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Effects of alumina trihydrate (ATH) on formation of biofilms settled on inert carriers of polyethylene terephthalate (PET)

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ABSTRACT

Alumina trihydrate (ATH), one of typical complexes of aluminum (Al), was selected to study its effects on the formation of biofilms settled on inert carriers made of polyethylene terephthalate (PET) filaments. Four identical biological reactors were developed to cultivate biofilms under a series of ATH concentrations. Three-dimensional elastic inert PET carriers were used as the biofilm substrata. The biofilm characteristics including the amounts of total biofilm, extracellular polymeric substances (EPS) productions, active biomass, and microbial activity, were investigated. The impact of Al toxicity on the formation of biofilms was also discussed. The results showed that the colloidal ATH was easily adhered to the inert carriers, and the effects of ATH on the formation of biofilms colonized on the inert carriers were clearly observed. Compared to the biofilm characteristics when ATH was absent, the presence of ATH could increase the amounts of total biofilm production, EPS production, active biomass, and microbial activity: when the ATH concentrations reached 0.1, 0.2, and 0.5 mg/L (as Al^{3+}), the total biofilm amount would increase to 1.44, 2.14, and 2.60 times, respectively, and the EPS production would enhance to 1.21, 1.60, and 1.85 times, respectively. Similar ratios for EPS production were also obtained for the corresponding active biomass and microbial activity. These results suggested that ATH is beneficial to the formation of biofilms settled on inert carriers, and the impact of Al toxicity seems to be insignificant in this study on the colonized microbes and the biofilm formation, probably due to the low ATH concentration (0.5 mg/L as Al^{3+} in maximum) in the reactors.

Keywords: Biofilm formation; Alumina trihydrate; Polyethylene terephthalate; Aluminum toxicity

1. Introduction

Biofilms are frequently observed at the solid/liquid interface throughout the natural aquatic environment [1,2]. Various factors, such as temperature, dissolved oxygen (DO), substrata materials, light intensity, nutrient concentration, and flow rate, can affect the formation of these biofilms [3–6]. In natural aquatic environment, such as rivers and lakes, there

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extensively exists a considerable amount of aluminum (Al) by virtue of various natural and anthropogenic factors [7–9]. The Al usually forms numerous mononuclear or polynuclear complexes as well as correlative intermediates with a variety of inorganic and organic ligands [10]. These Al complexes and intermediates can also impact the formation of the biofilms. Unfortunately, up to now, little is known about the effects of these complexes on the formation of the biofilms. Of all the Al complexes, the hydroxyl-containing aqueous groups (especially $AlOH^{2+}$, $Al(OH)_2^+$, $Al(OH)_3$, and $Al(OH)_4^-$; with the hydrating water omitted for simplicity) are one of the most important types of ligands, and they can transform in response to pH changes in ambient environment [11]. Colloidal Al trihydroxide is an important aqueous Al complex that is normally abundant and stable in typical pH of natural aquatic environment [12]. It is believed that such Al complexes have great a impact on the biofilm formation, while there is little study and results mentioned in literature. Hence alumina trihydrate (ATH) was selected in this study to test the effects of Al hydroxide complexes made in laboratory on the formation of biofilms.

In this study, four identical organic glass cylinders were used as biological reactors to cultivate biofilms. These four reactors were full of culture medium which contained four different concentrations of ATH. Three-dimensional elastic inert carriers of polyethylene terephthalate (PET) was selected to immerge in culture medium and used as biofilm substrata. The effects of ATH on the formation of biofilms were investigated, with four characteristics of biofilms, including total amounts of biofilm, extracellular polymeric substances (EPS), active biomass, and microbial activity, were determined and analyzed.

Moreover, there have been substantial studies during the last three decades on Al toxicity and its ecological effects, indicating that the toxicity of Al is one of the major factors that may affect the survival of aquatic organisms and communities in aquatic habitats [7,11]. The level of toxicity is intimately related to the ambient pH and temperature in aquatic environments and it is also influenced by the ability to form complexes with inorganic ligands and organic materials, such as humic acid and EPS [13–17]. It was, thus, reasonable to suspect that the toxicity of Al can also have some impact on the microbes of biofilms, which, thus, affects the formation of biofilms settled on the inert carriers. Hence, the effect of Al toxicity on the biofilm formation was also discussed in this study.

