

Full Length Research Paper

Study on the Modified triphenyl tetrazolium chloride – dehydrogenase activity (TTC-DHA) Method in Determination of bioactivity in the up-flow aerated bio-activated carbon filter

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The study applied triphenyl tetrazolium chloride-dehydrogenase activity (TTC-DHA) method to detect the activities of attached biofilm on bio-activated carbon (BAC) samples in the up-flow aerated biological activated carbon filter (UABACF) treating textile secondary effluent. Modification to the conventional TTC-DHA determination method was proposed. In the modification, BAC samples were used directly to measure TTC-DHA without pre-separating the attached biofilm from carbon samples. After modification, the mean values of biofilm TTC-DHA activities for the BAC samples at different heights of the biofilter were 25 to 193 times higher than those measured in conventional way. In addition, the microbial activity distribution related more closely to substrate removal along the height of the reactor after modification. The results indicated that high activity of the bacteria that are firmly fixed on the porous surface of the media would be ignored during pre-separation of the attached biofilm from media surface. The study also indicated the influence of granular activated carbon (GAC) adsorption on the bio-activity of attached biofilm. GAC adsorption was favorable in the improvement of the activities within the biofilter, especially when the attached films were destroyed. The modification of TTC-DHA determination method made this technique more convenient and accurate in activity measurement of biofilm fixed on porous surface structured activated carbon.

Key words: Up-flow, aerated bio-filter, BAC, TTC-DHA, bioactivity.

INTRODUCTION

Submerged up-flow aerated bio-filters are newly developed processes, which feature for the reactor compact size, high organic load and high efficiency in removing both the suspended solids and soluble organic

matters (Pujol et al., 1992). With increasing application in secondary or tertiary wastewater treatment, biofilters can complete the functions of carbonation, nitrification and de-nitrification. If granular activated carbon (GAC) served as support media in the biofilters, the biological activated carbon (BAC) filter would be formed. In BAC processes, the rough and porous surfaces of activated carbon (AC) are favorable for abundant active microbial growth: lots of macro-pores, crevasses and ridges provide the bacterial with not only large inhabitation area but also the shield from water and air flow flush. Enrichment of substrate, oxygen concentration and removal of the microbe-inhibitory matters by GAC adsorption are all advantageous for improving bioactivities of attached biofilm on BAC (Wang and Summers, 1995; Nishijima et al., 1992). Forming high active biofilms to bring out desired bioreactions is key to biofilters effective performances, and using quick, simple and accurate bio-

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Abbreviations: TTC, triphenyl tetrazolium chloride; DHA, dehydrogenase activity; TF, triphenylformazan; COD, chemical oxygen demand, indicator of oxygen equivalent of organic matter; UV254, ultraviolet absorbency at 254 nm, indicator of refractory and readily adsorbable organic matters; RDHA, ratio of maximum DHA to minimum DHA, indicator of bioactivity change; BAC, biological activated carbon; GAC, granular activated carbon; and TSE, textile secondary effluent.

activity determination method to monitor the biofilm activity is important for bioreactors operation. But the porous structures and adsorption property of activated carbon brings with it difficulties in quantifying the amount of biomass and bio-activity for the attached biofilm on BAC.

Techniques for estimation of bio-activity include adenosine triphosphate (ATP; Nouvion et al., 1987), deoxyribonucleic acid (DNA) (Jeffrey and Paul, 1988), (specific) oxygen up-take rate ((S)OUR; Andreottola, 2002) and triphenyl t̄trazolium chloride-dehydrogenase activity (TTC(INT)-DHA methods; Lazarova and Manem, 1995). Among them, TTC-DHA method is recommended as a very sensitive and simple methodology for bacterial activity determination (Lazarova and Manem, 1995). TTC □triphenyl tetrazolium chloride□ is a kind of colorless soluble dye, which serves as terminal acceptor in biochemical reactions. Red triphenylformazan (TF) salt forms in microbe cells when TTC irons react with H atoms and can be extracted from the cells using an organic solvent (Yang and Jiang, 2002; Yu and Wu, 1990). In previous studies using DHA assays to evaluate the bioactivities of biofilm in biofilters (Bihan, 1998, 2000; Zhou and Li, 1995; Zinbei and Henriette, 1994), the biomass is pre-detached from the support media to perform the measurements of biofilm activity, which tends to sacrifice certain bioactivity and biomass. Lazarova (1994, 1995) also pointed out the fixed biomass removal procedure can be one of the limiting steps in biofilm analysis especially for the porous surface media.

