Journal of Zhejiang University SCIENCE A
ISSN 1673-565X (Print); ISSN 1862-1775 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Applicability of anoxic-oxic process in treating petrochemical wastewater*

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Received Jan. 2, 2008; Revision accepted June 26, 2008; Crosschecked Oct. 29, 2008

Abstract: To explore the applicability of anoxic-oxic (A/O) activated sludge process for petrochemical wastewater treatment, the relationship between bacterial community structure and pollutants loading/removal efficiencies was investigated by gas chromatograph-mass spectrometry (GC-MS), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and other conventional techniques. It showed that when the concentrations of the influent chemical oxygen demand (COD) and ammonia nitrogen (NH_4^+-N) were 420~560 mg/L and 64~100 mg/L, respectively, the corresponding average effluent concentrations were 160 mg/L and 55 mg/L, which were 1.6 and 2.2 times higher than those of the national standards in China, respectively, demonstrating the inefficient performances of A/O process. Analysis of GC-MS indicated that refractory pollutants were mainly removed by sludge adsorption, but not by biodegradation. PCR-DGGE profile analysis suggested that the biological system was species-rich, but there was apparent succession of the bacterial community structure in different locations of the A/O system. Variations of bacterial community structure and pollutant loadings had obvious influences on pollutants removal efficiencies. Thus, A/O process was inapplicable for the treatment of complicated petrochemical wastewater, and strategies such as the reinforcement of pre-treatment and two-stage A/O process were suggested.

Key words: Petrochemical wastewater, Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), Anoxic-oxic (A/O) process, Applicability,

doi:10.1631/jzus.A0820006 **Document code:** A **CLC number:** X703

INTRODUCTION

The operation performance of wastewater treatment plants (WWTPs) is often unstable as they process petrochemical wastewater. This is mainly due to the complicated wastewater natures (Nyholm, 1996; Shi *et al.*, 2006) and the frequent shock loadings (Patel and Madamwar, 2002), especially under the conditions with low temperature at which the growth, reproduction and metabolism of the microorganisms (Margesin and Schinner, 1999; Ren and Ma, 2003) are further inhibited. In addition, insufficient dissolved oxygen (DO) leads to low biological activity followed by the degradation of activated sludge (Liu *et al.*, 2006). All these factors impact on the purification performances of WWTPs through various relationships between bacterial community structure and pollutants loading/removal efficiencies (Gao *et al.*, 2003). In the present study, by analyzing these relationships, the applicability of the existing full-scale anoxic-oxic (A/O) process was investigated to explore the optimal process for the treatment of petrochemical wastewater.

MATERIALS AND METHODS

Experimental facility

The test facility was an existing full-scale A/O

* Project supported by the National Basic Research Program (973) of China (No. 2004CB418505) and the Science and Technology Development Program of Heilongjiang Province (No. CC05S301), China

activated sludge system located in the petrochemical WWTP, which consisted of 5 compartments. The first compartment was an anoxic section (A in Fig.1) and the other compartments were oxic sections with aerators (O1~O4 in Fig.1). Effluent from the last compartment flew into the secondary settling basin for water and sludge separation. Partial settled sludge was recycled at a rate of R=1.0 and pumped back to the A/O system with the influent. Excess sludge was discharged into the sludge treatment system for further dewatering. The treatment capacity was 730 m^3/h and the design dimension of a single A/O tank was 60 m×40 m×7.2 m. The total hydraulic retention time (HRT) was 23 h, of which anoxic stage was 5 h and each oxic stage was 4.5 h. Mixed liquor suspended solid (MLSS) concentration in A/O system was 3500~4000 mg/L and the influent temperature was 17~19 °C.

Sample collection

Activated sludge samples were collected once per day for 3 d by aseptic apparatus at different locations of the A/O tank (Fig.1), labeled as S1, S2, S3, S4, S5 and S6, and stored under −20 °C immediately. Samples from the same locations were mixed together as the final for the extraction of bacterial DNA. Water samples were taken twice per day for 6 d and analyzed immediately for different water quality parameters.