2. Materials and methods

2.1. Cultivation of biofilms

In this study, four identical biological reactors named R1, R2, R3, and R4 were used to cultivate biofilms. For each culture reactor (Fig. 1), an organic glass cylinder with an internal diameter of 15 cm and a height of 180 cm, providing a total volume of 0.03179 m^3, was served as a water channel. A water pump was fixed for pumping culture medium from a feed tank into the cylinder. A circular perforated plate at the bottom of the cylinder was set to homogenize the water flow, and an effluent outlet at the top of the cylinder was designed to return the culture medium into the feed bank. In order to maintain a certain amount of DO in the culture medium, an aeration device was fixed to supply air directly to the feed tank through a gas distributor.

The flow rates of the culture medium in the four reactors were all identical and set at 0.3 $m^3/(m^2 s)$. The culture medium was uninterruptedly pumped into reactor cylinder from the feed tank and then flowed back into the feed tank again through the effluent outlets of the reactor. The culture medium contained nutrients including analytical reagents (AR, Guangzhou Chemical Reagent Factory) as following (all in mg/L): 17.7 of $(NH_4)_2SO_4$; 4.60 of $Na_3PO_4{\cdot}12H_2O$; 1.89 of $NaH_2PO_4{\cdot}2H_2O$; 2.54 of NaCl; 1.91 of KCl; 2.05 of $MgSO_4{\cdot}7H_2O$; and 0.74 of $CaCl_2{\cdot}2H_2O$. In order to accelerate biofilm's growth, glucose (Guangzhou Chemical Reagent Factory, Guangzhou, China) was added in the culture medium

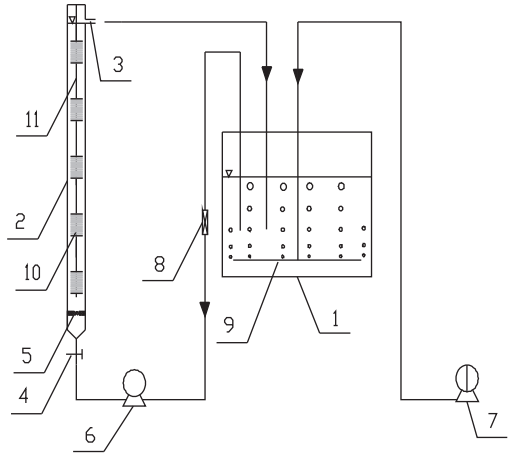

Fig. 1. Schematic diagram of biological culture reactor (1 feed tank; 2 organic glass cylinder; 3 effluent outlet; 4 water valve; 5 circular perforated plate; 6 water pump; 7 air pump; 8 flowmeter; 9 gas distributor; 10 three-dimensional elastic inert carrier; and 11 connecting string).

to maintain the chemical oxygen demand (COD) concentration at about 120 mg/L. For each biological reactor, 500 mL surface water taken from the campus river of Sun Yat-sen University was injected to the feed tank for microbial inoculation.

In this study, the three-dimensional elastic inert carriers made from PET were used as the culture substrata of biofilms. The carriers were made by Lijing Filter manufacturer (Guangzhou, China). Each carrier having a length of 12 cm and a diameter of 10 cm is composed of hundreds of 0.4 mm-diameter PET clamped by thin twisting stainless wire (Fig. 2). After being preweighed, five carriers were connected together by a string and submerged in the culture medium at the depths of 20, 50, 80, 110, and 140 cm from the effluent outlet of each reactor.