Wang and Summers (1995) used a phospholipid analytical technique to measure the amount of biomass attached to the surfaces of drinking water AC filter media. In their study, biomass was not pre-separated from media and phospholipids were directly extracted from the cells in the attached biofilm on the media to measure the biomass quantity colorimetrically. Enlightened by the idea of quantifying biomass without pre-separating the biofilm from the media, we applied TTC-DHA method to examine the bioactivity of attached biofilm in BAC filter in this research. Bioactivity determinations were directly done with biofilm not detached from the GAC particle. Experimental results showed that the modification of TTC-DHA determination method made this technique more convenient and accurate in activity measurement of biofilm that are fixed on porous surface structured activated carbon.

MATERIAL AND METHODS

Experimental apparatus and materials

The study was made in an up-flow aerated BAC filter (UBACF). The UBACF is cylinder of 2.5 m height and 0.1 m inside diameter with filter media packing height of 1000 mm. Pillar granular activated carbon (GAC) were used as filter media with the main characteristics of 3 mm diameter, volumetric weight of 487 kg/m³ and 850 mg/g iodine adsorption value. Four sampling ports were

mounted at the heights of 0.25, 0.45, 0.65 and 0.85 m for carbon or wastewater sampling during reactor operation.

The investigated waste stream was the textile secondary effluent (TSE), i.e. effluent of hydrolysis-acidification/aerobic oxidation biotreatment units (F2/O units) for treating simulated textile wastewater. Main item values of the investigated TSE stream are listed in Table 1.

The investigated TSE stream was injected to UBACF by metering pump from the bottom of the UBACF and treated by multi-function of GAC adsorption, microbial degradation and mechanical filtration. During reactor operation, the hydraulic loads were controlled within 0.5-1.0 m³/m²·h, organic loads 0.576 to 1.44 kg COD/m³·d and the dissolved oxygen level 3 to 6 mg/L. Items as influent and effluent COD, suspended solids (SS), turbidity, pH, color and UV254 were monitored daily according to standard methods (EPA of P.R. of China, 2002). The system was operated properly for 18 months, with the average COD_{Cr}, NH₄⁺-N, color and UV254 removal achieved being 55, 83, 45 and 37%, respectively. The schematic flow diagram of the UBACF is shown in Figure 1.

Filter bioactivity distribution monitoring was done when the filter was in steady operation, and activity monitoring continued for 6 months.

Table 1. Main item values of the investigated TSE stream.

Item	Range
COD _{Cr} /mg/l	45 – 119
pH	7 – 8
NH ₄ ⁺ -N/ mg/l	15 – 25
Color/dilution times	50 – 64
Turbidity/NTU	30 – 40
UV ₂₅₄ /m ⁻¹	28.0 – 56.6

Sample preparation

Activated carbon samplings were done upwardly from biofilter sampling ports every 3 days. Bioactivity measurements were immediately done after sampling. All the materials were pre-sterilized.

Conventional measurement with pre-separated biofilm: 10 ml activated carbon samples were placed in a cone flask and rinsed with 0.85% physiological saline three times to remove the loosely captured biofilm or organic matters adsorbed on the sample surfaces. Then, 50 ml distilled water and 3 ml (d=3 mm) glass beads were added to the flask. The flask was then capped with sterilized plug and shaken at 240 rpm for 10 min. During shaking operation, the attached biofilm were stripped off from AC samples, forming a mixture of liquid and the detached biofilm. The mixture was centrifuged and placed in a colorimetric tube for TTC-DHA bioactivity measurement.

