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# Responses of activities and communities of nitrifying bacteria to chromium (VI) in activated sludge

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ABSTRACT

In this study, the responses of activities and communities of nitrifying bacteria to toxic hexavalent chromium (Cr(VI)) in activated sludge were investigated in sequencing batch reactors (SBRs). The shock loading concentrations of Cr(VI) were 1, 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$ for five identical SBRs. The results showed that Cr(VI) resulted in more serious inhibition on nitrification than on COD removal. The activities of nitrifying bacteria (i.e. ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) were inhibited by greater than 5 mg Cr(VI) $L^{-1}$. With 10 and 20 mg Cr(VI) $L^{-1}$ loadings, the deterioration of nitrification was very significant in the Cr(VI) loading and subsequent recovery cycles. A loading of less than 10 mg Cr(VI) $L^{-1}$ caused few changes in communities of nitrifying bacteria. Higher loading of 10 and 20 mg Cr(VI) $L^{-1}$ reduced the densities of some nitrifying bacteria and changed the community structure of nitrifying bacteria. Twelve AOB and eight NOB species were identified by cloning and sequencing analyses. During the whole experiments period, *Nitrosomonas* sp. and *Nitrobacter* sp. were the major detected AOB and NOB species, respectively. New species of *Nitrosospira* sp. appeared in the recovery phase.

*Keywords:* Chromium; Nitrifying bacteria; PCR-DGGE; Cloning and sequencing analyses; Activated sludge

## 1. Introduction

Chromium, one of typical heavy metals frequently occurring in wastewater, is a toxic substance to microorganisms in biological wastewater treatment systems. Chromium is primarily released by effluent discharged from steelworks, chromium electroplating, leather tanning and chemical manufacturing processes [1,2]. The element chromium usually exists in the forms of trivalent and hexavalent (Cr(III) and Cr(VI)) in wastewater. Cr(III) is more stable and less toxic, while Cr(VI) has higher toxicity and easier transportation into the cells. Once entering the cells, Cr(VI) could be instantly reduced to Cr(III), which could react with some intracellular materials to cause toxic effects [3,4]. Therefore, toxic effects of Cr(VI) on microorganism in biological wastewater treatment systems are of much concern.

In biological treatment systems, nitrification is the key process for the removal of inorganic nitrogen from wastewater. Nitrification is mainly performed by

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nitrifying bacteria, which include ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). AOB convert ammonium-nitrogen ($NH_4^+$-N) to nitrite-nitrogen ($NO_2^-$-N), while NOB convert $NO_2^-$-N to nitrate-nitrogen ($NO_3^-$-N). Nitrification is generally considered as a rate-limiting step for nitrogen removal due to the low growth rate of nitrifying bacteria and their sensitivity to the environmental conditions, e.g. temperature, pH, dissolved oxygen, and toxic substances [5]. It has been found that nitrifying bacteria are more sensitive to Cr(VI) than heterotrophic microorganisms [5–12]. Stasinakis et al. investigated the effects of continuous loadings of 0.5, 1, 3 and 5 mg Cr(VI) $L^{-1}$ in a continuous-flow activated sludge reactor [11]. They found a significant inhibitory effect on nitrification with the addition of 0.5 mg Cr(VI) $L^{-1}$, whereas only a slight reduction on organic substrate removal efficiency was detected for up to 5 mg Cr(VI) $L^{-1}$. In another study, continuously feeding of 1, 3, 5 and 10 mg Cr(VI) $L^{-1}$ into four identical sequencing batch reactor (SBR) systems reduced the $NH_4^+$-N removal efficiencies from 99.5% to 48.5, 67.2, 60.0, and 49.9%, respectively [8]. Moreover, the inhibitory effects on nitrification efficiency still continued in a long time, even after the termination of the addition of Cr(VI) [8,11,12]. All these studies on toxicity of Cr(VI) were conducted by determine nitrification efficiency or respiratory activity, both of which were the external performances of metabolism reactions. These external performances can not give deep information on the inhibitory effects of Cr(VI) on community of nitrifying microbes.

Facing on heavy metal shock loadings, microorganisms can adjust their metabolic activity or community structure to adapt the harmful shock loadings. Original predominant microorganisms, which could often be very sensitive to heavy metals, would be inactivated or killed by heavy metals. Meanwhile, some resistant microorganisms to heavy metals remained and then could become new predominant species. Mertoglu et al. found that nitrifying bacterial population changed under loadings of cadmium by using slot–blot hybridization, denaturing gradient gel electrophoresis (DGGE), cloning and sequence analysis [13]. In their study, the AOB population were found to sharply shift from *Nitrosomonas* sp. and *Nitrosococcus* sp. to *Nitrosospira* sp. under gradually increasing cadmium loading, while the NOB population had no obvious changes. Another study reported that ions of nickel made the dominant AOB group shifted from a *Nitrosomonas*-like cluster to a *N.oligotropha* lineage, and *Nitrosospira* became the dominant AOB group [14]. However, Lee et al. observed that variations in zinc concentration did not cause visible shift or changes for AOB communities based on DGGE analysis [15]. Zhang et al. used fluorescence *in situ* hybridization analysis to find that Cr(VI) reduced the activities and populations of AOB and NOB, and AOB were more sensitive than NOB to Cr(VI) [8]. As far as we known, the response of nitrifying bacterial species community to Cr(VI) loading in activated sludge are seldom reported. Furthermore, a comprehensive discussion on the relationship of the external performances with nitrifying microbe species in activated sludge system was seldom reported. To obtain an insight into the inhibition mechanism of Cr(VI) on nitrification in activated sludge, it is very necessary to investigate the community/species of nitrifying bacteria.

Thus, the objectives of this study were to investigate the impact of different Cr(VI) loadings on metabolic activities and bacterial communities of AOB and NOB in activated sludge. The metabolic activities of heterotrophic microorganisms, AOB and NOB were determined by substrate removal efficiencies and specific oxygen uptake rate (sOUR) values. The changes in bacterial community structure were investigated by using DGGE, cloning and sequence analyses.

