Decolorization and degradation of cationic red X-GRL by upflow blanket filter
Bin Qiu, Yan Dang, Xiang Cheng and Dezhi Sun

ABSTRACT
An anaerobic upflow blanket filter (UBF) was employed for the treatment of dye wastewater containing cationic red X-GRL (X-GRL) in this study. The bacterial community in the UBF at its stable state was investigated by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The results showed that all color and the majority of chemical oxygen demand (COD) (92–74%) can be removed as the dye load increased from 33 to 134 g/(m³ d). The removal of color and COD were mostly attributed to the anaerobic activated sludge in the reactor. According to the DGGE fingerprints, the bacterial community in the biofilm was more diverse than that in the activated sludge. The bacterial diversity of the activated sludge and the biofilm both decreased with the dye load increasing. The dominant group was found to be phyla proteobacteria including β-proteobacteria, γ-proteobacteria, δ-proteobacteria and ε-proteobacteria, suggesting that these microbes might play an important role in X-GRL decolorization and degradation.

Key words | bacterial community, cationic red, upflow blanket filter

INTRODUCTION
Azo compounds, one of the largest groups of synthetic dyes, are widely used in a number of industries such as textile, food, cosmetics and paper printing, etc. Effluents from dye producing and dyeing industries are generally of high color and of high strength. The discharge of dye-containing wastewater into local environments could cause serious health and environmental problems due to the low biodegradability and high toxicity of dye compounds (Yoo et al. 2009).

Anaerobic biotreatment was shown to be a promising technology for azo dye removal because azo bonds can be reduced by microorganisms under anaerobic conditions (Plumb et al. 2001; Mohanty et al. 2006). In some instances, mineralization of dyes and their intermediates occur.

An anaerobic upflow blanket filter (UBF), which combines an upflow anaerobic sludge blanket (UASB) reactor and anaerobic filter, had been shown to be highly efficient in dye decolorization and degradation (Plumb et al. 2001; Bromley-Challenor et al. 2004). In UBF reactors, the contribution of activated sludge and biofilm to the total dye removal was different due to the difference in the bacterial community. A full understanding of the structure of the bacterial community and its dynamics in both activated sludge and biofilm would help optimize operational conditions during the treatment of dye wastewater by UBF. Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16s rRNA gene fragments was often adopted as a method to provide a fingerprint of the dominant strains in mixed culture biosystems and more especially to explore biological diversity and population dynamics in response to environmental changes in wastewater treatment works (LaPara et al. 2002).

The aim of this research was to evaluate the performance of a UBF reactor for the treatment of azo dye wastewater with different dye loads. The bacterial community was analyzed by PCR-DGGE to investigate the dominant strains responsible for the dye removal. Azo dye decolorization and degradation in the UBF reactor were then discussed in detail.

MATERIALS AND METHODS
Experimental setup
A plexiglass cylinder with a working volume of 3 L (Ø 10 × 35 cm) was used as a UBF (Figure 1) in this study. The reactor was seeded with 1 L of anaerobic activated sludge (volatile suspended solids (VSS): 8.5 g/L) from a
laboratory-scale UASB for the treatment of indigo wastewater. A volume of 1.5 L of plastic biocarriers (Φ 5 × 8 mm) were added to the reactor. The reactor was then fed with 20 mg/L of X-GRL solutions amended by 1,000 mg/L of sucrose for 4 weeks. The amount of biofilm on the biocarriers was observed to be 5.6 g/L in the form of VSS.

After the start-up period, the UBF reactor was operated with different X-GRL loads: stage I (day 1–42), 33 g/(m³ d); stage II (day 43–87), 46 g/(m³ d); stage III (day 88–132), 67 g/(m³ d); stage IV (day 133–180), 100 g/(m³ d); stage V (day 181–228), 134 g/(m³ d). The temperature of the reactor was kept at 25 ± 1 °C and the hydraulic retention time was 36 h for over 200 d. The influent of the UBF consisted of (per litre): X-GRL (as desired), sucrose (1,000 mg), NH₄Cl (96 mg), KH₂PO₄ (22 mg) and trace solution (1 mL), giving a COD: N: P ratio of 200:5:1. The trace solution contained the following components (mg/L): ZnCl₂ (0.05), MnSO₄ (0.05), CuCl₂ (0.05), CoCl₂·6H₂O (0.05), NiCl₂ (0.05), and HBO₃ (0.05). The concentrations of color and COD at sampling port 1 and port 2 were examined every 3 d. Color measurement was performed spectrophotometrically at 540 nm. COD was analyzed according to standard methods of APHA.