Modified measurement with undetached biofilm: Modification to conventional biofilm pre-separated based activity determination procedure was proposed. The biofilm-undetached samples were put in a colorimetric tube for TTC-DHA bioactivity measurement directly. Before measurement, the samples were washed with 0.85% physiological saline three times to remove loosely adsorbed biomass and organic matters.

DHA analytical measurement steps

The TTC-DHA analytical measurements were performed basing on the method proposed by Yu and Wu (1990). In the colorimetric

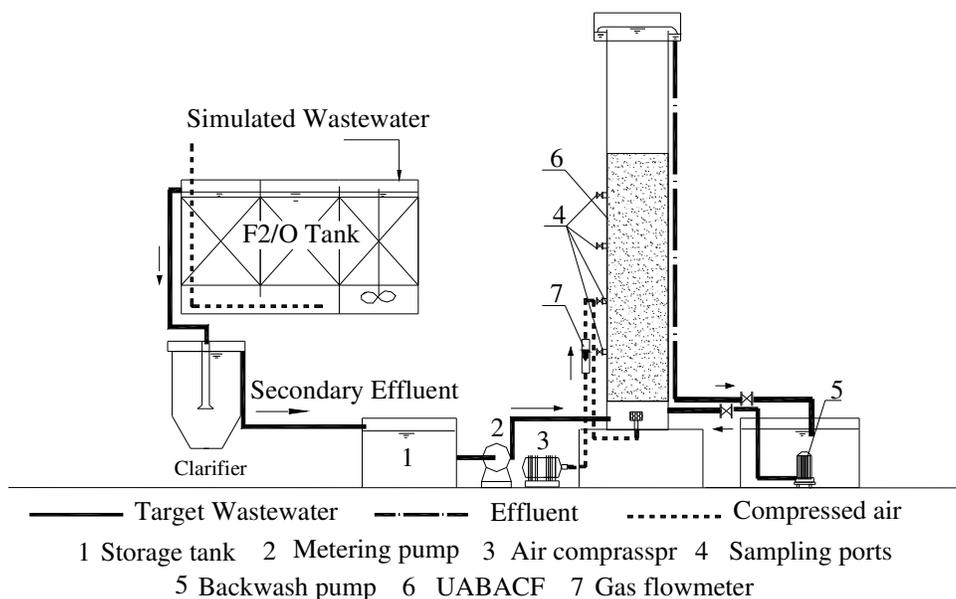


Figure 1. The schematic flow diagram of the pilot UABACF reactor.

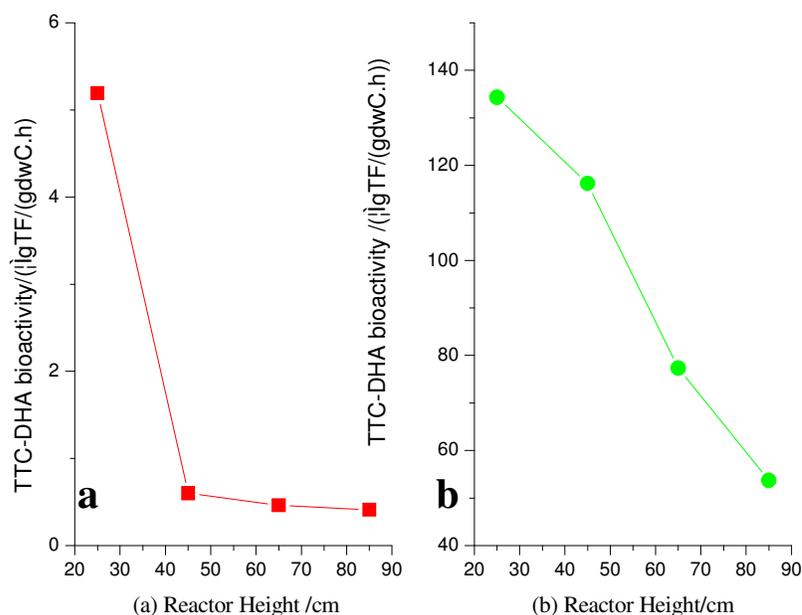


Figure 2. Comparison between TTC-DHA bioactivities determined by (a) conventional and (b) modified measurement methods in attached biofilm bioactivity determination with the same batch BAC samples at different heights of the biofilter.