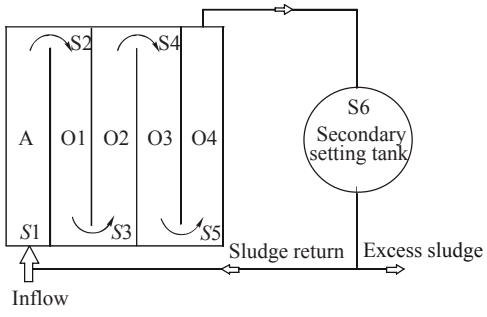

Fig.1 Locations of sample collection
S1~S6: sample locations; A: anoxic section; O1~O4: oxic sections

GC-MS analysis

The gas chromatograph-mass spectrometry (GC-MS) analysis was conducted by an MP5890GC/MS Chromatography-Mass machine. The chromatography was conditioned as follows: SE-54 capillary column was made of quartz (25 m×0.32 mm); the column temperature was retained at 40 °C for 2 min, and then increased to 250 °C with an increment of 3~5 °C/min and kept at 250 °C for 30 min. The mass conditions were set as follows: temperature for MS ion source was 250 °C; the multiplier voltage was 2400 V; the electron energy was 70 eV. The sample feeding amount was 0.2 μl.

PCR-DGGE (Polymerase chain reaction-denaturing gradient gel electrophoresis) analysis

1. Extraction of bacterial DNA

The 500 μl phosphate buffered saline (PBS) was used to suspend the bacteria contained in each activated sludge sample, and the mixture was then centrifuged at 12000 r/min for 10 min. Genomic DNA was extracted with the above supernatant by bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the supplier's instructions.

(1) The supernatant was added into another tube and mixed with 300 μl DNA extraction liquid (100 mmol/L tris, 100 mmol/L EDTA, 200 mmol/L NaCl, 1% polyvinyl pyrrolidone (PVP), 2% CTAB (cetyl trimethyl ammonium bromide), pH 8.0) (v/v, unless otherwise stated, the percentage referred to v/v in the rest of the paper). Then the tube was heated at 37 °C in water bath for 30 min and shook every 5 min for complete reaction.

(2) 80 μl of sodium dodecyl sulfate (SDS) (10%) was added into the tube and then heated at 65 °C in water bath for 1 h. Then the liquid was completely mixed and centrifuged at 12000 r/min for 10 min. The supernatant was collected in another tube.

(3) 30 μl NaAC was added and then cooled in ice bath for 10 min and then centrifuged at 12000 r/min for 5 min. The supernatant was collected in a new tube.

(4) 400 μl mixer of chloroform-isoamyl alcohol (24:1, v/v) was added and well mixed, and then centrifuged at 12000 r/min for 5 min to obtain the supernatant in another tube. Repeate these steps if there was too much protein.

(5) 0.6 times of volume of isopropanol was added and mixed. The samples were stored at −20 °C for 1 h or settled overnight and the supernatant were discarded after centrifuging at 12000 r/min for 20 min. The condensate was collected and exsiccated at room temperature for 6~8 h. The dry sample was then re-suspended in 20 μl tris-EDTA (TE) buffer.

2. Amplification of V3 region of the 16S rDNA

(1) The V3 region of 16S rDNA for most bacteria was amplified by using special primers F_{338}GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) and R_{518} (5′-ATT ACC GCG GCT GCT GG-3′).

(2) PCR amplification system. The PCR mixture (100 μl) contained 100 ng template, 20 pmol of each primer, 200 μmol/L dNTPs (10 mmol/L for each), 10 μl 10×PCR buffer ($MgCl_2$), 2.5 U Pfu DNA polymerases, and sterilized water was added to 100 μl.

(3) PCR reaction conditions. Using a touchdown PCR program (Eric *et al.*, 1999) (Long Gene MG48 PCR system), pre-denaturing at 95 °C for 5 min and then denaturing at 94 °C for 1 min, extending at 72 °C for 30 s. 30 additional cycles were carried out while the annealing temperature was lowered down by 0.5 °C each circle until it reached 55 °C and the primer extension was then performed at 72 °C for 8 min. The products of PCR reaction were detected by agarose gel (2%).