**Microbial community analysis**

**DNA extraction and amplification**

Duplicate biomass samples (activated sludge and biofilm) were taken from the UBF at the end of stage III and stage V. The samples were centrifuged at 13,000 g for 10 min and the supernatant was decanted. The remaining pellet was washed with 1 mL of deionized and distilled water and centrifuged again in the same manner to ensure a maximal removal of residual medium. Total DNA was extracted using a soil DNA isolation kit (Q-BIOgene, UK), and was then purified with a DNA purification kit (Biovation Biotechnology, China).

**PCR-DGGE**

The 16s rRNA fragments of the Eubacteria were amplified using primers EUB338F (5'-GACTCCTACGGGAGGCAGCAG-3') and EUB534R (5'-ATTACCGCGGCTGCTGG-3') with a GC clamp attached to the 5' end of the forward prime (Ovreas et al. 1997; Grobkopf et al. 1998) to enable the separation of the fragments using DGGE. The amplification reaction was conducted according to the Taq DNA polymerase protocol (Promega, Madison, Wisconsin, USA). The PCR conditions were as follows: predenaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min (decrease 0.5 °C/cycle till 58 °C), elongation at 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were then separated by DGGE, and conducted with the BioRad DCode™ Universal Mutation Detection System (BioRad). The PCR products were applied to 8% (m/v) polyacrylamide (37.5:1 acrylamide–bisacrylamide) gels in 1 × TAE buffer containing a 35–65% denaturing gradient. A 100% denaturing solution contained 7 mol/L urea and 40% (v/v) formamide. Electrophoresis was performed in 1 × TAE buffer, at 60 V and 60 °C for 16 h. The DNA was stained with SYBR Green I dye (Sigma) and visualized under UV transillumination.

**Cloning and sequencing**

The dominant DGGE bands of every profile were excised from the gels with a sterile blade, mixed with 40 μL of TE buffer in sterile Eppendorf tubes, and incubated overnight at 4 °C. The DNA was reamplified using the corresponding primers without the GC clamp. The amplicons were purified with a D6492 Cycle-Pure Kit (Omega). The final products were then ligated into the pGEM-T Easy vector system (Promega, Madison, Wisconsin, USA) before they were transformed into the competent E. coli DH5α cells. Ampicillin selection and blue/white screening were applied to select positive clones. The selected clones were sequenced by an ABI sequencer (Sangon Biotechnology Co. Ltd, China). The sequences obtained were...
compared to the 16s rDNA gene sequences in the National Centre for Biotechnology Information (NCBI) database using the BLAST search option.

Phylogenetic analysis

The sequences were aligned with those belonging to representative organisms of the domain bacteria. The phylogenetic trees were constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis package (MEGA, version 3) (Kumar et al. 2004). The stability of the phylogenetic trees was analyzed by Bootstrap (1,000 replicates). The similarity and diversity of bacteria populations in the activated sludge and biofilm were analyzed by Quantity One (BIO-RAD 4.6.2) and by the Shannon diversity index (Shannon & Weaver 1965), respectively.

RESULTS AND DISCUSSION

X-GRL decolorization and degradation

The UBF reactor was demonstrated to be effective for the treatment of X-GRL containing wastewater as revealed by the removal of color and COD during the 228 d (Figures 2(a) and 2(b)). Complete color removal was observed when the reactor became stable after every increase in dye load. The removal efficiency of COD decreased gradually from stage I (90%) to stage IV (85%) and was seen to drop severely to ~75% when the dye load was added up to 134 g/(m³ d) in stage V. The 'shock effect' from dye load rises on the reactor performance seemed to be reversible, whereas the time for system recovery extended obviously with a dye load of 134 g/(m³ d). The results suggested X-GRL and its degradation intermediates could have imposed an inhibitory effect on the microorganisms in the UBF (Van der Zee & Villaverde 2005). The color removal in the UBF was mostly attributed to the anaerobic activated sludge at the lower layer in the reactor during the whole operational period (Figure 2(a)). The biofilm in the carrier layer, however, had a noticeable contribution in the COD degradation, especially with higher dye loads (Figure 2(b)).