tubes, the detached biofilm samples and the biofilm-unfixed BAC samples were added with 2 ml of Tris-HCl buffering liquid (pH 7.6), 0.1 M glucose solution and 0.5% TTC solution. The tubes were lightly capped, then placed in water bath at 37°C and incubated for 24 h (48 h for naked BAC samples). After incubation, 2 ml concentrated sulfuric acid was added in the tubes to stop the reaction, and 5 ml of toluene was used to extract the TF crystal. The mixture was centrifuged at 600 rpm for 5 min and the

supernatant was separated to measure the TF content colorimetrically at 492 nm. The amount of TF was determined from the standard TF curve drawn with Na₂S as reduction agent. All samples were analyzed at least in duplicate. TTC-DHA activities were expressed in terms of g TF produced /g dry weight activated carbon·h.

RESULTS AND DISCUSSION

Results of TTC-DHA bioactivity distribution of attached growth biofilm in UABACF determined by the conventional measurement were shown in Figure 2a. TTC-DHA activities decreased as the biofilter height increased, which indicated TTC-DHA activity for attached biomass varied positively to the depth of the biofilter. Since organic load was the highest at the filter inlet, which induced the growth of bacteria, the bioactivity attained the highest at 0.25 m height of the biofilter. But sharp reduction in activity occurred from 0.25 m to 0.50 m. The minimum bioactivity appeared at the 0.85 m height of the reactor, and was only 7.9% of that at 0.25 m, suggesting significant variation in bioactivity existed in UABACF measured in conventional way.

In common biochemical analyzing tests (such as colony counting or stain separation; Yu and Wu, 1990), attached growth biomass has to be stripped off from the support media to perform measurements. But in TTC-DHA activity determination, the reaction essential is biological oxidation and reduction (Yang and Jiang, 2002). High concentration of TTC ions can penetrate deeply into the cells within biofilm during bioactivity detection, and react with hydrogen atoms (which were removed from organic matters catalyzed by dehydrogenase enzyme) to generate red TF crystal. No matter the organism cells fixed on the support media or kept free in liquid, the red TF product can be extracted by organic solvent from cells and measured quantitatively. Therefore, in TTC-DHA activity determination for attached biofilm, it is unnecessary to unfix the biofilm from the support media.

The TTC-DHA technique had been used largely for determining the bioactivities of biofilm undetached from the support media in bio-fluid bed reactor and soils (Lazarova et al., 1998; Wu et al., 2003). But in previous researches of biofilter bio-activities, the enzymatic assays were still performed with the biofilm pre-separated from support media (Bihan, 1998; 2000; Zhou and Li, 1995; Zinbei and Henriette, 1994). Monitoring the attached biofilm activities directly, with the biofilm still fixed on the support media (GAC), was tried in this research. The results of modified measurement of TTC-DHA bioactivity distribution in the UABACF are shown in Figure 2b. The mean bioactivity distribution profiles got from the two kinds of activity determination methods differ remarkably. Decreasing tendency was also found in the activity distribution profile along the height of reactor in Figure 2b, but the descending gradient was much lower than that of the profile in Figure 2a, with the minimum bioactivity at 0.85 m height of the reactor was 40% of that at 0.25 m after modification. Distinct differences existed between the results of conventional and modified measurements. After modification, the mean values of TTC-DHA activity from 0.25, 0.45, 0.65, 0.85 m reactor height for the same BAC sample were 53.29, 76.84, 115.6, 129.11 $\mu\text{gTF}/(\text{gdwC}\cdot\text{h})$, and higher than those of