3. DGGE analysis

The PCR products were separated by a Dcode^{TM} Universal Mutation Detection System produced by Bio-Rad, Hercules, CA, USA.

(1) Denaturing gradient gel. 8% polyacrylamide gels were prepared with denaturing gradient equipment with a linear denaturing gradient ranging from 35% denaturant at the top of the gel to 60% denaturant at the bottom (100% denaturant contains 7 mol/L urea and 40% formamide).

(2) PCR sample addition. After the denaturing gradient gel was totally solidified, put the gel to the electrophoresis slot which contained electrophoresis buffer. Then the mixture of 5 μl sample and 10 times volume loading buffer was added into the sample feeding hole.

(3) Electrophoresis and stain. The gels were run for 4 h at 150 V with 60 °C, and then the gels were stained with SYBR Green.

(4) Scanning. The stained gel was photographed by UMAX transillumination scanner. Put the promega on both sides of gel and fastened it by dry gel clip, then stored it after natural desiccation.

(5) Analysis of DGGE finger-print. A Bio-RADCEVANTIT YONE 4.3.0 software was used to process the lanes and the bands. Similarity coefficient was used to reflect the similarity between the bands in DGGE finger-print. Jaccard coefficient, $b=n_{xy}/(n_x+n_y-n_{xy})$, where n_x and n_y were bands number in lanes x and y, respectively; n_{xy} was the common bands number of the two lanes.

Analysis of wastewater quality

Chemical oxygen demand (COD) concentration was measured by the potassium dichromate method. Biochemical oxygen demand (BOD) was analyzed by dilution and the inoculating method. The ammonia nitrogen (NH_4^+-N) concentration was measured by Nessler's reagent spectrophotometer method (SEPAC, 2002).

RESULTS AND DISCUSSION

Performances of A/O process

As shown in Fig.2, the COD, BOD and NH_4^+-N concentrations of the influent varied widely in the range of 426~560, 160~310 and 64~100 mg/L, respectively. In response, effluent quality fluctuated in a wide range, while the COD, BOD and NH_4^+-N concentrations were 109~211, 7.3~38.8, 46.5~63.5 mg/L and their average value were 160, 18 and 55 mg/L, respectively. The effluent COD and NH_4^+-N concentrations were 1.6 and 2.2 times higher than those of the Level II criteria (100 and 25 mg/L, respectively) of the national discharge standards in China (SSB, 2002).

Efficiency analysis of different compartments in A/O system

From the results of the continuous monitoring, pollutants removal efficiency in different compartments of the A/O system varied erratically (shown in Fig.3). The average removal efficiencies of COD, BOD and NH_4^+-N in Section A (S1~S2) were 48%, 74% and 20%, respectively, which were relatively high compared with those in other sections. This may be due to the combined action of dilution by recycling sludge and adsorption of the activated sludge when the wastewater was introduced in the biological system. Another explanation was the cooperative removal ability of anaerobic bacteria and facultative bacteria contained in the anoxic tank (Wen *et al.*, 2006). Along the flow direction, the BOD removal efficiencies of Section O1 (S2~S3), Section O2 (S3~S4), Section O3 (S4~S5) and Section O4 (S5~S6)