## 2. Materials and methods

### 2.1. Synthetic domestic wastewater and activated sludge

Synthetic wastewater was prepared with 500 mg COD $L^{-1}$ and 75 mg $NH_4^+$-N $L^{-1}$ by dissolving the following chemicals in ultrapure water (mg $L^{-1}$): glucose (480), $NH_4Cl$ (288), $KH_2PO_4$ (30), $NaHCO_3$ (580), $MgSO_4{\cdot}7H_2O$ (20), $FeSO_4{\cdot}7H_2O$ (2.5), $ZnSO_4{\cdot}7H_2O$ (0.25), $CaCl_2{\cdot}2H_2O$ (10), $CoCl_2{\cdot}6H_2O$ (0.05 μg $L^{-1}$), and $MoO_3$ (1.50 μg $L^{-1}$). The influent pH was 7.5 ± 0.2. The influent with Cr(VI) was prepared by adding a potassium dichromate stock solution ($K_2Cr_2O_7$) into the synthetic wastewater.

Activated sludge was collected from an aerobic tank of a local sewage treatment plant, which employs an $A^2/O$ treatment system. The collected activated sludge was screened through a 2 mm sieve to remove coarse particles and then added to each SBR system.

### 2.2. SBR process

Six identical SBRs were used in the experiment. The schematic diagram of one SBR is shown in Fig. 1. The effective volume of the SBR is 5 L. The SBR was operated sequentially in a 6 h cycle: influent filling (20 min), aeration (240 min), settling (75 min), effluent withdrawal (15 min) and idle (10 min). During each cycle, 2.5 L of wastewater was treated. The SBR

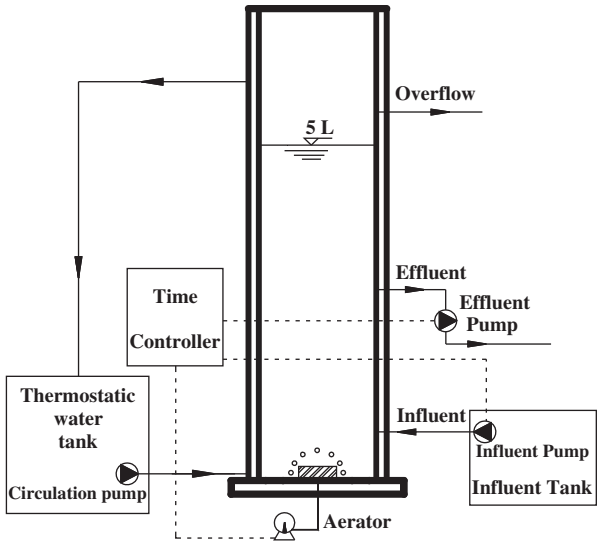


Fig. 1. Schematic of the SBR system.

system was operated automatically with a time-controller. The temperature and dissolved oxygen were kept at 25 ± 1℃ and above 2.0 mg $L^{-1}$, respectively.

The collected activated sludge was cultivated in the six SBRs. Mixed-liquor suspended solids (MLSS) were kept at around 4,500 mg $L^{-1}$ by periodically discharging excess activated sludge, and consequently a sludge retention time of 10 d was obtained in each reactor. When the effluent $NH_4^+$-N concentration was stable at less than 0.5 mg $L^{-1}$, the Cr(VI) loading experiment began. The six identical SBRs were operated simultaneously and numbered as SBR-1, SBR-2, SBR-3, SBR-4, SBR-5 and SBR-6, respectively. SBR-1, which received the synthetic wastewater without Cr (VI) throughout the experiments, was conducted as a control system. For the other five reactors, it was divided into three phases: phase I (acclimatization phase; cycles 1–20), the five SBRs were fed with the synthetic wastewater without Cr(VI); phase II (Cr(VI)-fed phase; cycles 21–28), SBR-2, SBR-3, SBR-4, SBR-5 and SBR-6 were fed with the synthetic wastewater containing a constant Cr(VI) concentrations of 1, 5, 8, 10 and 20 mg L-1, respectively; phase III (recovery phase; cycles 29–72), the five reactors were fed with the synthetic wastewater without Cr(VI) again to investigate the recovery capabilities of microbes in the SBRs.

At the end of the aeration period in cycles 28 and 72, activated sludge from the six reactors was collected for analyses of sOUR, Cr distribution, and species communities of AOB and NOB.

### 2.3. SOUR analyses

Heterotrophic sOUR ($sOUR_{org.C}$), ammonia oxidation sOUR ($sOUR_{NH4}$) and nitrite nitrogen oxidation sOUR ($sOUR_{NO2}$) were measured to show the activities of heterotrophic microorganisms, AOB and NOB. The values of $sOUR_{org.C}$, $sOUR_{NH4}$ and $sOUR_{NO2}$ were measured by a respirometer based on a previous study [16]. Duplicate samples were collected and measured. An inhibition rate of microorganism activity was calculated as the following equation:

$$\text{Inhibition rate}(\%) = (sOUR_{control} - sOUR_{Cr}) \,/\, sOUR_{control} \times 100\%. \quad (1)$$

where $sOUR_{control}$ was measured in the control system, and $sOUR_{Cr}$ was measured in the Cr(VI)-fed systems.

### 2.4. DNA extraction and nested PCR

DNA was extracted from the activated sludge samples using an EZNA™ soil DNA kit (D5625–01, Omega Bio-tek Inc., USA), and the extraction steps followed the manufacturer's instructions. The products from DNA extraction were examined by electrophoresis in 0.7% (wt/vol) agarose gel. To minimize the variation and contamination of DNA extraction, duplicate templates for each sample, which were extracted for nested PCR amplification and DGGE tests, were stored at a −20℃ refrigerator.