The colloidal ATH was prepared when it was ready for use and added to the feed tanks of R1, R2, R3, and R4 at the beginning of cultivation with the amounts of ATH controlled at the concentrations at 0, 1.0, 2.0, and 5.0 mg/L (as Al^{3+}), respectively, in the culture medium. The preparation method of ATH is as follows: weighed the required amounts of $Al_2(SO_4)_3{\cdot}18H_2O$ (Guangzhou Chemical Reagent Factory, Guangzhou, China), dissolved them into high purity water, and then adjusted the pH to 6–8 by adding appropriate amount of hydrochloric acid (0.50 M) or sodium hydroxide (0.50 M).

The DO in the culture medium was controlled at approximately 4.0 mg/L by aeration for about 10 min every 0.5 h, through a time switch (Hongjinda Electronics Technology Co. Ltd., Guangzhou, China). The two parameters, DO and pH, were monitored simultaneously using a SC100™ Universal Controller (Hach Company, USA). In order to ensure the normal life of micro-organisms, approximately 20% of culture medium in the feed tank was replaced with the fresh one in every two days, in which the corresponding amounts of ATH, as described above, were added simultaneously. The experiment was run at room temperature of about 28°C. The temperature in the reactors was also measured with the range of 24–26°C. The duration of experiment was about 1 month from July to August in 2009.

Fig. 2. A three-dimensional elastic inert carrier made from PET.

2.2. Sampling method

At the end of biofilm cultivation, each carrier was carefully removed into a 1000 mL beaker from the cylinder in sequence and was dispersed in phosphate buffered saline (PBS, pH = 7.2) containing (all in mg/L) 0.036 of K_2HPO_4, 0.092 of KH_2PO_4, and 0.493 of NaCl [18]. A glass rod was used to stir severely in order to shed off the biofilm from the carrier. This procedure was repeated for four times to remove the biofilms from the carrier surface. All the resulting suspension was transferred into a 500 mL volumetric flask and prepared 500 mL sample with PBS. This sample was subjected to the following experiments.

2.3. Evaluation of total amount of biofilms by dry weight (DW)

The total amount of biofilm can be evaluated by dry weight (DW) [19,20]. Mixed in a homogenizer, 50 mL homogeneous was prepared using biofilm sample and was put into a preweighed crucible. The crucible was placed in GZX-9140MBE electric oven (Boxun Industrial Co. Ltd., Shanghai, China) and dried at 105°C until constant weight was achieved. After cooling to room temperature, the crucible was weighed again. The biofilm DW was calculated in unit of g/kg carrier. The tests were in triplicate and took the average as the final results.

2.4. Extraction and analysis of EPS

The EPS were extracted using a cation exchange resin (CER) extraction method according to Frolund et al. [21]. The yields of EPS are represented by polysaccharide (PS) and protein (PN) which are the primary components of EPS in biofilm [18]. CER (Tianjin Reagent Co. Ltd., China), at a dosage of 60 g/g suspension solid, was added to the biofilm samples

and mixed in a homogenizer for 1 h at 4°C, allowing EPS in biofilm samples to be fully extracted. The residual solids were removed by a High-speed Refrigerated Centrifuge 5804R (Eppendorf, Germany) at 8,000 rpm for 15 min. The supernatant was used for PS and PN analysis. The PS and PN were determined by the phenol-sulfuric acid method and the Coomassie procedure, respectively [22,23]. The tests were in triplicate and took the averages as the final results with the units of mg EPS/g carrier.

2.5. Active biomass quantification by phospholipid

Phospholipids presented in bacterial membrane up to 90–98% do not form a part of the cell reserves and are easily degraded during bacteria lysis. Measurement of phospholipids can be used to estimate the active biomass [24]. In this study, the active biomass was quantified using the phospholipid method [25]. Ten milliliter suspension samples were used for the phospholipid test. A standard curve was established with K_2HPO4 (Shanghai Reagent Co. Ltd., China) to calculate the phospholipid content in each sample. The tests were in triplicate and took the average as the final results with the unit of nmol P/g carrier.

