooling water system. This study was therefore carried out to investigate the effect of petroleum products leakages on biofilm growth characteristics in a laboratory-scale circulating cooling water system.

II. Materials and Methods

A. Biofilm Formation

Representative industrial water from recirculating cooling water system of refining corporation was used as inoculums for research purposes. The inoculating bacteria from suspended microorganism in the recirculating cooling water system were cultivated in individual 2.0 L glass beaker filled with medium of nutritive proportion, which were prepared by adding to each liter of tap water with a measured amount of carbon source (glucose), ammonia nitrogen (ammonia sulfate) and TP (sodium hydrogen phosphate). In the medium, the ratio C/N/P was 50/10/1. All the media were sterilized at 121 ℃, 15 psi for 20 min. During experimental period, temperature of water bath for microorganism's growth was maintained at 32±1℃ by a thermostat, pH remained between 7 and 9, steady rotating speed of 100 rpm was implemented by mechanical stirrers attached to model device to ensure uniform nutrient solutions and shear on biofilm. Dissolved oxygen concentration was more than 2 mg/L consistently.

Standard stainless steel coupons (AISI 304, 50×25×2 mm^3) were used for microorganism's attachment and consequent biofilm formation. Before used, the coupons were washed in distilled water and air dried at room temperature. The cleaned coupons were stored in desiccators. Three stainless-steel coupons were used in every experiment. The biofilms were allowed to grow for 7 days in order to obtain steady-state biofilms.

B. Addition of Diesel

After steady-state biofilms were obtained, diesel oil was added into the reactors. The diesel additions were 0, 100 mg/L, 200 mg/L, 500 mg/L, 800 mg/L, 1000 mg/L, 1300 mg/L and 1500 mg/L, respectively. The diesel density is 0.84 g/cm^3 at 20℃ and the viscosity is 3.26 mm^2/s at 35℃. Zinc and iron contents in diesel are 2.38 μ g/g and 0.91 μ g/g, respectively.

C. Biofilm Sampling for Phenotypic Characterization

The biofilm on the stainless steel coupons was removed using a stainless steel scraper and afterwards resuspended in 50 mL of buffer solution (2 mM Na_3PO_4, 2mMNaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7) and homogenized by vortexing (Heidolph, model Reax top) for 30 s with 100% power input, according to the methodology described by Simoes [13]. The homogenized biofilm suspensions were then phenotypically characterized in terms of extracellular polymeric substances (EPS) content (proteins and polysaccharides).

D. EPS Extraction

EPS extraction of the biofilm was carried out using formaldehyde plus sodium hydroxide (NaOH) assay according to the procedure described by Liu and Fang [14].

E. Polysaccharide Content Quantification

The polysaccharide content in EPS was measured by the anthrone-sulfuric acid method using glucose as standard [15]. The data represented the mean of three measurements.

F. Protein Content Quantification

The protein content was determined by the method of Bradford using bovine serum albumin as standard [16]. The data represented the mean of three measurements.

G. Biofilm Detachment

Biofilm detachment was assessed by means of biofilm weight loss due to biofilm exposure in a rotating device. The stainless steel coupons with biofilm were removed from the 2.0 L bioreactors and then accurately weighted, marked W_1. Afterwards, the coupons were immersed into 0.5 L vessels containing 0.35 L distilled water. The coupons with biofilm were rotated at a constant speed of $300min^{-1}$ for 4 h in the rotating device. At 0.25h, 0.5h, 1h, 1.5h, 2h, 3h and 4h, the coupons with biofilm were removed from the device and then accurately weighted, marked W_2. In the end, after 4 hours, the coupons were removed from the device, and then washed away, accurately weighted, last marked W_3. So biofilm detachment ratio was described as the following equation:

$$S = \frac{W_1 - W_2}{W_1 - W_3} \tag{1}$$

H. Biofilm Wet Weight Quantification

The biofilm wet weight was calculated as the following equation:

$$m=(m_i - m_0)/A \tag{2}$$

In equation (2), m_i was the weight of coupon covering the biofilm, m_0 was the coupon weight, A was the coupon surface area. The results were expressed as mg per cm^2. The data represented the mean of three measurements.