A nested PCR approach was used to amplify ammonia-oxidizer and nitrite-oxidizer specific 16S rDNA for DGGE. This procedure was divided into two rounds. For the first round of PCR, the AOB specific primer was used to obtain DNA fragments 465 bp in length with an equimolar mixture of three forward primers (CTO189fA/B and CTO189fC) and with a reverse primer CTO654r [17]. The NOB forward and reverse primers used to obtain DNA fragments were FGPS872 and FGPS1269, respectively [18]. The primers, target and PCR conditions are listed in Table 1. After the first round of PCR amplification, PCR products were purified with a QIAquick® purification kit (Qiagen Inc., Valencia, CA) to get rid of the primers and other contaminants. For the second round of PCR, the universal primers (F357-GC and R518) were used to further amplify the first PCR amplicons under the conditions described in Table 1. All PCR products were verified by electrophoresis in 1.5% (wt/vol) agarose gel with ethidium bromide staining to ascertain the product size.

Table 1
Primer characteristics and conditions for nested PCR

| Primers | Target (specificity) | PCR conditions | Refs. |
|---|---|---|---|
| CTO189fA/B<br>CTO189fC<br>CTO654r | 16S rRNA gene (AOB β-*Proteobacteria*) | 3 min at 94℃, followed by 38 cycles of 30 s at 92℃, 30 s at 57℃, and 45 s at 72℃, followed by a 5 min final extension at 72℃ | [17] |
| FGPS872<br>FGPS1269 | 16S rRNA gene (NOB *Nitrobacter*) | 3 min at 95℃, followed by 35 cycles of 1 min at 95℃, 1 min at 50℃ and 1 min at 72℃, with a 3 min final extension at 72℃ | [18] |
| F357-GC<br>R518 | 16S rRNA gene (universal) | 5 min at 94℃, followed by 25 cycles of 1 min at 94℃, 1 min at 65℃ (−1℃ each 2 cycles and 5 cycles at 55℃), and 1 min at 72℃, followed by a 5 min final extension at 72℃ | [19] |

The PCR mixture was prepared in a total volume of 50 μL in 0.2 mL Eppendorf tubes and operated in a PCR Authorized Thermocycler (Eppendorf, Hamburg, Germany). The reaction system contained 2.5 U of Taq DNA polymerase (Promega, Shanghai, China), 1 × buffer (Promega, Shanghai, China), 2.0 mM $MgCl_2$, 2 μL template DNA, 0.2 mM dNTPs, and 0.25 μM of each primer.

### 2.5. DGGE analysis

The nested PCR amplicons were carried out on polyacrylamide gels (8%, 37.5:1 acrylamide-bisacrylamide) with a 35–55% linear gradient of denaturant (100% denaturant is 7 M urea and 40% formamide deionized). The gel was running for 6 h at 150 V in 1 × TAE buffer keeping at 60℃. Denaturing gradient gels were poured and run by using the DGGE-2001 System (C.B.S. SCIENTIFIC, Del Mar, CA, USA). After electrophoresis, the gel was stained by EB solution for 15 min, and photographed by gel imager. The gel images were analyzed with the software QuantityOne, version 4.62 (Bio-rad).

### 2.6. Cloning, sequencing and phylogenetic analysis

Prominent DGGE bands were excised and dissolved in a 50 μL TE solution overnight at 4℃. DNA was recovered from the gel by freeze-thawing three times. These obtained DNA band samples were amplified using the primer F357GC/R518, and the products again were run to DGGE to check their migration. The target DGGE bands were then excised and reamplified using the primer F357/R518 without GC-clamp, and subsequently these pure samples were prepared for the cloning and sequencing. The PCR products were cloned using a pGEM®-T Easy vector system (Promega, Madison, WI) and PCR amplicons were submitted for sequencing to Sangon Biotech. Sequence data from 16S rRNA fragments of AOB and NOB were used to phylogenetic analysis. Similarity search was performed using the GenBank server of the National Centre for Biotechnology Information and the BLAST algorithm. Based on the above results, phylogenetic trees were designed using the software Mega Version 5.10, and the neighbour-joining method was used for tree construction.

### 2.7. Other analysis methods

Concentrations of $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$-N and COD were detected according to the standard methods [20]. MLSS were measured with an MLSS analyzer (HACH Txpro-2).

## 3. Results and discussion

### 3.1. Effects of Cr(VI) on COD removal

The effects of Cr(VI) on the COD removal in the SBR systems are shown in Fig. 2. The six SBR systems run stably with the synthetic wastewater without Cr (VI) in the first 20 cycles. During the Cr(VI)-fed phase (cycles 21–28), SBR-1, -2, -3 and -4 maintained high COD removal efficiencies of 96.4 ± 1.2%, which indicates that Cr(VI) of up to 8 mg $L^{-1}$ cause no visible effect on the COD degradation. A loading concentration of 10 mg Cr(VI) $L^{-1}$ had a slight inhibition on the COD removal efficiency. After ceasing the addition of 10 mg Cr(VI) $L^{-1}$, the COD removal efficiency further decreased in cycle 33 and then gradually increased to the original level (96.4 ± 1.2%). With a loading concentration of 20 mg Cr(VI) $L^{-1}$, The COD removal efficiency drastically reduced to 71.8–69.6% in cycle 28–29. Subsequently, the COD removal efficiency recovered slowly to the original level in the following 34 cycles. The similar results were also observed by Cheng et al. [12]. In their study, they found that

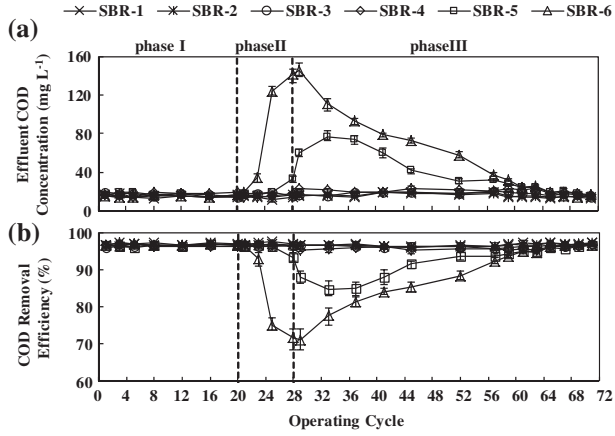


Fig. 2. Effects of Cr(VI) on COD removal: (a) effluent COD and (b) COD removal efficiency (SBR-1, -2, -3, -4, -5 and -6 were fed with 0, 1, 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$ in phase II, respectively).