conventional measurement by 25 to 193 times. The average TTC-DHA value of samples from the top part of the biofilter reached to 53.7 $\mu\text{gTF}/(\text{gdwC}\cdot\text{h})$ suggesting rather high activities existed on the upper part of the biofilter, which did not show in conventional measurement. RDHA (minimum bioactivity/ maximum bioactivity in the biofilter), the indicator of bioactivity change in the reactor, was high (up to 12.66) in conventional measurement, while it was only 2.5 in the modified measurement. This indicated that the TTC-DHA activity decay was not as significant as that determined in conventional measurement.

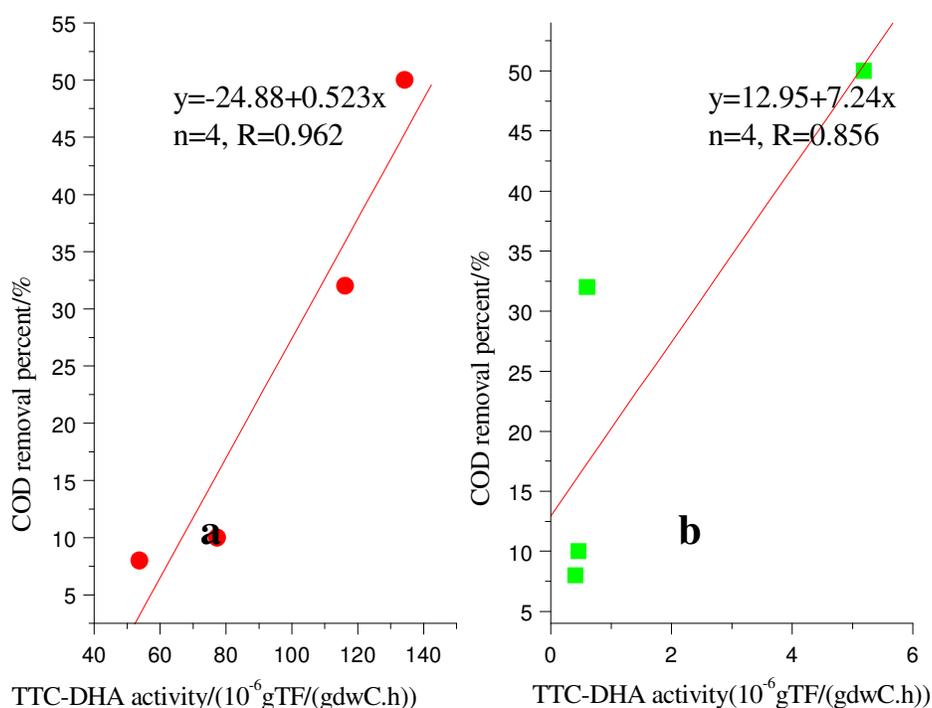
Substrate removal always indicated the corresponding microbe bioactivities indirectly. In the studied TSE wastewater, biodegradation was the dominant way for COD removal in the biofilter (Zhang, 2004). The magnitude of bioactivity within the reactor was key to COD removal along the height of the reactor. The ratio of COD section removal to reactor total COD removal at 0 to 0.25 m, 0.25 to 0.45 m, 0.45 to 0.85 m and 0.85 to 1.0 m sections of the reactor will indicate the corresponding microbial activity within the reactor. Figure 3 showed the comparisons of the COD removal proportions vs bioactivities determined in conventional way and in modified way, at different sections of the biofilter. A liner trend between COD removals and biomass activities can be observed both in the Figures 3a and 3b. R was the correlation parameter, value of R for Figure 3a (0.962) was much higher than that for Figure 3b (0.856). A better correlation between bioactivities and COD removal was observed in Figure 3a than Figure 3b. It demonstrated that after modification, the values of TTC-DHA activities were more accurate and reasonable. In TTC-DHA bioactivity measurement for BAC samples, it was more practical to determine the bioactivities with biofilm un-separated carbons than with the detached biofilm. In addition, determining the bioactivities with biofilm-unfixed samples was a more simple and time-saving way for batch and continual activities monitoring in biofilter.