I. Biofilm Biomass

The mixture of chloroform, methanol, and deionized water as the extractant to extract phospholipids from biofilm, and then added chloroform and deionized water to achieve stratification with a final ratio of 1:1:0.9. The lipid phase was separated and dried by nitrogen after stratification. The absorbance of the solvent was measured with a spectrophotometer after digestion. The detailed procedures of phospholipid analysis were referred to the reference [17].

J. Image Analysis of Scanning Electron Microscopy

The biofilm growing to the stable phase was selected. One group was the natural growth sample without diesel addition. And the other group was the sample with 500 mg/L diesel addition. The microstructure of biofilm was observed by S-4800 cold field emission scanning electron microscopy (SEM).

III. Results and Discussion

A. Effects of Diesel Concentration on Biofilm Chemical Composition

The effects of diesel concentration on biofilm polysaccharide, protein and EPS are shown in Fig. 1.

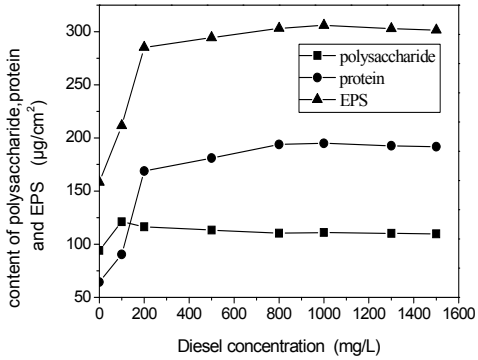


Fig. 1. Effects of diesel concentration on content of polysaccharide, protein and EPS.

Increase of diesel concentration led to increase of protein and EPS content when diesel concentration was less than 200 mg/L. Protein and EPS contents increased from 64.31 μg/cm^2 to 168.82 μg/cm^2 and from 158.42μg/cm^2 to 285.27μg/cm^2, respectively. Then protein and EPS content were kept relatively stable with diesel concentration more than 200 mg/L. While polysaccharide content reached the maximum (121.19 μg/cm^2) when diesel concentration was 100 mg/L, and then decreased slightly from 121.19 μg/cm^2 to 109.75 μg/cm^2 with diesel concentration increasing from 100 mg/L to 1500 mg/L .The obtained results suggested that diesel addition had the important impact on contents of polysaccharide, protein and EPS in biofilm at low diesel concentration. The ratio of protein to polysaccharide (PR/PS) was the key parameter for indicating biofilm structural characteristics [18]. Although polysaccharides have often been regarded as the most important extracellular components [19], proteins were found at relatively higher levels than polysaccharides in the biofilm with diesel concentration more than 200 mg/L in this study. PR/PS ranged from 0.68 to 1.75 when diesel concentration increased from 0 to 1500 mg/L (Fig. 2). This observation was consistent with work by CELMER who also observed that protein was the main component of biofilms [20]. Although diesel concentration has the important effect on biofilm chemical composition, PR/PS kept stable from 1.76 to 1.75 when diesel concentration increased from 800 to 1500 mg/L. The obtained results suggested that the biofilm structural was relatively stable after diesel concentration larger than 800 mg/L.

B. Effects of Diesel Concentration on Biofilm Detachment Ratio

Biofilm mechanical strength was assessed by biofilm detachment ratios on coupons at different times. Biofilm detachment ratios at different diesel concentration are shown in Fig. 3. The existence of shear stress force was higher than the one under which the biofilm was formed caused biofilm removal. It manifested that the biofilm removal was dependent on the hydrodynamic conditions. Fig. 3 shows that the increase in detachment time leads to an increase in biofilm detachment ratios, however, the biofilm detachment ratios keeps relatively stable after 2 hours. The biofilm mechanical stability, i.e., the behavior of biofilm facing external stress mechanical conditions, was of great impact for both wanted and unwanted biofilms [21]. In this study, the biofilm mechanical stability was assessed by submitting biofilm to the shear stresses, which could weaken the biofilm structure and promote detachment. The biofilm formed on the coupons of the rotating device prior to diesel addition was characterized in order to determine the inherent biofilm mechanical stability, since detachment processes may be dependent on it.