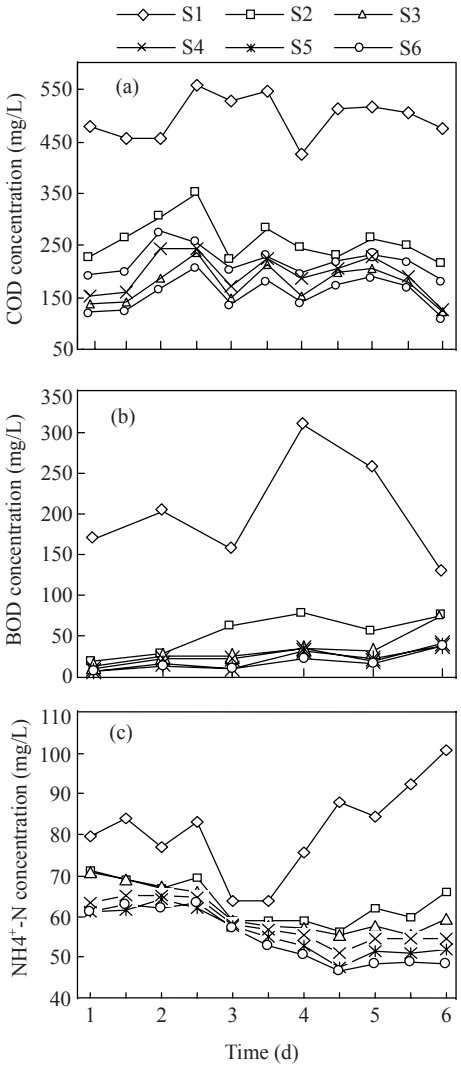

Fig.2 Concentrations of (a) COD, (b) BOD and (c) NH_4^+-N varied in different sections

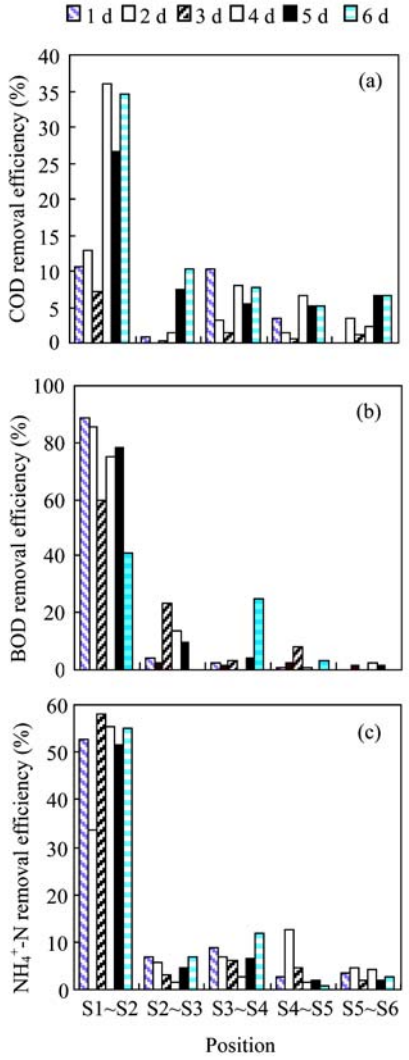

Fig.3 (a) COD, (b) BOD and (c) NH_4^+-N removal efficiency of different sections

decreased gradually 9.5%, 4.5%, 2.1% and 1.2%, respectively. This indicated that Sections O2, O3 and O4 were limited in removing BOD, which was caused by the gradually decreased biodegradability of the organics contained in the wastewater. Removal of COD and NH_4^+-N in oxic sections did not have a regular pattern, while that in Sections O1 and O2 were more efficient than that in Sections O3 and O4. Because of the possible impacts of unstable influent characteristics and operational conditions on bacterial community structure, the pollutants purification efficiencies of different compartments were extremely unstable in A/O biological system.

GC-MS analysis

GC-MS analysis of samples at different locations in A/O tank was conducted. The total ion chromatograms (TIC) were shown in Fig.4. It showed that the type (the number of TIC wave crest) and the amount (the area of TIC wave crest) of organics in the wastewater presented a decreasing trend along the flow direction. From S3, the areas of TIC wave crest were almost the same. This demonstrated that the removal abilities of the last three compartments on organics were extremely limited and most of the organics were removed in Section A and Section O1.