Since remarkable differences existed between the bioactivity values in conventional and modified measurements, speculations were made as follows: (1) Pre-separating biofilm from GAC support probably resulted in bioactivity loss and measurement error in conventional measurement. (2) After biofilm detachment, there still existed bacterial of high activities firmly fixed on the surface of GAC and its activities were excluded in conventional measurement, which influenced the results. To find out if there was firmly fixed bacterial with high activity when the attached biofilm had been stripped off from the BAC samples, TTC-DHA tests were performed on the naked BAC samples (biofilm having been removed from BAC). After detaching the biofilm from the surface of BAC samples, the naked activated carbon particles were washed with distilled water 10 times and added with TTC agent to find out if they can react with TTC agent. Tests were done on fresh raw GAC particles of the same type

Table 2. TTC-DHA activities of naked (biofilm detached) BAC samples at different heights of the reactor (detected 10 batches).

Sampling height (m)	Magnitude of TTC-DHA activity of naked BAC samples ($\mu\text{gTF}/(\text{gdwC}\cdot\text{h})$)		
	Range	Mean	SD*
0.25	22 – 41	34.8	16.5
0.45	27 – 65	42.2	16.6
0.65	33 – 71	39.8	21.8
0.85	3 – 42	19.6	14.8
Raw GAC as contrast	None -reaction	-	-

*SD standard deviation.

**Figure 3.** Correlations between COD removal proportions and bioactivities at different height sections of the biofilter: **a.** TTC-DHA activities determined in modified way. **b.** TTC-DHA activities determined in conventional way.

and size with tested BAC samples. These biofilm-removed BAC samples showed delayed reaction with TTC agent, thus the incubation time was prolonged to 48 h. The GAC samples in control test did not react with TTC at all. The results are in Table 2. After stripping off the closely attached biofilm, the naked BAC particle at different heights of the reactor still showed certain TTC-DHA activity whose magnitudes correlated to their height level in the reactor. A complication was that TTC-DHA activities of naked BAC samples were not directly related to the activities of attached biofilm. Data showed the maximum bioactivity of the naked BAC samples mainly

appeared at the height of 0.45 m and/or 0.65 m, which completely different from that appeared at the height of 0.25 m for attached biofilm. Since GAC media was different from other media in possessing good adsorption capacity, the differences in TTC-DHA activities for naked BAC at different filter heights probably linked with different GAC adsorption capacities.

Batch tests were carried out to examine the residual adsorption capacities of BAC samples at different heights of the biofilter according to the method proposed by Zhao (1999), at 6th, 12th and 18th months of biofilter operation time. Attached biofilms were first removed from BAC