addition of 5, 10 and 25 mg Cr(VI) $L^{-1}$ caused the COD removal efficiencies to decrease from 93.6 to 75.9, 66.2%, and 59.1% for the SBR systems, respectively; after the termination of Cr(VI) addition for 9 d, the COD removal efficiencies of the Cr(VI)-fed systems ascended up almost to that of the control system. Stasinakis et al. reported that a significant inhibition was observed at Cr(VI) concentrations equal or above 10 mg Cr(VI) $L^{-1}$ [10]. Altas demonstrated that the COD removal rate was inhibited by 25% and 50% at the presences of 18 and 40 mg Cr(VI) $L^{-1}$, respectively [6]. Thus, the heterotrophic microorganisms have a relatively high tolerance to Cr(VI), and only a high loading concentration of Cr(VI) (equal or greater than 10 mg $L^{-1}$) was able to affect the COD removal efficiency.

### 3.2. Effects of Cr(VI) on nitrification

The control system (SBR-1) exhibited a high and stable removal efficiency of 99.8 ± 0.1% for $NH_4^+$-N (Fig. 3). It indicates a high activity of the nitrifying bacteria and a good operating stability of the SBR systems. The formation of $NO_3^-$-N at 38.7 ± 0.5 mg $L^{-1}$ accounted for 50.9% of the consumed $NH_4^+$-N. The bio-reaction of heterotrophic bacteria through aerobic and anoxic conditions in SBRs contributed to the other portion of the consumed $NH_4^+$-N. In SBR-2, with a shock loading of 1 mg Cr(VI) $L^{-1}$, nitrification performance was not affected during the whole experimental period. However, a rapid increase of $NH_4^+$-N concentrations and a drastic drop of $NO_3^-$-N concentrations in the effluent could be observed in other four

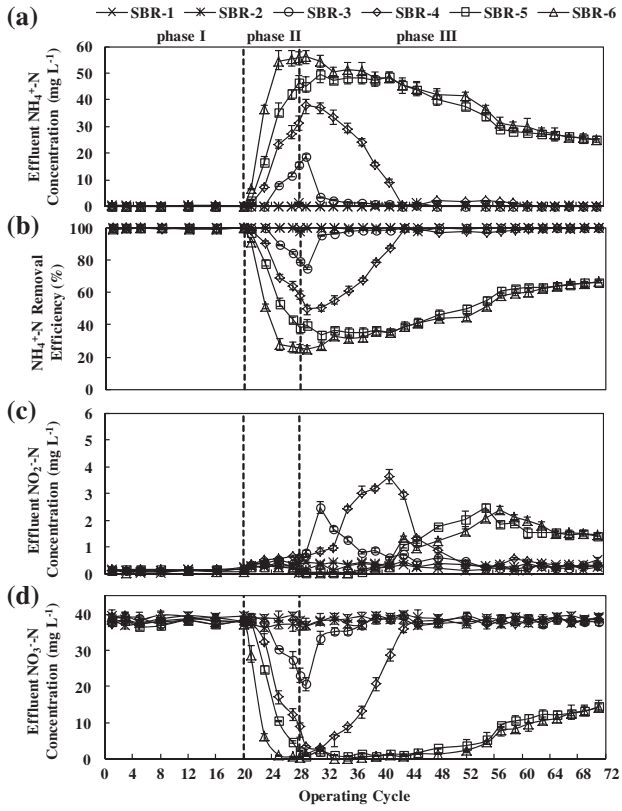


Fig. 3. Effects of Cr(VI) on (a) effluent $NH_4^+$-N concentration, (b) removal efficiency of $NH_4^+$-N, (c) effluent $NO_2^-$-N concentration, and (d) effluent $NO_3^-$-N concentration in SBR (SBR-1, -2, -3, -4, -5 and -6 were fed with 0, 1, 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$ in phase II, respectively).

Cr(VI)-fed SBR systems (Fig. 3(a) and (c)). Higher dosage of Cr(VI) caused lower removal efficiencies of $NH_4^+$-N from cycle 21 to 28. After the termination of the Cr(VI) addition, the $NH_4^+$-N removal efficiencies further slightly decreased in SBR-3, -4, -5 and -6 and then increased. It has been reported that Cr(VI) could be effectively up-taken by cells via anion channels, and reduced to Cr(III) [21]. Then Cr(III) subsequently reacted with intracellular reductants (e.g. glutathione, ascorbate, or nicotinamide adenine dinucleotide (phosphate) (NAD(P)H)) to generate highly reactive and potentially DNA-damaging intermediates [22,23]. Once the protein and DNA of nitrifying bacteria were damaged by Cr(III), they were hard to be repaired in short time. This could be one reason why the activities of nitrifying bacteria were still low during the beginning of the recovery phase in 10 and 20 mg $L^{-1}$ Cr(VI)-fed SBR systems. The $NH_4^+$-N removal efficiencies in SBR-3 and SBR-4 totally recovered in 16 cycles, but in SBR-5 and SBR-6 only recovered to 66.5–66.7% in 44 cycles. The results show that higher shock loadings of 10 and

20 mg Cr(VI) $L^{-1}$ caused severe inhibition on the $NH_4^+$-N removal, and the recovery of $NH_4^+$-N removal efficiency was a rather slow and difficult process. Stasinakis et al. also observed that $NH_4^+$-N removal efficiency increased from 30 to 57% after ceasing the addition of Cr(VI) in 12 d [11].

The effluent concentration of $NO_2^-$-N increased little in Cr(VI)-fed reactors in comparison with SBR-1 (Fig. 3(c)). Accumulations of $NO_2^-$-N occurred in phase III in SBR-3, -4, -5 and -6. In SBR-3 and -4, $NO_2^-$-N began to accumulate when the activity of AOB began to recover. When the $NH_4^+$-N removal efficiency recovered to 96.7% in SBR-3 (in cycle 33) and 93.5% in SBR-4 (in cycle 42), the accumulation of effluent $NO_2^-$-N began to decrease. In SBR-5 and -6, accumulation of $NO_2^-$-N occurred late at cycle 43 and slowly increased until cycle 54. At the end of the experiment, a level of 1.45 ± 0.3 mg $L^{-1}$ $NO_2^-$-N still remained in the effluent.