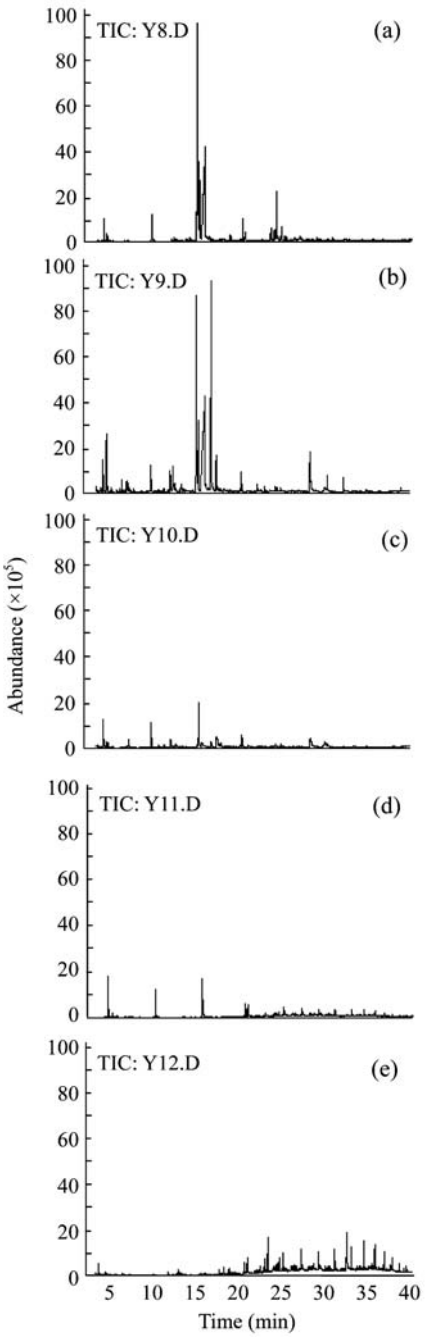

Fig.4 Chromatogram of different samples. (a) Influent; (b) S1; (c) S3; (d) S5; (e) S6

Further analysis of the types and the relative amounts of organics were shown in Table 1. It showed that there were 7 main types of organics in the influent. The number of organics in influent was 112, and increased to 185 (at S1) after the influent mixed with the return sludge in Section A. Then, the number decreased to 113 (at S3) and 48 (at S5), but increased again to 116 (at S6) in the secondary settling basin. The number of organics in the secondary settling basin was close to that in the influent. Thus, along the water flow direction, the types of organics first increased, then gradually decreased, and finally increased abruptly. The sudden increased substances were mainly such refractory organics as alcoholic aldehyde, ketone acid, hydrocarbons and halogenated hydrocarbons. It was inferred that these refractory organics were adsorbed initially by the activated sludge under aeration conditions. And then under the anoxic conditions of the secondary settling basin, the activity of the aerobic bacteria and zoogloea reduced dramatically. Accordingly, the activity of activated sludge deteriorated gradually and began to disintegrate. As a result, the refractory substances adsorbed originally by the activated sludge released back into the supernatant, leading to the abundance of these organics in the effluent. The similar result had also been proved by Xue (2002), which demonstrated that refractory organics in wastewater were mainly removed due to the effects of adsorption of sludge, while the degradation was minor.

The content ratio of different organics also had dissimilar changing trend as shown in Fig.5. Phenols were completely discomposed and could not be detected in the effluent; the content ratio of alcoholic aldehydes and ketone acids diminished gradually, while rose suddenly in the secondary settling basin. The proportion of aromatic hydrocarbons and ether esters increased gradually, but declined suddenly in the secondary settling basin. The reduction of alcoholic aldehydes and ketone acids as well as

Table 1 Analysis of wastewater component in different section

Sample	Hydrocarbons	Alcoholic aldehydes and ketones acid	Aromatic hydrocarbons	Ether esters	Phenols	Halogenated hydrocarbons	Others	Total
Influent	38 (38%)	23 (22%)*	18 (15%)	4 (5%)	2 (2%)	2 (1%)	22 (17%)	112
S1	52 (32%)	23 (11%)	20 (13%)	18 (7%)	5 (3%)	16 (10%)	51 (24%)	185
S3	44 (37%)	15 (13%)	14 (14%)	14 (12%)	3 (4%)	14 (11%)	9 (9%)	113
S5	14 (21%)	1 (1%)	15 (40%)	14 (35%)	−	2 (2%)	2 (2%)	48
S6	51 (33%)	22 (20%)	19 (19%)	9 (6%)	−	10 (11%)	5 (11%)	116

* Data in parentheses represent the percentage of wastewater volume concentrations

hydrocarbons in wastewater was due to the adsorption of activated sludge but not aerobic degradation. Under the anaerobic condition in the secondary settling basin, these substances released back into the supernatant, and resulted in their sudden content rising. Aromatic hydrocarbons and ether esters could hardly be biodegraded or adsorbed by activated sludge, thus their quantities remained still and the content ratios changed according to the decrease and increase of other organics.

