Performance and microbial characteristics of integrated fixed-film activated sludge system treating industrial wastewater

C. Li, X. L. Li, M. Ji and J. Liu

ABSTRACT

Integrated fixed-film activated sludge (IFAS) is a modification of conventional activated sludge processes used for wastewater treatment consisting of biofilm attachment surfaces added to suspended-growth reactors, thereby creating hybrid suspended attached-growth systems. In this work, an IFAS system, with a total working volume of 560.7 L, was employed to investigate the performance of pollutants removal in industrial ef fluent. Microbial communities of suspended-growth and attached-growth biomass were investigated by a combination of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescence in situ hybridization (FISH). In the system, suspended-growth and attached-growth biomass worked together to maintain a stable COD, NH4+-N and TN removal efficiency of 74, 93 and 76%, respectively. PCR-DGGE results showed that Proteobacteria was the dominant genera of bacteria in both the suspended and attached growth sludge. Some of the bacterial species (Firmicutes and Pseudomonas) may play an important role in the organic degradation of this water. Denitrifying bacteria were found to be accumulated in the biofilms. FISH results showed that there were more nitrifying bacteria in the suspended-growth biomass than the attached-growth biomass but fewer denitrifying bacteria in the former biomass.

Key words | FISH, IFAS, industrial park wastewater, non-biodegradation, PCR-DGGE

INTRODUCTION

In recent years, rapid economic development has led to over-development of industrial parks in China (Geng & Zhao 2009). In general, wastewater treatment plants (WWTPs) with traditional biological processes were simultaneously built at the beginning of the construction of most industrial parks. Wastewater from industrial parks always contains chemicals, a great portion of which are non-biodegradable and/or toxic to the microbial system in common WWTPs. As a result, the effluent from the WWTPs cannot often meet the Discharge Standard for Pollutants for Municipal Wastewater Treatment Plant (GB 18918–2002, China), and therefore put the receiving water bodies or other environments at great risk. Thus, the WWTPs in a large amount of industrial parks in China urgently need upgrading and/or retrofitting (Lei et al. 2010).

Integrated fixed-film activated sludge (IFAS, also known as hybrid biological reactor) is an aerobic hybrid process that combines suspended sludge with the attached growth biomass by incorporating biofilm carriers into aeration tanks of the activated sludge process. It contains both suspended-growth and attached-growth biomasses, so the total biological treatment capacity of the hybrid reactor is the sum of the two types of biomass (Wang et al. 2000). Integrated fixed-film activated sludge (IFAS) can retain a considerable amount of the attached-growth biomass, which increased along with organic loading rates (Wang et al. 2000). When the bio-carrier concentration increased, the average biomass concentration of suspended sludge decreased (Wang et al. 2005). IFAS also exhibited high efficiencies of simultaneous nitrification and denitrification with the operational parameters of hydraulic retention time (HRT) 4 h and dissolved oxygen (DO) 2 mg/L (Xia et al. 2008). Kim et al. (2010) reported that the attached-growth biomass in IFAS has lower density than that of the suspended-growth biomass.
biomass, due to the lower amount of polyphosphate storage. Furthermore, IFAS is an attractive option for retrofitting many existing facilities, in part because the inclusion of an attached biomass phase enriches the slow-growing autotrophs responsible for ammonia and nitrite oxidation (nitrifying bacteria), which are central to nitrogen removal processes in wastewater treatment, thus providing improved nitrification capacity without the construction of new reactors (Randall & Sen 1996). By means of incorporating bio-carriers, it is possible to obtain a two-fold increase in biomass concentration in the aeration tanks without increasing its volume (Seetha et al. 2010). However, there are few studies available in the literature on industrial park wastewater treatment by IFAS, and there is still a lack of knowledge about the microorganisms in suspended-growth and attached-growth biomasses at the molecular level.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), cloning-sequencing and fluorescence in situ hybridization (FISH) are popular in the analysis of microorganisms in microbial sludge samples. PCR-DGGE has been developed to analyse bacterial community structures without the inherent biases of cultivation (Lapara et al. 2002). The FISH method allows the direct identification and enumeration of microbial populations active in both suspended-growth and attached-growth biomasses (Fu et al. 2010).