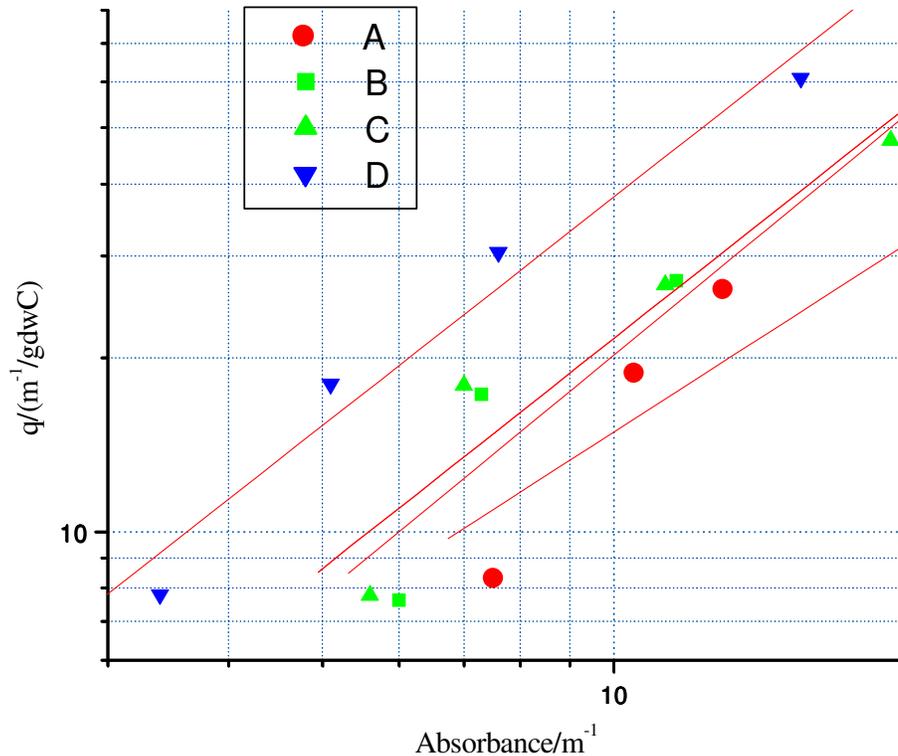


Figure 4. Freundlich adsorption isotherm lines for UV₂₅₄ of the GAC samples at different heights of the biofilter. A, B, C and D: BAC samples taken from different heights of the biofilter; at 0.25, 0.45, 0.65 and 0.85 m, respectively.

samples, and these biofilm detached samples were then rinsed with distilled water, dried, mashed and screened into powder activated carbon with the size of 200 mesh. Carbon of different weights of 10, 50, 100, 250, 500 mg and 100 ml tested wastewater sample were added into the 250 ml flasks and shaken for 24 h at room temperature to achieve adsorption equilibrium. The carbon contained wastewater samples were filtered with 0.45 μm membrane to separate AC powder, and the filtrate was tested for COD, color and UV₂₅₄. As one of collective parameters surrogated for organic matter content in wastewater, UV₂₅₄ was the main indicator of refractory and readily adsorbable organic matters in wastewaters (Ferhan and Aktas, 2001; Seo, 2001). Therefore, the isotherm lines for UV₂₅₄ can best indicate the residual adsorption capacities for tested AC samples. The Freundlich- adsorption isotherm lines for UV₂₅₄ in the batch adsorption tests for the BAC samples at different filter heights were shown in Figure 4. These samples were taken at 18th month of the biofilter operation time. As indicated in Figure 4, the slopes for these Freundlich adsorption isotherm lines decreased, while the ordinate interceptions increased, with the rise of tested BAC sampling height. This demonstrated the residual adsorption capacities of naked BAC samples increased with biofilter height.

The specific relations of naked BAC TTC-DHA bioactivity, attached biofilm DHA bioactivity and GAC adsorption capacity at different heights of the biofilter were indicated qualitatively in Figure 5. It can be seen from the figure that the TTC-DHA activities of naked BAC samples were influenced by GAC residual adsorption capacity and attached biofilm bioactivity simultaneously. The curve shape tended to be resultant of the two curves: one corresponding to the depletion of DHA activity of attached biofilm with the rise of biofilter height, and the other corresponding to the GAC residual adsorption capacity augment with the increase of biofilter height. Thus, the naked BAC sample with the maximum DHA activity appeared at the middle part of the biofilter. From Table 2 and Figure 5, it was evident that even the attached biofilm was broken or destroyed, certain activity could still be hold on BAC surface. This phenomenon is important for UABACF in continual and effective removing organic matters, especially in the occasions of backwashing. This mainly resulted from the existence of firmly fixed microbes adsorbed on the porous GAC surface, and their activity cannot be detected by biofilm pre-separated method. Therefore, compared with non-adsorbable support media such as anthracite and sands, GAC was advantageous in holding closely fixed high active bacterial by adsorption. And the bioactivity