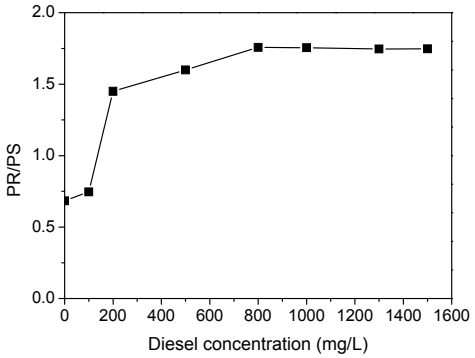


Fig. 2. Effects of diesel concentration on the ratio of protein to polysaccharide (PR/PS)

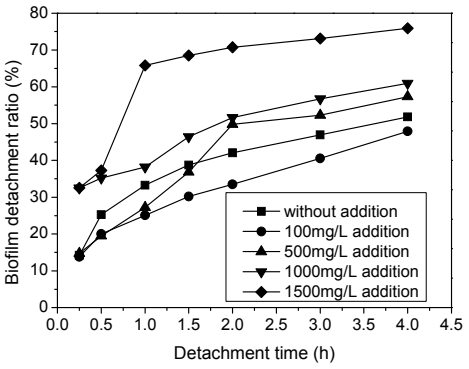
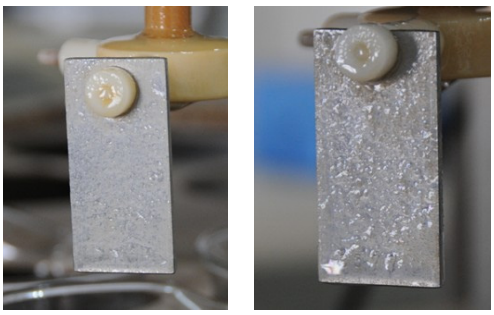


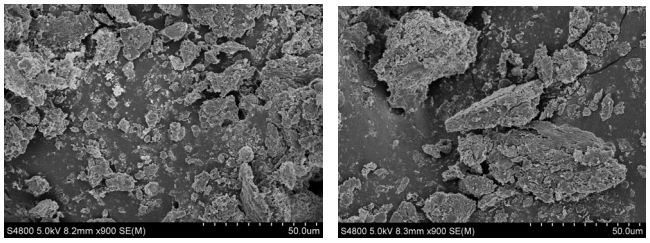
Fig. 3. Effects of diesel concentration on biofilm detachment ratio

The comparison of biofilm detachment ratios at different diesel concentrations, for the same shear stress force, showed that diesel had the important effect on biofilm detachment. Expect for 100 mg/L diesel concentration, increasing diesel concentrations enhanced biofilm detachment ratios with respect to the control test. It reflected that small content of diesel mainly made the adhesion between biofilm and coupons closer. When diesel of larger concentration was added, diesel and biofilm joined together into massive structure, which was easy to be peeled off. Moreover, larger diesel concentration may lead to the increase of biofilm thickness, which resulted in anaerobic conditions development within the biofilm. Because of the biofilm thickness and the activity of anaerobic species, the biofilm detached and sloughed off from the coupons. The results obtained from Fig.3 can also be manifested by image analysis of the biofilm (Fig. 4. to Fig. 6.).



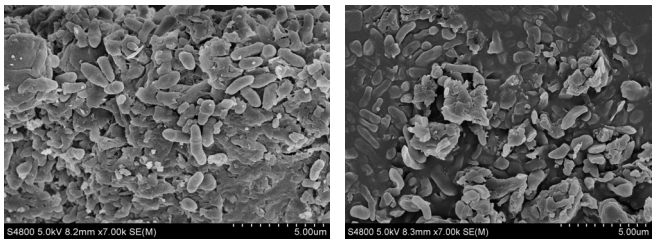
(a) coupon without diesel addition (b) coupon with 500mg/L diesel addition

Fig. 4. Coupon pictures at different diesel addition



(a) without diesel addition (b) 500mg/L diesel addition

Fig. 5. Biofilm SEM pictures at 900 times magnification



(a) without diesel addition (b) 500mg/L diesel addition

Fig. 6. Biofilm SEM pictures at 7000 times magnification

It was obvious from Fig. 4 (a) to Fig. 6(a) that without diesel addition, the structure of biofilm was loose and fragmentary. After adding 500mg/L diesel, the structure became large blocks and porosity became smaller (Fig. 4(b) to Fig. 6(b)), which might be explained that diesel could adsorb and adhere to biofilm. According to Fig.3, in a short period of stirring, the detachment ratios of biofilm with 500mg/L diesel were smaller than that in control test. However, when detachment time was longer than 2 h, the detachment ratios became larger, even exceeded that of without diesel addition.

