Water Research 92 (2016) 254–261

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Nitrification inhibition by hexavalent chromium Cr(VI) – Microbial ecology, gene expression and off-gas emissions

Young Mo Kim [1], Hongkeun Park, Kartik Chandran*, [2]

Department of Earth and Environmental Engineering, Columbia University, 500 West 120th Street, New York, NY 10027, USA

ARTICLE INFO

Article history:
Received 3 November 2015
Received in revised form
14 January 2016
Accepted 19 January 2016
Available online 1 February 2016

Keywords:
Hexavalent chromium (Cr(VI))
Nitrification
Inhibition
Microbial ecology
Gene expression
Nitrous oxide

ABSTRACT

The goal of this study was to investigate the responses in the physiology, microbial ecology and gene expression of nitrifying bacteria to *imposition* of and *recovery* from Cr(VI) loading in a lab-scale nitrification bioreactor. Exposure to Cr(VI) in the reactor strongly inhibited nitrification performance resulting in a parallel decrease in nitrate production and ammonia consumption. Cr(VI) exposure also led to an overall decrease in total bacterial concentrations in the reactor. However, the fraction of ammonia oxidizing bacteria (AOB) decreased to a greater extent than the fraction of nitrite oxidizing bacteria (NOB). In terms of functional gene expression, a rapid decrease in the transcript concentrations of *amo*A gene coding for ammonia oxidation in AOB was observed in response to the Cr(VI) shock. In contrast, transcript concentrations of the *nxr*A gene coding for nitrite oxidation in NOB were relatively unchanged compared to Cr(VI) pre-exposure levels. Therefore, Cr(VI) exposure selectively and *directly* inhibited activity of AOB, which *indirectly* resulted in substrate (nitrite) limitation to NOB. Significantly, trends in *amoA* expression preceded performance trends both during *imposition* of and *recovery* from inhibition. During recovery from the Cr(VI) shock, the high ammonia concentrations in the bioreactor resulted in an irreversible shift towards AOB populations, which are expected to be more competitive in high ammonia environments. An inadvertent impact during recovery was increased emission of nitrous oxide (N_2O) and nitric oxide (NO), consistent with recent findings linking AOB activity and the production of these gases. Therefore, Cr(VI) exposure elicited multiple responses on the microbial ecology, gene expression and both aqueous and gaseous nitrogenous conversion in a nitrification process. A complementary interrogation of these multiple responses facilitated an understanding of both *direct* and *indirect* inhibitory impacts on nitrification.

1. Introduction

Biological nitrogen removal (BNR) in wastewater treatment plants (WWTPs) is achieved by complete or partial oxidation of ammonia to nitrate or nitrite, respectively through the process of nitrification followed by the reduction to dinitrogen gas via denitrification. Nitrification is mediated mainly by two distinct classes of autotrophic bacteria: (a) ammonia oxidizing bacteria (AOB), which oxidize ammonia to nitrite; and (b) nitrite oxidizing bacteria (NOB), which oxidize nitrite to nitrate.

In engineered BNR systems, nitrification is often the rate-limiting step due to the inherently low specific growth rates and biomass yields of nitrifying bacteria compared to heterotrophic bacteria. In addition, nitrifying bacteria are typically sensitive to physical, chemical and environmental perturbations such as pH, temperature, dissolved oxygen levels and chemical toxicants (Painter and Loveless, 1983; Kim et al., 2008). Therefore, it is important to understand the effects of such perturbations on nitrification.