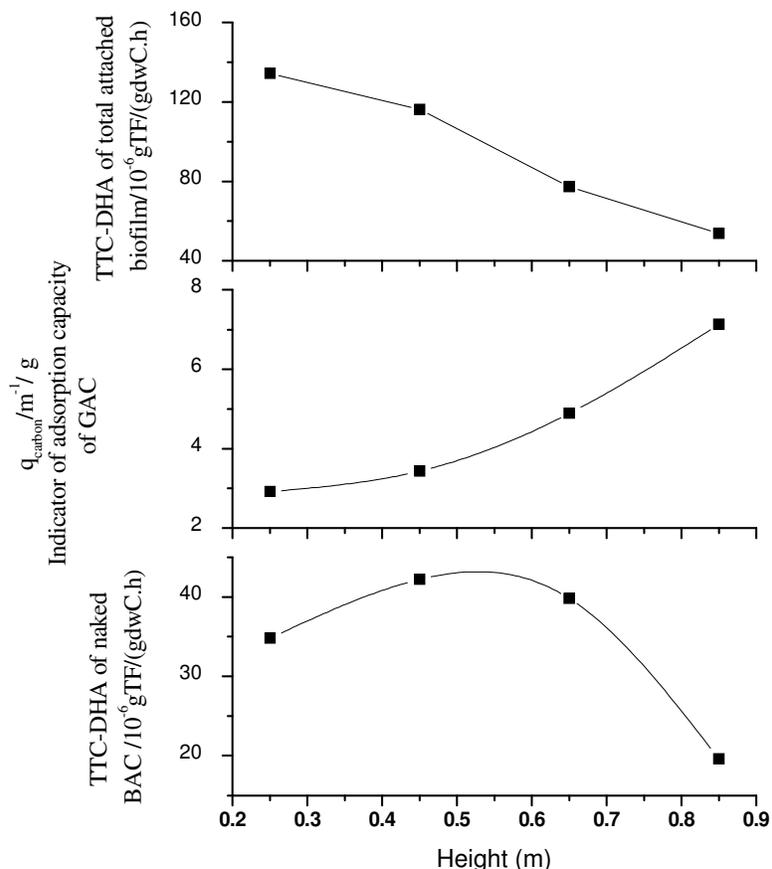


Figure 5. Multi-relations of GAC adsorption capacity, TTC- DHA activities of the naked BAC samples and corresponding TTC- DHA activities of attached biofilm changing with the height of biofilter.

magnitudes of these firmly fixed microbes at different heights of the reactor were influenced by GAC residual adsorption capacity and attached biofilm bioactivity simultaneously.

In the modified TTC-DHA bioactivity measurement, no extra activity loss occurred during biofilm sample preparation, because the biofilm was not pre-separated from BAC samples. The reaction product TF crystals can be easily extracted from the cells in the attached biofilm on porous surface structured GAC and measured quantitatively. After modification, the mean values of TTC-DHA activity for the same BAC sample were 53.29 to 129.11 $\mu\text{g TF}/(\text{gdwC}\cdot\text{h})$ or 25 to 193 times higher than those in conventional measurement. Bioactivities determined by the modified method showed closer relation than the bioactivities determined in conventional way, to COD removal. The significant difference resulted from the existence of high active bacterial firmly fixed on the surface of the activated carbon when the attached biofilm were removed from porous GAC surface. Experimental results proved that the biofilm stripped BAC samples were able to retain certain bioactivity, and these

bioactivities related to the GAC residual adsorption capacities and bioactivities of the attached biofilm.

In conclusion, in TTC-DHA bioactivity measurement for BAC samples, it is more practical to determine the bioactivities with biofilm unseparated carbons than with the detached biofilm. Determining the bioactivities with biofilm-unfixed samples was a more simple and time-saving way for batch and continual activities monitoring in biofilter.

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