The purpose of this study was to investigate the performance of IFAS on the pollutant removal rates of industrial park wastewater and bacterial community structures in the suspended-growth and attached-growth biomasses. The results obtained in this study may aid in the design of full-scale processes.

**MATERIALS AND METHODS**

**IFAS system**

A schematic diagram of IFAS is shown in Figure 1. The reactors were made of UPVC sheets, including an anaerobic reactor (65.6 L), an anoxic reactor (112.5 L), two aerobic reactors (191.3 L each) and a settling tank. The equipment was located at the West Zone WWTP of Tianjin Economic-Technological Development Area (TEDA), China, and operated in an anaerobic–anoxic–aerobic configuration. Influent wastewater was pumped directly into the top of the anaerobic reactor. The flow rate (Q) was $1.10 \pm 0.05 \ \text{m}^3/\text{d}$, resulting in a total HRT of $12.08 \pm 0.53 \ \text{h}$. The process was fed with industrial wastewater and air continuously. The other operational parameters were as follows: water temperature 11–24 °C, pH 7.0–8.0, DO concentration 3–4 mg/L.

The bio-carriers used in the IFAS are made of polyurethane plastic (Figure 1). Several pieces of bio-carriers were fixed in the aerobic reactors with a carrier concentration of 8.6% (the volume of cumulated carriers to the volume of aeration basin). The specific surface area of the bio-carrier is about 2,000 m$^2$/m$^3$, which is much larger than that of other bulk media such as Bioportz (Kim et al. 2011). Another advantage is the bio-carrier can swing with the flow in the reactors and will not be discharged.

**Industrial wastewater**

Wastewater gathered from West Zone of TEDA had low BOD$_5$ values and contained a variety of non-biodegradable organic chemicals. More than 90% of the organic matter in
all collected water samples belonged to different types, which showed the instability of the water quality. Among them, 2,6-di-tert-butyl-methylphenol, benzothiazole, N-(methyl)aniline, cholesteryl alkyl and cholesterol are of high frequency according to the gas chromatography–mass spectrometry (GC-MS) results. The characteristics of the industrial wastewater (influent load increased from experiment day 95) used as the influent for IFAS are listed in Table 1.

### Startup and operation of IFAS

The IFAS system was inoculated with activated sludge, which came from the aeration tank of the West Zone WWTP. As shown in Table 2, IFAS was operated for 160 days including four successive phases: Stage 1, only bio-carriers were fixed in the reactor without suspended-growth biomass. Suspended biomass was washed out to avoid competing with the attached microorganisms for substrates after 3 d inoculation (Guo et al. 2013); Stage 2, sucrose was added into IFAS as external carbon source in this period; Stage 3, the suspended-growth biomass was added into the system working together with attached-growth biomass and the solids residence time (SRT) of the suspended-growth biomass was maintained at 25 d (the concentration of suspended-growth biomass was 3,500 mg/L); Stage 4, concentrations of all the influent pollutants increased significantly and the external carbon was no longer added.

### DNA extraction and PCR-DGGE

Sludge samples from attached-growth biomass were collected by scraping the biofilm from the media surface with a spatula and diluting it with distilled water. Suspended sludge samples were collected from the aerobic reactor of IFAS. The sludge samples were used for PCR-DGGE and FISH analysis in the following steps. The total DNA was extracted by using an EZNA™ Soil DNA kit (D5625–01, Omega Bio-tek Inc., USA) according to the manufacturer’s instructions. The primer pair F341GC and R534 was used to amplify the V3 region part of 16S rDNA genes and to obtain DNA fragments 240 bp in length (Li et al. 2013; Zhang et al. 2014). The touchdown PCR strategy included an initial 10 min denaturation at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min at 65°C (touchdown for 0.5°C every cycle) and 1 min at 72°C, then 10 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and 8 min final extension at 72°C. DGGE was performed on a DGGE-2001 System (CBS SCIENTIFIC, USA). Finally, gels were stained with AgNO₃ and then visualized and scanned by a scanner (HP LaserJet M1005 MFP, China). The gel images were analysed using the software Quantity One, version 4.6.2 (Bio-rad, USA).