Heavy metals are typical toxic pollutants in sewage as well as internally generated wastewater streams such as anaerobic digester centrate (from centrifugation of digested biosolids) or filtrate (from filtration of digested biosolids). Of these heavy metals, chromium is most often encountered in centrate from municipal sludge digestion as well as the wastewater effluents of steel manufacturing, leather tanning and leachates (Cheng et al., 2011; Chen et al., 2014). Chromium in wastewater is primarily found in two oxidation states Cr(III) and Cr(VI). While Cr(III) accumulates in

* Corresponding author.
E-mail address: kc2288@columbia.edu (K. Chandran).
[1] Present address: School of Environmental Science and Engineering, Gwangju Institute of Science and Technology, Buk-gu, Gwangju 500-712, Republic of Korea.
[2] www.columbia.edu/~kc2288.

http://dx.doi.org/10.1016/j.watres.2016.01.042
0043-1354/

the cell membrane and poses limited toxic impacts, Cr(VI) crosses cellular membranes reacting with intracellular biomolecules (Stasinakis et al., 2003).

Although a number of studies have revealed effects of Cr(VI) on substrate removal, respiration activity and bacterial growth in activated sludge systems (Cheng et al., 2011; Vaiopoulou and Gikas, 2012; Novotnik et al., 2014), the effects on activated sludge described vary in severity and are dependent on a number of confounding factors, which frequently hinder clear conclusions. In addition to these unclear results, many questions remain unanswered. Especially, the effects of Cr(VI) on the nitrification process related to *direct* inhibition by Cr(VI) and *indirect* effects related to the accumulation of nitrification substrates or intermediates require further clarification. Potential selectivity in inhibition of AOB and NOB also needs to be examined, which might be possible through direct molecular inspection of DNA and mRNA profiles. Longer term, direct exposure studies in continuous reactor systems are especially lacking, perhaps owing to the analytical effort involved. Such studies are particularly needed to infer any sequential or hierarchical nature in the multiple process indicators (including process performance, kinetics and microbial ecology). It is imperative to recognize the relative rapidity of different process indicators and select the appropriate ones to facilitate effective process monitoring and control both during inhibition and recovery. Along this direction, increased production of nitrous oxide (N_2O) by AOB has been suggested as an indicator of nitrification upsets (Yu et al., 2010; Ahn et al., 2011; Jiang et al., 2015; Ma et al., 2015) and merits evaluation during entry into and recovery from transient inhibitory events.

Thus, the primary objective of this study was to assess the effects of Cr(VI) on nitrification process performance (both *aqueous* and *gaseous* nitrogen species), microbial ecology and gene expression related to ammonia and nitrite oxidation in a lab-scale bioreactor operated in full-nitrification mode.

2. Materials and methods

2.1. Reactor operation and monitoring

A nitrifying enrichment consortium was developed in a reactor (V = 6 L; hydraulic retention time (HRT) = solids retention time (SRT): 6.7 days) fed with a nutrient medium containing 2000 mg-N–NH_4^+/L and devoid of any organic carbon as previously described (Ahn et al., 2008). Dissolved oxygen (DO) was maintained higher than 3.0 mg/L using filtered lab-air. The pH was automatically controlled at 7 ± 0.2 with 0.1 M sodium bicarbonate. The temperature of the reactor was maintained in the range of 23–25 °C. The reactor performance was monitored by periodically measuring the ammonia (potentionmetry), nitrite (colorimetry) and nitrate (potentionmetry) concentrations, all according to Standard Methods (APHA, 2005). Reactor biomass concentrations were approximated using total chemical oxygen demand (tCOD) since no organic carbon was added to the feed. The tCOD measures are approximate due to the likely presence of microbially derived COD in the reactor. Upon attaining a performance-based steady state (0.6 ± 0.3 mg-N/L of effluent ammonia, 1.5 ± 0.5 mg-N/L of effluent nitrite and 1990 ± 10.0 mg-N/L of effluent nitrate), Cr(VI) was supplied on day 53 at an influent concentration and flow rate of 10,000 mg/L and 8.6 mL/day, respectively, using a separate influent bottle to achieve a reactor concentration of 100 mg/L at 3*SRT. This dosing regime was selected based on discrete Cr(VI) exposure tests on the reactor biomass, during which, specific oxygen uptake rates of ammonia oxidation were reduced by about 50% at a Cr(VI) dose of 100 mg/L. Concurrently, minimal reduction in nitrite oxidation rates were observed even at an exposure concentration of 200 mg Cr(VI)/L (data not shown). After 3*SRT (days 54–73), the supply of Cr(VI) was discontinued to monitor recovery of the reactor. Soluble reactor Cr(VI) concentrations (after filtration of reactor biomass through 0.2 μm nominal pore size syringe filter) were measured colorimetrically using the method of Park et al. (2005). Gaseous N_2O (gas filter correlation, Teledyne API 320E, San Diego, CA) and NO (Chemiluminescence, CLD 64, Ecophysics, Ann Arbor, MI) concentrations in the off gas were also measured.