C. Effects of Diesel Concentration on Biofilm Wet Weight and Biomass

Fig. 7 shows that the biofilm wet weight tends to increase with diesel concentration changing from 0 to 1500 mg/L. Because of its viscosity, diesel could adsorb suspended matters and biofilm, which resulted in a mixture of material fouling and bacterial biofilm formation. As mentioned in Fig.1, the increase of diesel concentration resulted in EPS rising. EPS was highly hydrated because it can incorporate large amounts of water into structure by hydrogen bonding [22]. The water increase also led to biofilm wet weight increasing.

The diesel addition has the obvious effect on the lipid phosphorus content (Fig. 8). The lipid phosphorous content in biofilm reached the largest value (about 1.0 μgP/cm^2) when diesel concentration was 200 mg/L. Diesel oil is a compound found as a component of petroleum hydrocarbons. It is a complex mixture of paraffins, cyclic alkanes and aromatic compounds having low water solubility, high adsorption coefficient and high stable aromatic ring [23]. The obtained results in Fig.8 showed that hydrocarbons in diesel at low concentration provided microorganism's carbon sources to promote their growth. And then, there was a marked reduction in the lipid phosphorus content with increase of diesel concentration from 200 to 1500 mg/L. The reduction, which indicated that diesel was toxic to part of the microbial population in biofilm, was related to the intensity of diesel concentration. Several authors have indicated that the hydrocarbons in petroleum had a toxic effect on the soil microbial community, especially presenting at high concentrations, which caused changes in the structure of the microbial community and a general decrease in the diversity and number of microorganisms [24],[25].

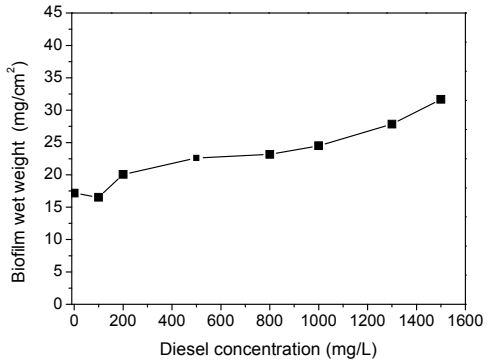


Fig. 7. Effects of diesel concentration on biofilm wet weight

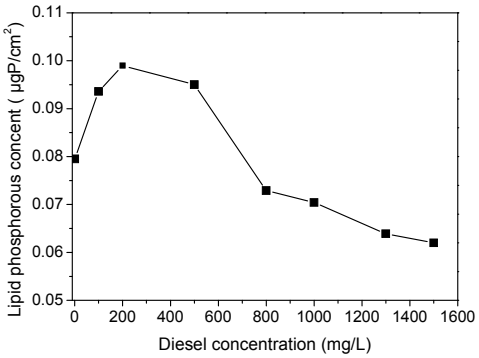


Fig. 8. Effects of diesel concentration on lipid phosphorus in biofilm

IV. Conclusions

The diesel addition has the important impact on biofilm EPS composition. This implied that EPS composition was variable and was related to bacteria physiological state and the operating conditions under which biofilm was developed. The small content of diesel made the adhesion between biofilm and coupons closer. When diesel of larger concentration was added, diesel and biofilm joined together into massive structure, which was easy to be peeled off. Although the biofilm wet weight tended to increase with diesel addition, the lipid phosphorous content in biofilm reached the largest value when diesel concentration was 200 mg/L, and then there was a marked reduction in the lipid

phosphorus content with increase of diesel concentration from 200 to 1500 mg/L. The reduction, which indicated that diesel was toxic to part of the microbial population in biofilm, was related to the intensity of diesel concentration.

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