### 3.3. Effects of Cr(VI) on sOUR

Respiration rates, determined as sOURs, were more sensitive to the toxicity of Cr(VI) than the substrate removal efficiency. The loading of 1 mg Cr(VI) $L^{-1}$ resulted in a positive stimulation on the activities of heterotrophic bacteria (Fig. 4(a)), while higher loadings of Cr(VI) led to inhibitory effects. With the shock loadings of 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$, the inhibition ratios of $sOUR_{org.C}$ were 7.2, 14.3, 30.2 and 42.0%, respectively. At cycle 72 in the recovery phase, the values of $sOUR_{org.C}$ in SBR-3 and 4 were close to that in the control system. It indicates that the inhibitory effect of 5 and 8 mg Cr(VI) $L^{-1}$ on the respiration activity of heterotrophic microorganisms can be naturally recovered. The values $sOUR_{org.C}$ in SBR-5 and 6 were still inhibited by 11.3% and 12.7% at cycle 72.

Being consistent with literatures [11,12,24], Cr(VI) was found to cause a higher toxicity to nitrifying bacteria than to heterotrophic microorganisms. The loading of 1 mg Cr(VI) $L^{-1}$ did not affect the levels of $sOUR_{NH4}$ and $sOUR_{NO_2^-}$. With the loadings of 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$, the inhibition ratios were 17.7, 29.6, 78.5 and 79.5% for $sOUR_{NH4}$, respectively; 33.3, 38.4, 85.4 and 89.4% for $sOUR_{NO2}$, respectively. At the end of the recovery phase, the respiration activity of AOB and NOB were naturally recoverable in SBR-2, -3 and -4. In SBR-5 and -6, $sOUR_{NH4}$ were still inhibited by 64.3% and 65.1%, and $sOUR_{NO2}$ were inhibited by 72.5% and 73.0%.

The same Cr(VI) loading led to a higher inhibitory effect on NOB respiration than on AOB respiration. However, the inhibition degrees of Cr(VI) on the respiration activities of AOB and NOB were not consistent with the inhibition degrees on their substrate removal efficiencies. For example, in SBR-3, the decrements of effluent $NO_3^-$ were almost equal to the increments of effluent $NH_4^+$-N in cycles 25, 27 and 28. It implies that the NOB transformed all the AOB-produced $NO_2^-$. That is to say that the transformation efficiency from $NO_2^-$ to $NO_3^-$ by NOB was not lower than that from $NH_4^+$-N to $NO_2^-$ by AOB, despite higher inhibition of Cr(VI) on NOB respiration than on AOB respiration. In the recovery phase, the activity of AOB recovered faster than that of NOB, leading to the accumulation of $NO_2^-$. NOB suffered not only from the Cr(VI) toxicity but also from a lack of $NO_2^-$-N. Another possible reason for the faster recovery rate of AOB than NOB might be that the activities and abundance of AOB are higher than that of NOB in the activated sludge. Carvalho et al. reported that AOB had a higher volume specific activity and biomass volume in the flocs than NOB, which resulted in a temporary accumulation of $NO_2^-$-N [25]. They also pointed out that the activity of NOB would be limited by the AOB activity, unless $NO_2^-$-N was supplied from the bulk liquid.

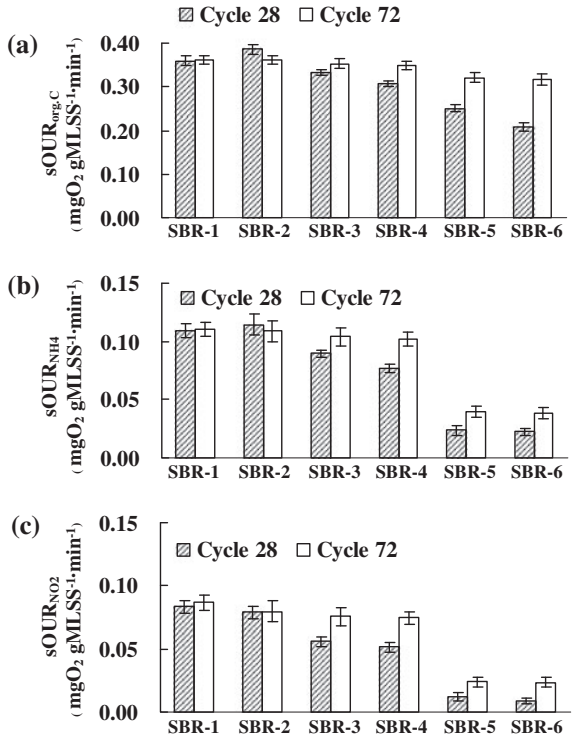


Fig. 4. Effects of Cr(VI) on (a) $sOUR_{org.C}$, (b) $sOUR_{NH4}$, and (c) $sOUR_{NO2}$ in cycles 28 and 72 (SBR-1, -2, -3, -4, -5 and -6 were fed with 0, 1, 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$ in phase II, respectively).