2.2. Cell collection, DNA and RNA extraction

At each sample point, 1.0 mL cell suspensions were collected from the reactor and immediately centrifuged at 4 °C and 13,000 rpm for 5 min. The resulting cell-pellets were stored at −80 °C for further molecular interrogation of the sample DNA or mRNA (with added RNA protect Bacteria Reagent, Qiagen, CA). DNA and RNA were extracted (QIAcube, Qiagen, USA) and the total concentrations and quality of the resulting DNA and RNA were measured by a NanoDrop spectrophotometer (NanoDrop lite, Thermo Scientific, USA).

2.3. DGGE and sequencing

The impact of Cr(VI) on the diversity of nitrifying bacteria was qualitatively inferred via PCR-denaturing gradient gel electrophoresis (DGGE) using the primer set 1055F/1392R with a GC-clamp as previously described (Ferris et al., 1996; Park et al., 2010a). DGGE was conducted at 60 °C in 1X TAE buffer at 75 V for 13 h on a Dcode system (BioRad Laboratories, CA) on a 8% polyacrylamide gel with 30–60% (M/V) gradient of urea-formamide denaturant. Gels were stained with ethidium bromide and visualized under UV transillumination. Specific gel bands were excised with a sterilized scalpel. Upon confirmation of the excisions as single bands via a secondary DGGE run, the bands were re-amplified, purified with QIAEX II (Qiagen, CA), and sequenced (ABI3730XL DNA analyzer, Applied Biosystems, CA). Sequences were aligned using MEGA 4.0 and analyzed using BLAST.

2.4. Quantitative PCR

The concentrations of AOB and NOB were quantified via SYBR® Green chemistry quantitative PCR (qPCR) assays targeting, ammonia monooxygenase subunit A (*amoA*) (Rotthauwe et al., 1997), *Nitrobacter* 16S rRNA (Graham et al., 2007) and *Nitrospira* 16S rRNA (Kindaichi et al., 2006), respectively. The concentrations of total bacteria were quantified using eubacterial 16S rRNA targeted primers (Ferris et al., 1996) (Table 1). qPCR assays were conducted on a iQ5 real-time PCR thermal cycler (BioRad Laboratories, Hercules, CA) via previously documented protocols (Park et al., 2010b; Ahn et al., 2011). Standard curves for qPCR were generated via serial decimal dilutions of plasmid DNA containing specific target gene inserts. Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis (data not shown). Copies of target genes were translated to cell equivalents assuming the following conversions: 2.5 copies of *amo*A gene per cell in AOB (Norton et al., 2002), 1 copy of 16S rRNA gene per cell in *Nitrobacter* sp. and *Nitrospira* sp. cell (Dionisi et al., 2002; Navarro et al., 1992) and 3.6 copies of 16S rRNA gene per cell in eubacteria as a whole (Klappenbach et al., 2001).

2.5. Functional gene expression

Expression of the functional genes coding for ammonia oxidation (*amo*A) and nitrite oxidation (*nxr*A) in AOB and NOB, respectively, was quantified by reverse-transcriptase PCR (RT-qPCR) (Park

Table 1
Summary of primers employed for qPCR and PCR-DGGE.