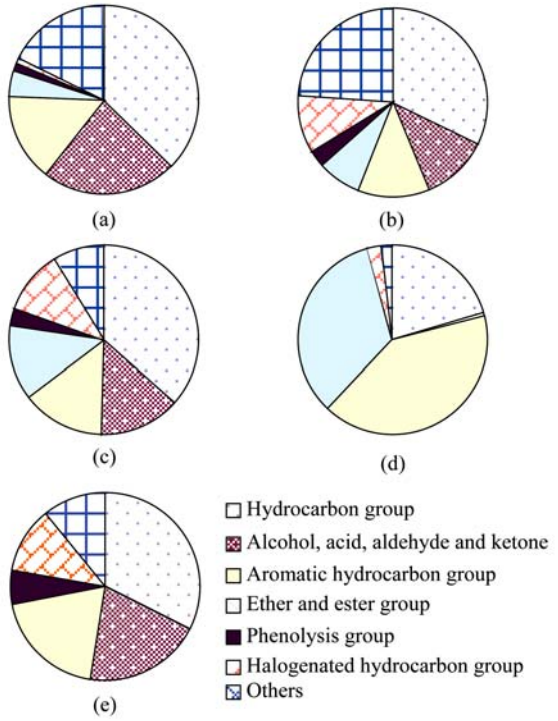

Fig.5 Content ratio variation of different organics
(a) Inflow; (b) S1; (c) S3; (d) S5; (e) S6

Analysis of PCR-DGGE

The stability of bacterial community structure of activated sludge would directly affect the purification efficiency of the biological reaction tank (Marsh *et al.*, 1998; Eichner *et al.*, 1999). To investigate the bacterial community structure in the A/O biological system and its impacts on the process performances, the dynamic changes of bacterial community of different sludge samples were analyzed through PCR-DGGE.

Similarity analysis of sludge samples

Bacterial community fingerprints for each sample collected from different sections were presented in Fig.6. From a qualitative perspective, the community fingerprints of the 5 sections varied as the total bacterial bands of S1, S2, S3, S4, S5 and S6 were 30, 19, 12, 31, 23 and 31, respectively (mean=24.3). Similarity coefficient was adopted to analyze the microbial community structure in 6 different locations of the wastewater treatment system (shown in Table 2).

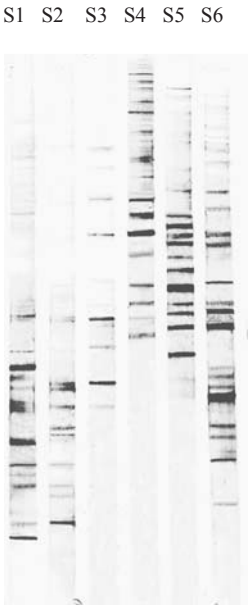

Fig.6 DGGE fingerprints of different samples

Table 2 Similarity coefficient of the DGGE profiles (%)

	S1	S2	S3	S4	S5	S6
S1	100.00					
S2	36.11	100.00				
S3	16.67	29.17	100.00			
S4	27.27	8.16	16.22	100.00		
S5	10.32	6.25	9.38	22.73	100.00	
S6	32.60	16.30	10.26	16.98	35.00	100.00

According to cluster analysis of the similarity between each lane in DGGE profiles, the similarity coefficients of the predominant bacterial community structure in each section were low, with the highest similarity coefficient (S1 and S2) of 36.11% and the lowest similarity coefficient (S2 and S5) of 6.25%. The cluster analysis also demonstrated that S1 and S6 had high similarity, which indicated that the recycling of the activated sludge had certain side impacts on the bacterial community structure.