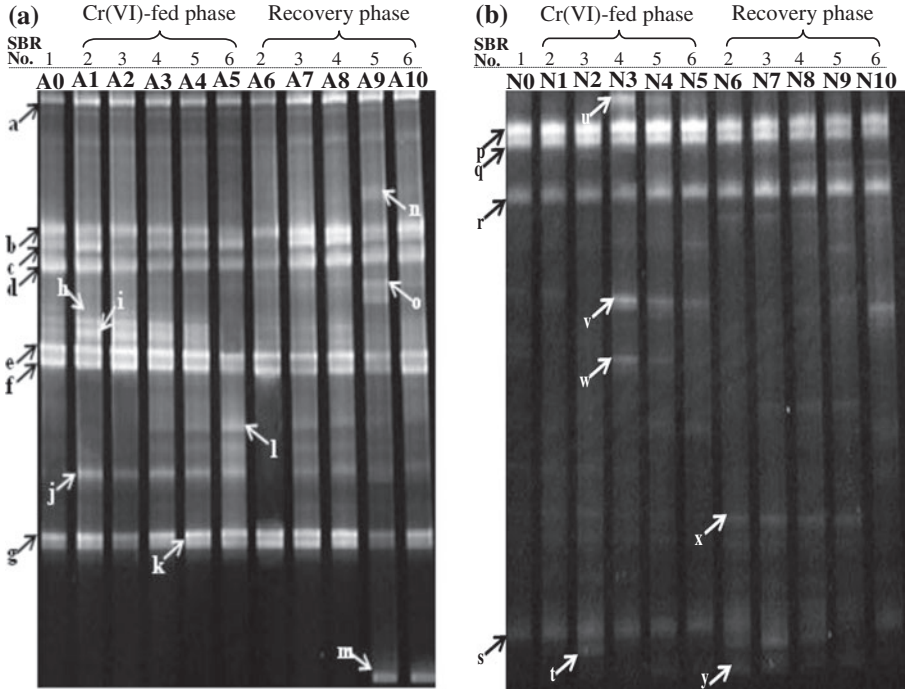


Fig. 5. DGGE profiles of (a) AOB and (b) NOB in the activated sludge (SBR-1, -2, -3, -4, -5 and -6 were fed with 0, 1, 5, 8, 10 and 20 mg Cr(VI) $L^{-1}$ in phase II, respectively).

### 3.4. Effects of Cr(VI) on communities of AOB and NOB

Specific PCR amplification of 16S rDNA genes were used to reveal the variations of AOB and NOB population in the activated sludge systems during the Cr(VI)-fed and recovery phases. The major bands were excised and labeled with the lowercase letters. Each band represented a unique species and the brightness of each band symbolized the species abundance. Many

Table 2
Results of some partial 16S rDNA sequences using BLASTN in GenBank

| Band | Accession | Most closely related sequence by BLASTN | Identity (%) |
|---|---|---|---|
| a | FR828478 | *Nitrosomonas oligotropha* 16S rRNA gene, strain Nm45 | 99 |
| b | JX391861 | Uncultured *Nitrosomonas* sp. clone W1_ASTS62, partial16S rRNA gene | 100 |
| c | GQ325296 | Uncultured *Nitrosomonas* sp. isolate DGGE gel band 42, partial 16S rRNA gene | 100 |
| d | HQ221942 | Uncultured *ammonia-oxidizing bacterium* clone LS-3, partial 16S rRNA gene | 99 |
| e | FM997816 | Uncultured *Nitrosomonas* sp. clone LEQUIA_R0CTO61, partial 16S rRNA gene | 100 |
| f | FN429865 | Uncultured *Nitrosomonas* sp. DGGE7/R0/February07, partial 16S rRNA gene | 99 |
| g, k | JF514818 | Uncultured *β-proteobacterium* clone AOB47, partial 16S rRNA gene | 100 |
| h | FM997789 | Uncultured *Nitrosomonas* sp. clone LEQUIA_R0CTO21, partial 16S rRNA gene | 99 |
| i | AJ245756 | Uncultured *Nitrosomonas* DGGE band 6c, partial 16S rRNA gene | 99 |
| j | EU661951 | Uncultured *Nitrosomonas* sp. clone M2c, partial 16S rRNA gene | 99 |
| m | EU661952 | Uncultured *Nitrosospira* sp. clone M2d, partial 16S rRNA gene | 98 |
| p | NR074313 | *Nitrobacter hamburgensis* X14 strain X14, partial 16S rRNA gene | 99 |
| q | AM292286 | *Nitrobacter* sp. strain 263 | 100 |
| r | HQ424484 | Uncultured *Nitrobacter* sp. clone 9, partial 16S rRNA gene | 99 |
| s | AB748628 | Uncultured *beta proteobacterium* clone Anjo-5–4, partial 16S rRNA gene | 99 |
| t | CU926914 | Uncultured *beta proteobacteria bacterium* clone QEDN7BF02, partial 16S rRNA gene | 100 |
| u | HQ821468 | Uncultured *gamma proteobacterium* clone N11, partial 16S rRNA gene | 100 |
| v | JN371623 | Uncultured *beta proteobacterium* clone C3–64, partial 16S rRNA gene | 96 |
| w | AB635876. | Uncultured *gamma proteobacterium* clone DG-KM-B11, partial 16S rRNA gene | 100 |

AOB (bands a–g) and NOB (bands p–s) population were detected with relatively high intensities on the DGGE profiles of all the SBRs in Cr(VI)-fed and recovery phases (Fig. 5). They should play an important role in nitrification. The major bands excised from DGGE gel were identified and nucleotide sequences were compared to the GenBank database by BLASTN. All sequences show high homologous (96–100%) with previously identified 16S rRNA gene sequences. The sequencing data are shown in Table 2, which exhibited that there were many uncultured AOB and NOB species. Based on the results listed in Table 2 and other closely related 16S rRNA genes in Genbank, phylogenetic trees which revealed the genetic relationships between different species were drawn (Fig. 6).

The results in Fig. 6(a) denoted that a close genetic relationship existed among AOB species. These AOB species mainly belonged to uncultured *Nitrosomonas* sp. The uncultured *Nitrosospira* sp. (i.e. band *m*) appeared in 10 and 20 mg $L^{-1}$ Cr(VI)-fed systems in the recovery phase. Many previous studies have debated whether *Nitrosomonas* or *Nitrosospira* sp. is more important AOB species in wastewater treatment systems [26–29]. It has been reported that *Nitrosomonas* sp. was suitable to grow on the condition of adequate nutrients, whereas *Nitrosospira* sp. was a better competitor in environments contaminated with toxic metals [26]. In this study, *Nitrosospira* sp. had a high tolerance to Cr(VI).