2.6. Development of microbial activity measurement with 2,3,5-triphenyl tetrazolium chloride (TTC)

A potential method of monitoring metabolic activity of biofilm is through the measurement of dehydrogenase activity (DHA) [26]. The biofilm microbial activity was evaluated by using 2,3,5-triphenyl tetrazolium chloride-dehydrogenase activity (TTC-DHA) method [20]. The TTC-DHA method was based on the method proposed by Mathew and Obbard, which was simple and sensitive [27]. The microbial activity was assessed by TTC-formazan (TF, the reductant of TTC) production. A calibration curve established using Na_2S as reducing agent was used to convert the absorbance of each tube to μg TF/g carrier at 486 nm with a UV-250 spectrophotometer (Shimadzu, Japan). The tests were in triplicate and took the average as the final results.

3. Results and discussion

3.1. Observations on biofilms growth

At the beginning of biofilm formation, it was apparent that the colloidal and flocculent ATH was easily attached onto the carriers soon after they were immersed in the culture medium. The attached amount increased with increasing ATH contents at the corresponding depths of R2, R3, and R4, i.e. R4 > R3 > R2 in the total amount of biofilms growth. The biofilms in R1 grew slowly and some biofilm appeared on the carriers about 2 days later than in other reactors. It was also noted that the biofilms formed in different reactors showed completely different at the end of the cultivation when the biofilms are mature, the biofilms in R1 was filamentous and sparse, whereas those in R2, R3, and R4 were flocculent and dense, which could be easily identified in Fig. 3. From the cultivation process, the biofilms growth in the four reactors showed significant differences, which was possibly mainly attributed to the colloidal ATH showing strong absorptance and easy adherence to the carriers. It was noticeable that ATH facilitated the biofilms formation.

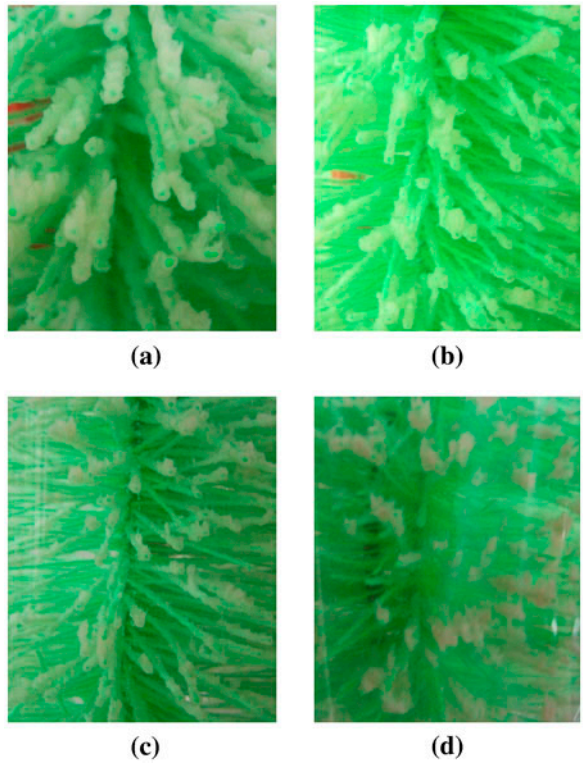

Fig. 3. Biofilms cultured in four reactors (a), (b), (c), and (d) indicated the biofilms on inert carriers at depth of 50 cm in R4, R3, R2, and R1, respectively. Photographs were taken by Canon DIGITAL IXUS 55 before sampling. The attachment of biofilm on internal surface of cylinders of reactors made the photographs a bit obscure.

3.2. Effects of ATH on total amounts of biofilms

As indicated in Fig. 4, the total amounts of biofilms measured by DW (g/kg carrier) increased with increasing ATH contents at the corresponding

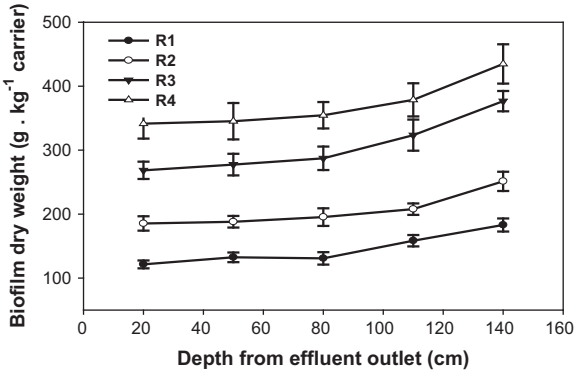

Fig. 4. Total amounts of biofilms by DW.