Characteristics analysis of each sampling location

For further analysis of the differences between

bacterial community structures, characteristics analysis of each sampling location was conducted (shown in Table 3).

Table 3 Characteristic analysis of sampling locations

Location	DO (mg/L)	pH	BOD/COD	COD removal efficiency (%)
S1	0.12	8.33	0.41	
S2	0.66	6.79	0.20	47.78
S3	4.50	6.71	0.15	7.86
S4	4.47	6.61	0.13	5.33
S5	4.34	6.53	0.11	4.03
S6	0.61	6.47	0.11	3.41

As shown in Table 3, S1 was in anaerobic and alkalescent (pH=8.33) conditions with low DO concentration (0.12 mg/L) (less than 0.3 mg/L), and its biodegradability was satisfying as BOD/COD=0.41. S2 was in anoxic condition with 0.66 mg/L DO (less than 0.7 mg/L) and weakly acidic (pH=6.79), and had poor biodegradability as BOD/COD<0.25. S3, S4 and S5 were in aerobic condition with DO concentrations all exceeded 4.3 mg/L, and were extremely poor in biodegradability as BOD/COD<0.15. S6 was in anoxic condition with 0.61 mg/L DO and low BOD/COD ratio (<0.15).

The above analysis indicated that the environments at each sampling location were quite different. The activated sludge flew from S1 through S2, S3, S4 and S5 to S6 (secondary settling basin), and then recycled back to S1. This go-round-and-round sludge reflux led to the frequent changes of the bacterial population of the activated sludge from anaerobic, anoxic to aerobic conditions. The original activated sludge existed in the tank was constantly replaced by the newly introduced activated sludge. Therefore, the predominant bacterial community structure was also in continuous variation and was unstable. In addition, the biodegradability of the wastewater declined along the flow direction. Thus, the substrates could be utilized by aerobic bacteria gradually decreased, resulting in the community structure adjustment in response to the constantly changing influent characteristics. This finally led to relatively low similarity of community structure. The COD removal efficiency of the first two compartments (55%) was far higher than that of the last three compartments (13%), which indicated that the degrading ability to refractory organic pollutants of anaerobic bacteria and facultative bacteria was better than that of the aerobic bacteria. Previous researches (Ling and Xiao, 2003) had also proved that anaerobic and anoxic conditions were more suitable for the treatment of refractory organic pollutants involved in industrial wastewaters.

DISCUSSION

Impacts of microbial community structure variations on pollutants removal efficiency

Since the ecological function of microorganisms depended on its community structure, the operational performances and degrading efficiencies of the treatment system could be reflected by the variations of its microbial community structure. Each organism had its inherent niche and optimum substrates, thus the microbial community would adjust its structure in response to the ever-changing environment. As shown in Fig.2, shock loadings occurred as the influent quality greatly varied. In addition, the constant changes of DO concentration, the dramatic reduction of biodegradability and the go-round-and-round sludge recycling also contributed to the constant variation of the environmental conditions, which led to the repeating alterations of bacterial community structure. As shown in Fig.6, the obvious distinction of the bacterial populations proved the adjustment and succession process of bacterial community structures. Unstable community structure would directly result in unfavorable removal efficiency. The high removal efficiency in Section A was due to the similar environment in the secondary settling basin and Section A; the removal efficiency in Section O1 was lower, while in Sections O2, O3, O4 were even worse, only 20% compared with those of Sections A and O1. Thus, bacterial community in activated sludge was in constant changing in response to the variations of environment, and unstable bacterial community structure would lead to poor pollutants removal efficiency. These points were consistent with the conclusion drawn by Lapara *et al*.(2002).

In this study, PCR-DGGE technique used to assess bacterial community structure does not necessarily provide a completely accurate and unbiased fingerprint. These biases have been discussed elsewhere in detail (Myers, 1985; Buchholz-Cleven *et al.*, 1997; Hansen *et al.*, 1998; Orphan *et al.*, 2000).