Bacterial community analysis

Figure 3 shows the DGGE profiles of activated sludge and biofilm samples from the UBF in stage III and stage V when the reactor stabilized under a lower dye load of 67 g/(m³ d) and a higher load of 134 g/(m³ d). The similarity of DGGE profiles between sludge/biofilm samples in the UBF and the seed was lowered with an increase in the dye load (Table 1), which suggests that the microbes in the system were subject to selective pressure from increases in X-GRL in the influent. The similarity between the activated sludge and the seed decreased more significantly under a high dye load than that in the case of biofilm. This could be explained by the facts: (1) activated sludge at the lower layer in the UBF exposed directly to high concentrations of X-GRL; (2) mass transfer in biofilm was in general slower than that in activated sludge, which reduces the inhibition effect of hazardous substances on microorganisms. The similarity between the activated sludge and the biofilm shows a marked decrease with the dye load increasing, revealing that the functions of the two ecosystems tend to differ from each other with a high input of X-GRL.

Figure 2 | Removal efficiencies of color (a) and COD (b) in UBF.
The bacterial community in the activated sludge/biofilm was more diverse in stage III than stage V, as shown by the SDI values ($A_{III}$: 3.51; $B_{III}$: 3.93; $A_{V}$: 2.81; $B_{V}$: 3.04). This indicates that some of the bacterial populations were washed out from the reactor under a high dye load. The SDI of the biofilm was noticeably greater than that of the activated sludge, demonstrating a higher capability of toxicity resistance for biofilms compared with sludge flocs, as mentioned above. The lowered bacterial diversity in the UBF could have led to a rise in COD in the effluents.

Five bands (8, 9, 10, 15 and 16) were observed to be dominant in the DGGE profile of activated sludge at stage III ($A_{III}$ in Figure 3). These bacterial strains could be the functional species in anaerobic decolorization of X-GRL in the UBF. At a dye load of 134 g/(m$^3$ d), three new bands (13, 14 and 17) were observed to be dominant in the sludge ($A_{V}$ in Figure 3), which were probably related to X-GRL reduction under the high dye load. The variation of the bacterial community was, however, smaller in the biofilm ($B_{III}$ and $B_{V}$ in Figure 3) because of a higher resistance to shock loads.

The results of phylogenetic analysis showed that the bacterial populations of the UBF reactor were affiliated to proteobacteria, Firmcutes, Actinobacteria, Spirochaetes, OP8 and Bacteroidetes (Table 2 and Figure 4). For the activated sludge layer in the UBF, band 10 (within $A_{III}$ and $A_{V}$ in Figure 3) was closest to *Synergistes* sp. (HQ133193), which is a $\delta$-proteobacterium. The bacterium has a high utilization efficiency for simple substrates, e.g. sucrose in this study, under anaerobic conditions (Ariesyady et al. 2007). Degradation products of sucrose, mainly volatile fatty acids, could have served as electron donors in the reduction of azo bonds, generating colorless aromatic amines (Van der Zee & Villaverde 2005).

The microorganisms shown by bands 6, 8, 9 and 16 were specific in sample $A_{III}$, in which band 8 and band 9 belong to phylum Actinobacteria with a 100% similarity to *Bifidobacterium minimum* (GU361826) and *Bifidobacterium* sp. GC61 (EF990663) respectively, while band 6 and band 16 were identified to be phylum Firmicutes. Most of phylum Actinobacteria and phylum Firmicutes are heterotrophs, which could catalyze the generation of electron equivalents for the reduction of azo dye when sucrose was degraded (Raman et al. 2011). The bacteria represented by band 11 had a 100% similarity to an uncultured *Arcobacter* sp. (HQ245948). The strain was reported to be a strong anaerobic reducer (Wong & Yuen 1996; Garcia-de-Lomas et al. 2007). The apparent
band 13 and band 17 at a higher dye load (Stage Aₕ) could be attributed to Comamonas sp. p19 (JN674090) and an uncultured bacterium (HQ848035) (100% similarity). These strains belong to β-proteobacteria and ε-proteobacteria, respectively and had been found to decolorize azo dyes through oxidative reactions (Jadhav et al. 2009).