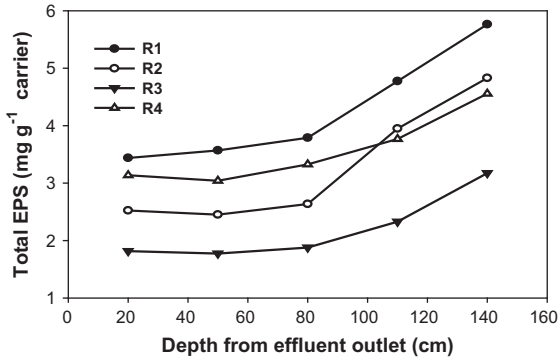

Fig. 5. EPS productions in biofilms.

depths. Specifically, the total amounts of biofilms represented by the average of DW in R2, R3, and R4 were 0.44, 1.14, and 1.60 times than that of R1, respectively (Table 1).

The increase in the total amounts of biofilms with increasing ATH contents was most likely due to the ATH characteristics that was colloidal, and absorbed or adhered to more nutrients from the culture medium during the cultivation process. It was clear that richer nutrients will be conducive to more microbial growth and reproduction in biofilms. In addition, the attachment of ATH immensely and effectively enlarged the surface areas of carriers, as remarkably favored the adhesion or attachment of nutrients and microbes onto the carriers provided with an extra chance. Furthermore, the increasing EPS productions, one part of biofilms, caused progressive increase in total amounts of biofilms in the reactors, which would be discussed in more detail as below.

3.3. Effects of ATH on EPS productions

It is shown in Fig. 5 that EPS productions represented by the sum of PS and PN increased with increasing ATH contents at the corresponding depths of the four reactors. The average EPS productions in R2, R3, and R4 were approximately 1.21, 1.60, and 1.85 times as much as that in R1 at the equivalent depths, respectively (Table 1). In addition, the average ratios of PS/PN in R1, R2, R3, and R4 were 2.66, 2.87, 2.95, and 2.77 (Table 2), respectively, indicating that the ratios increased with increasing ATH contents, except for that in R4 with a decreasing ratio. The ratio decreased may be due to the extreme high concentration of ATH that exceeds a certain limit.

A matrix of EPS secreted by microbes can enhance the attachment of biofilm community and facilitate biofilm formation [28]. As noted above, a higher content of ATH produced a larger total amount of biofilm, leading to more active biomass produced and more EPS excreted proportionately. Meanwhile, more EPS adhered to or absorbed more nutrients from the bulk culture medium, which would furthermore promote the reproduction and growth of microbes in biofilms.

The increase of EPS with increasing ATH concentration was probably induced by the addition of active biomass of the biofilms in the four reactors. Nevertheless, the PS/PN ratios were not constant for the four reactors, and it varied as ATH content increased (Table 2). This was most likely related to the toxicity

Table 1
Average ratios of biofilm characteristics in four reactors

Ratio	Average ratio			
	Total amount of biofilm (DW) $g\,kg^{-1}$carrier	EPS $mg\,g^{-1}$ carrier	Active biomass (phospholipids) nmol $P\,g^{-1}$ carrier	Microbial activity (TF) $\mu g\,g^{-1}$ carrier
R2/R1	1.44*	1.21	1.21	1.23
R3/R1	2.14	1.60	1.61	1.64
R4/R1	2.60	1.85	1.85	1.87

*The average ratio of total amount of biofilm (DW) 1.44 denoted the average ratio of total amounts of biofilms at five depths in R2 and R1 reactors, i.e. $(R2/R1)_{\text{Total amount of biofilm}}(1.44) = \sum(R2/R1)_{\text{Total amount of biofilm, } i}/5 = 1.46$, $i = 1, 2, 3, 4, 5$.