*Nitrobacter* is one of the domain nitrite oxidizing bacteria (NOB) within the phylum *Proteobacteria*, and has been widely used as a biomark of NOB in DGGE analysis [30,31]. The primer for NOB in this study was specific to *Nitrobacter*. The Cr(VI) loadings also caused a variation in NOB species (Fig. 5(b)). Bands v, w, and x were found to be brightened after the activated sludge systems subjected to Cr(VI) shock loadings. Some new bands u, t and y appeared in Cr(VI)-fed systems, but they showed a relatively low abundance. The sequencing and phylogenetic analysis found that

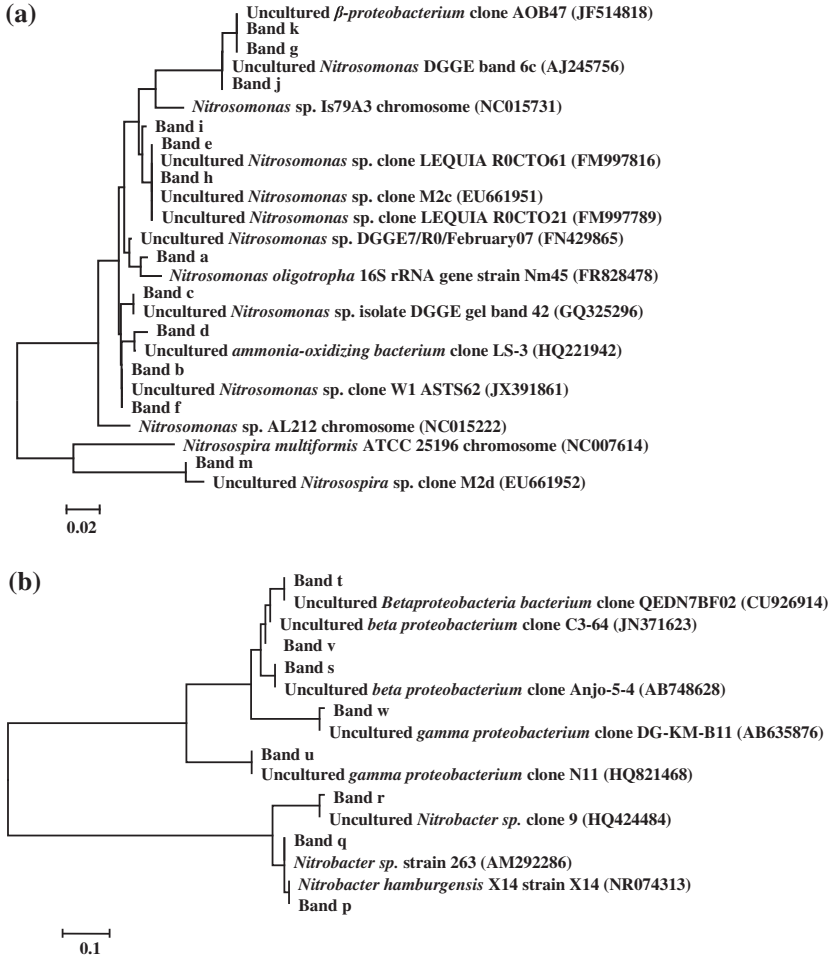


Fig. 6. Phylogenetic trees of (a) AOB and (b) NOB in the SBR systems.

the detected NOB was mainly belonged to *Nitrobacter* sp., *beta-proteobacterium* and *gamma-proteobacterium*, three of which had a close genetic relationship (Fig. 6(b)). The bands of *Nitrobacter* (e.g. bands p and q) had high intensities in DGGE profiles of N0-N5. It suggested that the population of *Nitrobacter* changed little under the shock loading of Cr(VI). However, the population of *Nitrobacter* decreased in the recovery phase. Mertoglu et al. reported that *Nitrobacter* sp., as one group of major NOB, had no variation under gradually increasing cadmium loadings [13].

The Cr(VI) loading of 5–8 mg $L^{-1}$ reduced the nitrification efficiencies and respiration activities of AOB and NOB, but caused little variation in the DGGE profiles (Fig. 5). It was inferred that 5–8 mg Cr(VI) $L^{-1}$ could mainly inhibit the activities of the major nitrifying bacteria rather than totally eliminate them. That could be one of reasons why the nitrification efficiency recovered quickly in the 5–8 mg $L^{-1}$ Cr(VI)-fed systems. The high Cr(VI) loading of 10–20 mg Cr(VI) $L^{-1}$ caused reductions in abundance intensity of some major nitrifying bacterial bands (e.g. bands b, c, d, p and q), leading to a severe deterioration in nitrification efficiency in phase II and low degrees of recovery.

## 4. Conclusion

The shock loading of Cr(VI) generated inhibitory effects on microorganism activities and nitrifying bacterial communities in activated sludge systems during Cr(VI) loading phase and still in the following recovery phase. Higher Cr(VI) loadings led to higher inhibitory effects and slower recovery rates for microorganism activities. A loading of less than 10 mg Cr(VI) $L^{-1}$ inhibited the activities of nitrifying bacteria, but did not significantly change the community structure of nitrifying bacteria. Higher loadings of 10 and 20 mg Cr(VI) $L^{-1}$ reduced the densities of the major detected nitrifying bacteria and slightly changed the community structure of nitrifying bacteria. New species of *Nitrosospira* sp. appeared in the recovery phase.