Target gene	Primer	Sequence (5′–3′)	Refs.
Universal 16S rRNA	1055f	ATGGCTGTCGTCAGCT	Ferris et al. (1996)
	1392r	ACGGGCGGTGTGTAC	
AOB 16S rRNA	CTO 189fA/B	GGAGRAAAGCAGGGGATCG	Hermansson and Lindgren (2001)
	CTO 189fC	GGAGGAAAGTAGGGGATCG	
	RT1r	CGTCCTCTCAGACCARCTACTG	
Nitrospira 16S rRNA	NTSPAf	CGCAACCCCTGCTTTCAGT	Kindaichi et al. (2006)
	NTSPAr	CGTTATCCTGGGCAGTCCTT	
Nitrobacter 16S rRNA	Nitro 1198f	ACCCCTAGCAAATCTCAAAAAACCG	Graham et al. (2007)
	Nitro 1423r	CTTCACCCCAGTCGCTGACC	
*amo*A	amoA 1f	GGGGTTTCTACTGGTGGT	Rotthauwe et al. (1997)
	amoA 2r	CCCCTCKGSAAAGCCTTCTTC	
*nxr*A	nxrA f1	CAGACCGACGTGTGCGAAAG	Poly et al. (2008)
	nxrA r2	TCCACAAGGAACGGAAGGTC	
Universal 16S rRNA	1055f	ATGGCTGTCGTCAGCT	Ferris et al. (1996)
	1392r	[GC-Clamp]ACGGGCGGTGTGTAC	

[GC-Clamp sequence] = [CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC].

et al., 2010b; Poly et al., 2008). The mRNA concentrations of the two functional genes were normalized to the concentrations of AOB 16S rRNA (Hermansson and Lindgren, 2001) and *Nitrobacter* sp. 16S rRNA (Graham et al., 2007), respectively. DNA removal and reverse transcription from total RNA were performed using the QuantiTect Reverse Transcriptase kit (Qiagen, Valencia, CA). RT-qPCR was performed with SYBR® Green chemistry in triplicate on an iCycler iQ5 (Bio-Rad Laboratories, Hercules, CA) with appropriate no-template controls. Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis performed for each RT-qPCR assay conducted (data not shown).

3. Results and discussion

3.1. Reactor performance

During steady state full-nitrification (days 30–52), ammonia was predominantly oxidized to nitrate (98 ± 5.8%, n = 10). After Cr(VI) was introduced to the system (days 53–73, Fig. 1), the nitrification process performance was significantly affected as indicated by a reduction in effluent nitrate and an increase in effluent ammonia concentrations without nitrite accumulation (ammonia increased to 1703 ± 7.9 mg-N/L; nitrate decreased to 301 ± 6.5 mg-N/L after 3*SRT). The corresponding soluble Cr(VI) concentrations in the reactor during this period (days 53–73) ranged from 13.8 ± 0.17–90.5 ± 0.81 mg/L (Fig. 1).

Although Cr(VI) loading was discontinued on day 73, ammonia continued to accumulate to a maximum concentration of 1946 ± 99.4 mg-N/L (days 75–93), corresponding to a minimal ammonia removal efficiency of 2.7 ± 2.0%. These results can reflect a residual inhibitory impact of the remaining Cr(VI) in the bioreactor. The corresponding soluble Cr(VI) concentrations in the reactor during this period (days 75–93) ranged from 68.6 ± 0.4–3.6 ± 0 mg/L (Fig. 1). After a further decrease in soluble reactor Cr(VI) concentrations (around day 105, Fig. 1), rapid recovery of ammonia oxidation was observed although accompanied by transient nitrite accumulation. Although AOB could be more *directly* susceptible to heavy metal inhibition, in our study, NOB were actually slower in recovering from the effects of Cr(VI) exposure. This delay could be perhaps due to *direct* impact of Cr(VI) on NOB or due to lack of availability of nitrite (*indirectly* due to inhibition of AOB). These factors are distinguished based on microbial concentrations and functional gene expression below. After day 119, full-nitrification performance had eventually recovered to