Table 2
PS and PN content in biofilms at different depths

Reactor	Depth from effluent outlet cm	PN mg/g carrier	PS mg/g carrier	PS/PN[b]
R1	20	0.588 ± 0.047[a]	1.518 ± 0.129	2.58
	50	0.614 ± 0.029	1.555 ± 0.105	2.53
	80	0.605 ± 0.035	1.610 ± 0.127	2.66
	110	0.650 ± 0.042	1.762 ± 0.119	2.71
	140	0.715 ± 0.058	2.016 ± 0.134	2.82
R2	20	0.688 ± 0.043	1.859 ± 0.135	2.70
	50	0.689 ± 0.031	1.891 ± 0.098	2.75
	80	0.683 ± 0.036	1.969 ± 0.132	2.88
	110	0.759 ± 0.055	2.197 ± 0.125	2.89
	140	0.798 ± 0.067	2.480 ± 0.143	3.11
R3	20	0.893 ± 0.049	2.432 ± 0.122	2.72
	50	0.925 ± 0.073	2.532 ± 0.142	2.74
	80	0.896 ± 0.038	2.676 ± 0.149	2.98
	110	0.938 ± 0.064	2.874 ± 0.154	3.06
	140	1.015 ± 0.067	3.256 ± 0.187	3.21
R4	20	1.079 ± 0.057	2.765 ± 0.161	2.56
	50	1.057 ± 0.081	2.893 ± 0.149	2.74
	80	1.101 ± 0.074	3.073 ± 0.175	2.79
	110	1.174 ± 0.083	3.319 ± 0.157	2.83
	140	1.238 ± 0.098	3.607 ± 0.194	2.91

[a]The number 0.588 ± 0.047 denoted that the average is 0.588, and its standard deviation is 0.047.[b]PS: the average content of polysaccharide; PN: the average content of protein.

of dissolved Al in water [11]. Within a certain limit of ATH content, the toxicity of Al in culture medium could stimulate colonized microbes to secrete more PS and PN for preventing them from being poison. However, beyond a certain limit identified as 5.0 mg/L as Al^{3+} in this study, it was likely that the Al toxicity inhibited the reproduction and growth of microbes in biofilms and decreased the productions of PS and PN. This inhibition could be illustrated by decreasing PS/PN ratios, when ATH content increased above the given threshold. However, the mechanisms underlying opposite relationship between PS/PN and Al toxicity has not completely understood, and needs further investigation in the future study.

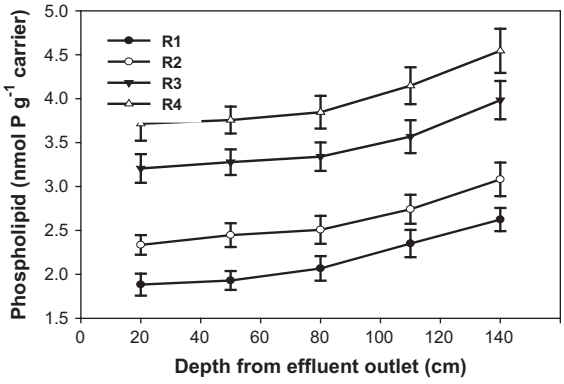

Fig. 6. Active biomasses (phospholipids) in biofilms.

3.4. Effects of ATH on active biomass in biofilms

It was observed that the active biomasses increased with increasing ATH contents in the four reactors. This trend was similar to EPS productions as described above. From the Fig. 6, the average ratios of active biomasses in R2, R3, and R4 were 1.21, 1.61, and 1.85 times as much as that of R1, respectively, which had the same ratios as the EPS productions (Table 1).

The total amount of biofilm mainly consists of two parts: one part comes from active biomass and the other part comes from inactive components, such as EPS and other inorganic and organic substances

attached onto biofilms [29]. As EPS were secreted by the active microbes in biofilms, it could be implied that the EPS production was directly related to the active biomass, which has been verified by the observation in this study that the average ratios of active biomasses was similar to that of EPS productions in the four reactors (Table 1). From this point of view, the active biomass is a more suitable and useful parameter than total amount of biofilm for describing and evaluating the characteristics of biofilm.