Prominent DGGE bands were excised and purified by using a PolyGel Extraction Kit (CoWin, China). The target DNA fragments were then reamplified using the primer set F341/R534 without the GC-clamp, thus obtaining a pure sample for the cloning and sequencing step. The PCR products were cloned using the pGEM®-T Easy vector system (Promega, Madison, WI). The positive colonies were amplified with F341/R534. PCR amplicons were submitted for sequencing using ABI 3730 capillary sequencers (PE Applied Biosystems, Invitrogen, Beijing, China). Sequence data from 16S rRNA gene fragments were submitted to NCBI (National Center for Biotechnology Information, USA) for homology searching.

### Table 1: Characteristics of the industrial wastewater (mg/L)

<table>
<thead>
<tr>
<th></th>
<th>COD</th>
<th>BOD₅</th>
<th>NH₄⁺-N</th>
<th>TN</th>
<th>TP</th>
<th>NO₃⁻-N</th>
<th>TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1–3 value</td>
<td>97–202</td>
<td>12–35</td>
<td>5.7–18.5</td>
<td>11.6–27.2</td>
<td>2.5–10.2</td>
<td>1.4–14.0</td>
<td>24–1,102</td>
</tr>
<tr>
<td>Stage 4 value</td>
<td>179–458</td>
<td>39–127</td>
<td>5.4–50.0</td>
<td>17.8–48.2</td>
<td>4.3–48.5</td>
<td>3.8–15.9</td>
<td>118–1,840</td>
</tr>
</tbody>
</table>

### Table 2: Operating conditions of IFAS

<table>
<thead>
<tr>
<th>Step</th>
<th>Day</th>
<th>Suspended-growth biomass</th>
<th>Attached-growth biomass</th>
<th>External carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 (biofilm culturing)</td>
<td>1–28</td>
<td>×</td>
<td>√</td>
<td>×</td>
</tr>
<tr>
<td>Stage 2 (external carbon)</td>
<td>29–48</td>
<td>×</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Stage 3 (suspended sludge)</td>
<td>49–94</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Stage 4 (high organic loading rate)</td>
<td>95–160</td>
<td>√</td>
<td>√</td>
<td>×</td>
</tr>
</tbody>
</table>

*Does not include this condition.*
FISH analysis

Sludge samples of suspended sludge and biofilm biomass were hybridized for 3 h with a 5 ng/μL oligonucleotide probe at 46 °C (Coskuner et al. 2005). After that, the samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI) in the dark for 10 min. The oligonucleotide probes used for FISH and the corresponding hybridization conditions are given in Table 3. All the probes were provided by Invitrogen (China). The samples were visualized with a fluorescence microscope (OLYMPUS IX71, Japan) immediately. The number of probe-hybridized cells was determined by software Image-Pro Plus 6.0 (Media Cybernetics, USA) (Fu et al. 2010).

Other analytical methods

Influent and effluent samples were collected three times a week and analysed for soluble COD, NH4+-N, TN, NO3, NO2 and mixed liquor suspended solids (MLSS) according to standard methods (APHA 1998). Suspended-growth and attached-growth biomass samples were collected and analysed by the method mentioned in the literature (Wang et al. 2005).

RESULTS AND DISCUSSION

Performance of IFAS

COD removal

Figure 2(a) shows the influent and effluent chemical oxygen demand (COD) variation under different stages. Sucrose was added into IFAS as external carbon source since Stage 2, and influent COD increased by 66 ± 10 mg/L. Suspended sludge was added to IFAS in Stage 3; as a result, the sludge loading rate dropped to 0.11 ± 0.04 kg COD/kg MLSS per day and the average COD removal efficiency was significantly increased to 49.6%. In Stage 4, although the influent COD concentration significantly increased to a range of 142–458 mg/L, IFAS still maintained a relatively stable COD removal efficiency of 39.5–82.5%, and the effluent COD was below 80 mg/L from day 150, even when the COD increased a lot. That was because after 100 days' operation, the biomass in IFAS had adapted to the system environment and the total biomass concentration in the reactor was relatively high. It showed that the IFAS system was capable of absorbing organic shock loads, which was also reported in the literature (Seetha et al. 2010).

Nitrogen elimination

The effectiveness of NH4+-N removal in IFAS is depicted in Figure 2(b). The average NH4+-N concentration in the effluent was 5.16 mg/L with an average removal efficiency of 55.6% in Stage 1–2. After adding suspended sludge into the system, removal efficiency of NH4+-N increased to 93.5%. Even though the NH4+-N load was nearly doubled on day 100, the removal efficiency of NH4+-N was as high as 98%, which indicated that nitrification was complete in the reactor and IFAS was not operated at a maximum load.