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## References

[1] M. Chuan, J. Liu, Release behavior of chromium from tannery sludge, Water Res. 30 (1996) 932–938.
[2] A. Zahoor, A. Rehman, Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater, J. Environ. Sci. 21 (2009) 814–820.
[3] A.S. Stasinakis, N.S. Thomaidis, D. Mamais, M. Karivali, T.D. Lekkas, Chromium species behaviour in the activated sludge process, Chemosphere 52 (2003) 1059–1067.
[4] R. Bencheikh-Latmani, A. Obraztsova, M.R. Mackey, M.H. Ellisman, B.M. Tebo, Toxicity of Cr(III) to *Shewanella* sp. strain MR-4 during Cr(VI) reduction, Environ. Sci. Technol. 41 (2007) 214–220.
[5] E. Vaiopoulou, P. Gikas, Effects of chromium on activated sludge and on the performance of wastewater treatment plants: A review, Water Res. 46 (2012) 549–570.
[6] L. Altaş, Effects of chromium (VI) on the activities of ureolytic mixed culture, J. Chem. Technol. Biotechnol. 84 (2009) 229–235.
[7] F. Çeçen, N. Semerci, A.G. Geyik, Inhibition of respiration and distribution of Cd, Pb, Hg, Ag and Cr species in a nitrifying sludge, J. Hazard. Mater. 178 (2010) 619–627.
[8] H. Zhang, H. Zhai, M. Ji, X. Su, Z. Du, J. Liu, Long-term effect of Cr(VI) on ammonia-oxidizing and nitrite-oxidizing bacteria in an activated sludge system, Desalin. Water Treat. 54 (2015) 1981–1989.
[9] J. Mazierski, Effect of chromium (CrVI) on the growth rate of denitrifying bacteria, Water Res. 28 (1994) 1981–1985.
[10] A.S. Stasinakis, D. Mamais, N.S. Thomaidis, T.D. Lekkas, Effect of chromium (VI) on bacterial kinetics of heterotrophic biomass of activated sludge, Water Res. 36 (2002) 3341–3349.
[11] A.S. Stasinakis, N.S. Thomaidis, D. Mamais, E.C. Papanikolaou, A. Tsakon, T.D. Lekkas, Effects of chromium (VI) addition on the activated sludge process, Water Res. 37 (2003) 2140–2148.
[12] L. Cheng, X.C. Li, R.X. Jiang, C. Wang, H.B. Yin, Effects of Cr(VI) on the performance and kinetics of the activated sludge process, Bioresour. Technol. 102 (2011) 797–804.
[13] B. Mertoglu, N. Semerci, N. Guler, B. Calli, F. Cecen, A.M. Saatci, Monitoring of population shifts in an enriched nitrifying system under gradually increased cadmium loading, J. Hazard. Mater. 160 (2008) 495–501.
[14] C.H. Yeung, C.A. Francis, C. Criddle, Adaptation of nitrifying microbial biomass to nickel in batch incubations, Appl. Microbiol. Biotechnol. 97 (2013) 847–857.
[15] S. Lee, K. Cho, J. Lim, W. Kim, S. Hwang, Acclimation and activity of ammonia-oxidizing bacteria with respect to variations in zinc concentration, temperature, and microbial population, Bioresour. Technol. 102 (2011) 4196–4203.
[16] J. Surmacz-Gorska, K. Gernaey, C. Demuynck, P. Vanrolleghem, W. Verstraete, Nitrification monitoring in activated sludge by oxygen uptake rate (OUR) measurements, Water Res. 30 (1996) 1228–1236.
[17] G.A. Kowalchuk, J.R. Stephen, W. De Boer, J.I. Prosser, T.M. Embley, J.W. Woldendorp, Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of

PCR-amplified 16S ribosomal DNA fragments, Appl. Environ. Microbiol. 63 (1997) 1489–1497.
[18] V. Degrange, R. Bardin, Detection and counting of *Nitrobacter* populations in soil by PCR, Appl. Environ. Microbiol. 61 (1995) 2093–2098.
[19] G. Muyzer, E.C. De Waal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.
[20] APHA, AWWA, WEF, Standard Methods for the Examination of Water and Wastewater, APHA, Washington, DC, 1998.
[21] A.S. Stasinakis, N.S. Thomaidis, D. Mamais, T.D. Lekkas, Investigation of Cr(VI) reduction in continuous-flow activated sludge systems, Chemosphere 57 (2004) 1069–1077.
[22] M.R. Bruins, S. Kapil, F.W. Oehme, Microbial resistance to metals in the environment, Ecotoxicol. Environ. Saf. 45 (2000) 198–207.
[23] A. Levina, I. Mulyani, P.A. Lay, Redox chemistry and biological activities of chromium(III) complexes, in: J.B. Vincent (Ed.), The Nutritional Biochemistry of Chromium(III), Elsevier, Amsterdam, 2007, pp. 225–256.
[24] P. Madoni, D. Davoli, L. Guglielmi, Response of sOUR and AUR to heavy metal contamination in activated sludge, Water Res. 33 (1999) 2459–2464.
[25] G. Carvalho, R. Meyer, Z.G. Yuan, J. Keller, Differential distribution of ammonia- and nitrite-oxidising bacteria in flocs and granules from a nitrifying/denitrifying sequencing batch reactor, Enzyme Microb. Technol. 39 (2006) 1392–1398.
[26] A. Schramm, D. de Beer, J.C. van den Heuvel, S. Ottengraf, R. Amann, Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira spp.* along a macroscale gradient in a nitrifying bioreactor: Quantification by *in situ* hybridization and the use of microsensors, Appl. Environ. Microbiol. 65 (1999) 3690–3696.
[27] U. Purkhold, A. Pommerening-Röser, S. Juretschko, M.C. Schmid, H.P. Koops, M. Wagner, Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys, Appl. Environ. Microbiol. 66 (2002) 5368–5382.
[28] B. Zhang, B.S. Sun, M. Ji, H.N. Liu, X.H. Liu, Quantification and comparison of ammonia-oxidizing bacterial communities in MBRs treating various types of wastewater, Bioresour. Technol. 101 (2010) 3054–3059.
[29] X. Zhang, D. Li, Y. Liang, H. Zeng, Y. He, F. Dan, J. Zhang, Start-up, influence factors, and the microbial characteristics of partial nitrification in membrane bioreactor, Desalin. Water Treat. 54 (2015) 581–589.
[30] Y. Takasaki, H. Satoh, M. Onuki, T. Mino, K. Ito, O. Miki, Behavior of nitrite oxidizers in the nitrification/denitrification process for the treatment of simulated coke-oven wastewater, J. Water Environ. Technol. 5 (2007) 29–36.
[31] A. Cébron, T. Berthe, J. Garnier, Nitrification and nitrifying bacteria in the lower Seine River and Estuary (France), Appl. Environ. Microb. 69 (2003) 7091–7100.