3.5. Effects of ATH on microbial activity

The profiles in Fig. 7 show the microbial activity represented by TF contents (μg/g dry carrier) in a series of depths of the four reactors. It was showed that the microbial activity was enhanced with increasing ATH contents at the corresponding depths in the four reactors. The average ratios of microbial activity in R2, R3, and R4 were determined to be 1.23, 1.64, and 1.87 times as much as that of R1, respectively, which were nearly the same as those of the EPS production and the active biomass (Table 1).

It has been reported that the microbial activity highly correlates with the microbial biomass in biofilm [30], which was consistent with the results in this study that the average ratios of the active biomass and the microbial activity were similar, according to (Table 1). Mei et al. [31] also discovered this relationship when studying the growth characteristics of photoautotrophic biofilm in Donghu Lake of Wuhan Province. In fact, the microbial activity was mainly reflected by the active biomass in biofilms.

3.6. Impact of Al toxicity on biofilms

From the variability of PS/PN ratios in Table 2, the Al toxicity did have some effects on the biofilms.

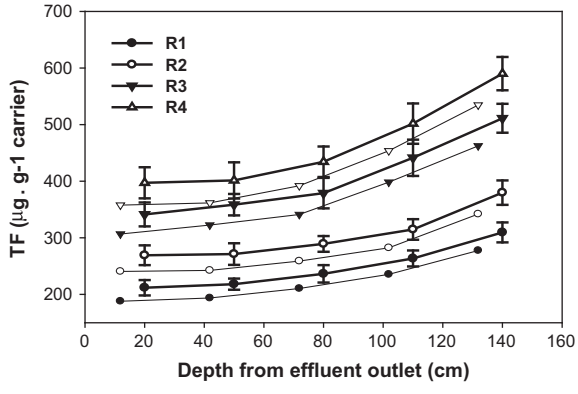

Fig. 7. Microbial activity by TTC-formazan (TF) presented in biofilms.

However, the toxicity effects from ATH were alleviated greatly probably due to the following reasons. First of all, Al was thought to be unavailable to utilize by microbes in biofilms owing to the formation of insoluble Al particulates and complexes in circumneutral aquatic environment [17,32]. Although Al is highly toxic, its biological toxicity is virtually driven by aquatic pH [33,34]. When the pH of the medium was in the range of 6.0–8.0, Al was only sparingly soluble and the microbes in biofilms would hardly absorbed Al. Secondly, the dissolved organic matter (DOM) secreted by the organisms in biofilms increased when the biofilms grew on carriers. DOM typically formed complexes with 50–70% of the dissolved Al in water [35]. Thus, there was generally more organic Al existed when DOM concentration increased, leading to increasing the solubility of Al, and at the same time decreasing the Al toxicity [11,36,37]. In addition, the EPS layer formed by the secretes of the microbes provided protection to the live micro-organisms in biofims [38,39]. In summary, the impacts of Al toxicity from ATH were small on microbes in biofilms, and thus the effect of Al toxicity would be negligible on the formation of biofilms in our study.

4. Conclusions

In this study, four identical biological reactors and three-dimensional elastic inert carriers were employed to cultivate biofilms in the presence of various concentrations of ATH. The characteristics of ATH on the formation of biofilm were investigated. The results indicated that when a serial concentrations of ATH reached 0.1, 0.2, and 0.5 mg/L (as Al^{3+}), the total amounts of biofilms were increased to approximately 1.44, 2.14, and 2.60 times, respectively, as much as that when ATH was absent. The corresponding percentage increase of EPS productions, active biomass, and microbial activity were similar, with the ratios of 1.21, 1.60, and 1.85 times, respectively. The EPS productions and the microbial activity were mainly depended on the active biomasses but not on the total amount of biofilms. In addition, the toxicity of Al induced by ATH decreased markedly by forming insoluble particulates or complexes, such as DOM in aquatic environment, leading to the insignificant effect of Al toxicity on microbial activity colonized in biofilms and the formation of biofilms in our study.

Acknowledgments

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