Effluent NO3 concentration ranged from 6.02 to 18.55 mg/L in Stage 1. Most NH4+-N was oxidized to NO3, but there was not enough biodegradable carbon source for denitrification (BOD5/TN = 1.27). As a result, the TN concentration in effluent was high (Figure 2(c)). In order to improve the removal efficiency of NO3, sucrose (66 ± 10 mg/L) was added into the anaerobic reactor. Denitrification increased continuously, and the TN concentration in the effluent decreased to approximately 3.7 mg/L with the exception from Day 47 to Day 60, when the suspended sludge was first added into the system. As influent COD increased in Stage 4, there was no need to dose the external carbon source any more. The mean effluent TN was 6.56 mg/L during this period, which showed that IFAS had been enriched with denitrifying bacteria.

Biomass microbial community structure analysis

PCR-DGGE

The PCR-DGGE fingerprints of bacterial community acclimated by different stages are presented in Figure 3. 16S rDNA bands in a DGGE gel might link to different bacteria

Table 3 | FISH Probes

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequence (5′-3′)</th>
<th>Specificity (rRNA target, position)</th>
<th>Fluorescein</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSO190</td>
<td>CGATCCCTGCTTTTCCTCC</td>
<td>Ammonia-oxidizing Betaproteobacteria (190–208)</td>
<td>Cy5</td>
<td>Coskuner et al. (2005)</td>
</tr>
<tr>
<td>TBD121</td>
<td>CTCGGTCCTCACCAGC</td>
<td>Thiobacillus denitrificans</td>
<td>Cy5</td>
<td>Beristain et al. (2010)</td>
</tr>
</tbody>
</table>
species and the intensity of the bands might correspond with the abundance of the corresponding species (Li et al. 2009). Different bacterial community structures were found during acclimation of wastewater by analysing DGGE profiles. Although the dominant species in the inoculating sludge were preserved throughout the process (band 1, 2, 3), many more bands were observed in the sludge by 150 d compared with the seed sludge. There was a noticeable difference in microbial population between suspended samples and biofilm samples (band 12, 13, 14, 15). DGGE analysis suggested that biofilm contained a greater microbial diversity than that of suspended sludge.
Bands from the DGGE analysis were sequenced to determine the identity of bacterial strains present in the sludge (Table 4). As determined by comparison of the 16S rRNA sequences, the majority of the bacteria grouped with members of Proteobacteria, with one in α-subdivision, three in β-subdivision and two in γ-subdivision. Proteobacteria also played an important part in other biological treatment systems (Wang et al. 2009). *Pseudomonas putida* has a similarity of 91% with band 5, which was able to degrade phenol, phenylacetic acid, salicylic acid and toluene and other aromatic compounds (Hu et al. 2011). The bacterium in band 6, which was widely distributed in the sewage and demonstrated a strong ability to adapt to environmental changes, was present during the whole experiment. A denitrifying bacterium was observed in band 13 (Betaproteobacterium clone DF), which was only present in IFAS biofilm samples. To amplify ammonia oxidation bacteria (AOB) and nitrite oxidizing bacteria (NOB) specific 16S rDNA for DGGE, the AOB and NOB-specific primer pairs were used to obtain DNA fragments 465 and 397 bp in length, respectively, with a nested-PCR strategy. Detailed results will be discussed in further research.