However, compared to the conventional cultivation-based approaches, the adaptation of the community structure at different locations of the biological treatment system was evident only because of the presence of a few bands that were not detectable among different samples by PCR-DGGE through identical methods. Thus, it could be inferred that relatively stable bacterial community structure is necessary in improving the performances of the biological system for treating petrochemical wastewater.

Influence of sludge recycling on operational performance of WWTP

It is known that activated sludge recycle could remain sufficient biomass in the biological system, which was crucial to maintaining the stable operational performance. However, for the complex characteristics of petrochemical wastewater, such sludge recycle seemed to have negative effects. The removal efficiency of pollutants in the first two compartments accounted for almost 80% of the total removal efficiency and functioned far better than the last three compartments. This demonstrated that the dilution and adsorption of the activated sludge and the degradation of bacteria were vital to the removal of pollutants. The degradation of the recycling sludge was mainly attributed to the relatively high community structure similarity (32.6%) of the recycling activated sludge (anoxic) and the sludge in Section A (anaerobic). The environment of the activated sludge was in constant variation when it flew into the following aerobic compartments, which directly affected the stability of bacterial community structure and finally influenced the performances and the stability of the WWTP. Accordingly, for sludge recycling, compared to its advantages such as the retention of sufficient biomass, more attention should be paid to its side impacts on the A/O system's stability and purification efficiency.

Process improvement strategies

To stabilize the bacterial community structure and the purification efficiency to petrochemical wastewater of the A/O process, the following strategies may be considered: (1) to strengthen the pretreatment units for reducing the fluctuation of the wastewater and avoiding the shock loadings to the activated sludge system; (2) to transform the original O3 compartment into the anoxic compartment and packed A/O tank with immobilized carriers at different locations, i.e., to adjust the original A/O process into two-stage A/O (A1-O1-O2-A2-O3) biofilm process. Thus, after most of the organics that are liable for aerobic bacteria are depleted, the anaerobic bacteria and facultative bacteria could again become dominant for pollutants degradation by the alteration of DO concentration. By this step-by-step biodegradation, the diversity of the microbial population and the stability of the community structure would be enhanced. Microorganism in different biological compartments would display its unique functions and the removal efficiency of pollutants will be improved. Further study on purifying efficiency of the improved process is underway through on-site pilot research.

CONCLUSION

By adopting conventional physicochemical analysis, GC-MS analysis and PCR-DGGE techniques, the purification efficiency of a full-scale A/O process treating petrochemical wastewater and its stability of microbial community structure were investigated; the applicability of A/O process in treating petrochemical wastewater was also discussed. The results from this work lead to the following conclusions:

(1) The present A/O process had poor removal efficiency of pollutants, especially the refractory organics contained in the petrochemical wastewater. While COD and NH_4^+-N concentrations of the influent were 420~560 mg/L and 64~100 mg/L, respectively, the corresponding average concentrations in the effluent were 160 mg/L and 55 mg/L, which were 1.6 and 2.2 times higher than those of the national standards in China, respectively.

(2) GC-MS technique was applied to analyzing the variation of pollutants' content ratios and their migration and transformation patterns along the water flow direction. The number and amount of organics in the wastewater exhibited a collectively decreasing trend; the removal of pollutants was mainly carried out in Sections A and O1 primarily through sludge adsorption but not biodegradation.

(3) The bacterial community structure of the activated sludge in A/O biological wastewater treatment

system was species-rich but unstable, and apparent succession of community structure was observed in the system. The variation of bacterial community structure had great influences on the pollutants removal efficiency, thus the key for the petrochemical wastewater treatment with high removal efficiency was to maintain a relatively stable bacterial community structure.

(4) Both the purification efficiency and community structure stability of the A/O process were poor and A/O process was unsuitable for the treatment of complicated petrochemical wastewater. The sludge recycling would directly affect the stability of microbial community structure, which was a crucial factor affecting the wastewater purification efficiency. The anaerobic and anoxic conditions are preferable for the treatment of recalcitrant pollutants in petrochemical wastewater. Therefore, transforming the original process into the two-stage A/O biofilm process may greatly enhance the purification efficiency of the WWTPs, and this awaits further study.

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