As for the biofilm phase in the UBF, bands 2, 20 and 21 were found to be specific in the DGGE gel (BIII and BV in Figure 3). These bands were attributed to stains of phylum proteobacteria which are common habitants in anaerobic reactors (Plumb et al. 2001). The dominant population represented by band 21 in BIII and BV was an uncultured Sulfuricurrum sp. (HQ162722) belonging to ε-proteobacteria and had been reported as a strong anaerobic reducing bacterium (Yoo et al. 2000b). Three specific bacteria (bands 3, 7 and 19) in sample BIII were demonstrated to be phylum Firmicutes. These bacteria in the biofilm could be responsible for the degradation of aromatic amines as X-GRL decolorization products, leading to a greater reduction in the COD of the effluent (Tan et al. 2009). Band 4 was another dominant population in the biofilm with an increased dye load (BV in Figure 3), which showed 100% similarity to Pseudomonas sp. TN301 (JN800352), a strain of γ-proteobacterium. Pseudomonas was previously documented to be capable of degrading or even mineralizing azo dyes and the aromatic intermediates from dye reduction (Kalyani et al. 2008; Lin et al. 2010). Band 5 seen in BV sample was an uncultured Spirochaetes bacterium (HQ245948), belonging to phylum Spirochaetes. This group of bacteria are able to degrade complex organic compounds such as amines (Schmidtova & Baldwin 2011).

According to the significant differences observed in the phylogenic analysis for the microbial community, the degradation of X-GRL in the UBF reactor approximately followed a two-step mechanism: decolorization reactions by the anaerobic activated sludge in the lower layer and a further degradation of COD (mostly decolorization products) by the biofilm in the biocarrier layer. This is consistent with the results of the removal of color and COD discussed earlier in this paper.

### Table 2

<table>
<thead>
<tr>
<th>Band</th>
<th>Phylum, class, or order</th>
<th>Closest species or taxon (accession number)</th>
<th>Similarity (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>OP8</td>
<td>Uncultured bacterium (DQ682544)</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>ε-proteobacteria</td>
<td>Uncultured bacterium (CU921669)</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Firmicutes</td>
<td>Uncultured bacterium (CU925611)</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>γ-proteobacteria</td>
<td>Pseudomonas sp.TN301 (JN800352)</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Spirochaetes</td>
<td>Uncultured Spirochaetes bacterium (GQ242900)</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td>Firmicutes</td>
<td>Uncultured Clostridium sp. (GU000276)</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>Firmicutes</td>
<td>Clostridiales bacterium JN18_A24_M (DQ168655)</td>
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</tr>
<tr>
<td>8</td>
<td>Actinobacteria</td>
<td>Bifidobacterium minimum (GU361826)</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Actinobacteria</td>
<td>Bifidobacterium sp.GC61 (EP990065)</td>
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</tr>
<tr>
<td>10</td>
<td>δ-proteobacteria</td>
<td>Synergistes sp. (HQ133193)</td>
<td>99</td>
</tr>
<tr>
<td>11</td>
<td>γ-proteobacteria</td>
<td>Klebsiella sp.F-12 (JN180128)</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>Bacteroidetes</td>
<td>Bacteroides graminisolvens (AB547643)</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>β-proteobacteria</td>
<td>Comamonas sp.p19 (JN674090)</td>
<td>100</td>
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<tr>
<td>14</td>
<td>ε-proteobacteria</td>
<td>Uncultured Arcobacter sp. (HQ245948)</td>
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<td>15</td>
<td>δ-proteobacteria</td>
<td>Uncultured Syntrophaceae bacterium (GU202945)</td>
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<td>16</td>
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<tr>
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<td>98</td>
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<tr>
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<td>Uncultured bacterium (HQ438805)</td>
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<tr>
<td>19</td>
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<td>Lactococcus lactis sub sp. lactis (JN194197)</td>
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<td>20</td>
<td>γ-proteobacteria</td>
<td>Uncultured bacterium (HQ282016)</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>ε-proteobacteria</td>
<td>Uncultured Sulfuricurrum sp. (HQ162722)</td>
<td>100</td>
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</table>

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CONCLUSIONS

Decolorization and mineralization of X-GRL can be achieved efficiently by a UBF reactor with a dye load up to 134 mg/(m³ d). The removal of color and COD was mostly attributed to the anaerobic activated sludge in the lower layer in the reactor. The bacterial diversities in the activated sludge and biofilm both decreased with the dye load increasing, indicating a selective pressure from the higher dye input. The bacterial community in the biofilm was more diverse than that in the activated sludge, revealing a higher resistance ability against shock loads. From the phylogenetic analysis, the microbial ecosystems in the activated sludge and the biofilm in the UBF differed from each other, and were responsible for the decolorization and mineralization of X-GRL respectively.

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