### Table 4 | Species identification of selected DGGE bands

<table>
<thead>
<tr>
<th>DGGE band</th>
<th>Closest species in GenBank</th>
<th>GenBank No.</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>DQ444188</td>
<td>Uncultured bacterium clone cs90</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>EU300338</td>
<td>Uncultured <em>Firmicutes bacterium</em> clone GASP-KC33S_E10</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>EU620069</td>
<td><em>Pseudomonas putida</em> strain GNA5</td>
<td>91</td>
</tr>
<tr>
<td>6</td>
<td>FJ393072</td>
<td>Uncultured <em>Aeromonas</em> sp. clone MFC-B162-B02</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>FJ529954</td>
<td>Uncultured bacterium clone MABRDTU1</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>DQ182217</td>
<td>Uncultured bacterium clone KRF7</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>AB354655</td>
<td>Uncultured Gammaproteobacterium clone HF009</td>
<td>98</td>
</tr>
<tr>
<td>11</td>
<td>AY144356</td>
<td>Uncultured <em>candidate</em> division TM7 bacterium clone SBG4</td>
<td>94</td>
</tr>
<tr>
<td>12</td>
<td>EF419216</td>
<td>Uncultured <em>Arcobacter</em> sp. clone P5–5</td>
<td>98</td>
</tr>
<tr>
<td>13</td>
<td>AY823968</td>
<td>Uncultured Betaproteobacterium clone DF AW-04</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>CU926935</td>
<td>Uncultured Betaproteobacteria bacterium 16S rRNA gene from clone QEDN3DG10</td>
<td>99</td>
</tr>
<tr>
<td>15</td>
<td>AF204254</td>
<td>Uncultured Betaproteobacteria clone SBR 1022</td>
<td>90</td>
</tr>
<tr>
<td>16</td>
<td>DQ640676</td>
<td>Uncultured <em>Bacteroidetes</em> bacterium clone Skagenf 93</td>
<td>99</td>
</tr>
<tr>
<td>18</td>
<td>FJ204548</td>
<td>Uncultured bacterium clone ISA006</td>
<td>96</td>
</tr>
</tbody>
</table>
FISH analysis

Figure 4 shows the bacterial distribution ratios of suspended-growth and attached-growth biomass in different operation periods. The distribution ratios of Betaproteobacteria AOB, Nitrobacteria NOB and Thiobacillus denitrifying bacteria were displayed by the relative distribution of total bacteria.

As shown in Figure 4, the initial distributions of each bacterium in the inoculated sludge were 2.8 ± 0.7, 2.4 ± 1.0 and 0.7 ± 0.3%, respectively. Betaproteobacteria AOB population at day 70 had increased to 3.7 ± 0.7 and 3.9 ± 0.6% in the suspended-growth and attached-growth biomasses, respectively. Population of Nitrobacteria NOB increased to 3.0 ± 0.6 and 2.8 ± 0.4% in the two types of biomasses, respectively. The corresponding NH₄⁺-N removal efficiency increased to 93.5% in the same period. The distribution ratios of denitrifying bacteria increased to 1.0 ± 0.4 and 3.0 ± 0.6% in the suspended-growth and attached-growth biomasses at day 150, which suggested that more denitrifying bacteria accumulated in the attached-growth biomass. The bio-carriers in IFAS provided a better habitat for denitrifying bacteria. The detection differed from results by Kim et al. (2011), who reported much higher AOB and NOB contents in the attached-growth biomass than in the suspended-growth biomass by quantitative PCR.

A typical pollutant variation of IFAS on day 150 is shown in Figure 5. Influent and recycled NO₃⁻ was eliminated almost completely in the anoxic tank, which contributed to denitrifying bacteria in suspended-growth biomass. During the aerobic period, NH₄⁺-N was oxidized to nitrate, and the accumulated denitrifying bacteria in attached-growth biomass continued to play a role in denitrification. Suspended-growth biomass and attached-growth biomass worked together to achieve aerobic denitrification so as to reach high total nitrogen (TN) removal efficiency.

CONCLUSIONS

1. In IFAS, suspended-growth and attached-growth biomasses worked together to maintain stable COD, NH₄⁺-N and TN removal efficiencies even during a high pollutant load period. COD, NH₄⁺-N and TN in the effluent were around 65.3, 0.86 and 5.48 mg/L, respectively. The remaining COD was refractory.

2. PCR-DGGE results showed that the microbial community accumulated both in suspended-growth biomass and attached-growth biomass and microorganisms were more abundant in attached-growth biomass. Proteobacteria was found to be the dominant genera of bacteria in the sludge. Some of the bacterial species (Firmicutes and Pseudomonas) may play an important role in organic degradation. FISH results showed that there were more
nitrifying bacteria in the suspended-growth biomass than in the attached-growth biomass but fewer denitrifying bacteria in the former biomass. Denitrifying bacteria were found enriched in the biofilms.

3. Aerobic denitrification was achieved in IFAS due to the accumulation of denitrifying bacteria in the bio-carriers.

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REFERENCES


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