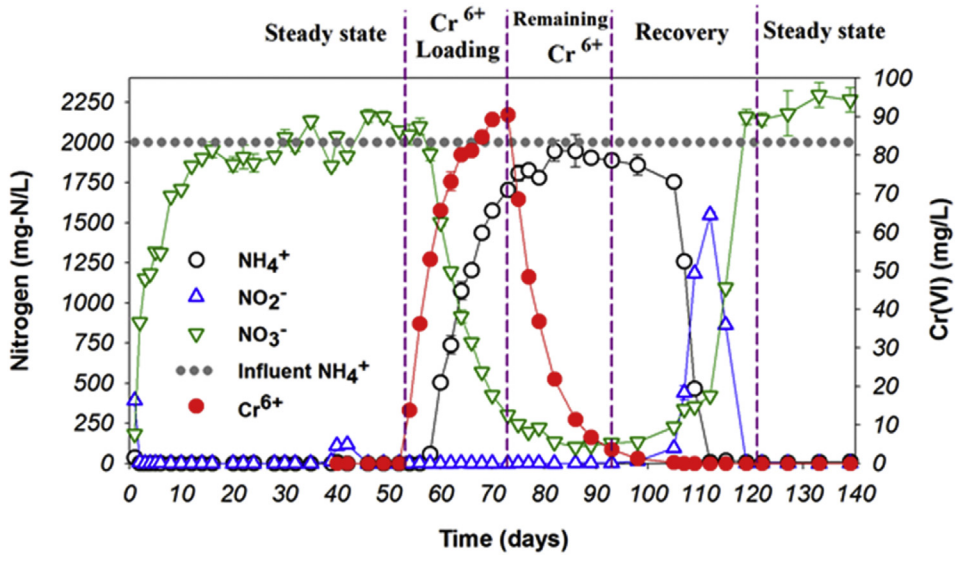

Fig. 1. Effect of Cr(VI) on nitrification performance in the test reactor. Symbols represent average and standard deviation of duplicate measurements.

the same-steady state levels as before Cr(VI) loading.

3.2. Emissions of N_2O and NO

Production of nitrous oxide (N_2O) and nitric oxide (NO) by AOB can occur during nitrification under variable nitrogen loads or transient switching between oxic and anoxic conditions (Chandran et al., 2011). Emissions of N_2O and NO during steady-state full-nitrification (days 30–52) were in the range of almost 0 ppm V and less than 30 ppb V, respectively (Fig. 2). During the period of Cr(VI) mediated inhibition (days 58–89), emission of NO decreased to less than 10 ppb V with non-detectable emission of N_2O. This reduction in N_2O and NO production was likely associated with the low specific ammonia oxidation rate of AOB, as reported by several authors (Ahn et al., 2011; Jiang et al., 2015; Law et al., 2012; Ma et al., 2015; Yu et al., 2010).

During the recovery period, which coincided with a decrease in soluble reactor Cr(VI) concentrations to non-detectable levels(days 105–115), high N_2O and NO emissions were temporarily observed in parallel with partial recovery in nitrification performance. During this time, emissions of N_2O and NO were 0.02 ± 0.01% and 0.05 ± 0.04%, respectively, of the influent ammonia loading. Especially, considering sufficient DO concentrations in the reactor during our study (range of DO value of 3.8–4.2 mg/L during pre-Cr(VI) shock, 6.0–7.6 mg/L during Cr(VI) shock and 4.0–4.3 mg/L during recovery, respectively), the imposition of ammonia loads on AOB with a high metabolic rate might trigger high production of N_2O and NO (Chandran et al., 2011). Therefore, these higher emissions during the recovery phase are consistent with previous findings that ammonia oxidation under non-limiting ammonia and DO concentrations lead to production of N_2O and NO by AOB (Yu et al., 2010; Ahn et al., 2011). Subsequently, the N_2O and NO emissions stabilized as nitrification performance recovered to pre-Cr(VI) exposure levels. Upon recovery, N_2O and NO concentrations in the off-gas were in the range of 0 ppm V and approximately 30 ppb V, respectively, similar to levels observed prior to the imposition of the Cr(VI) shock.

3.3. Molecular ecology of nitrifying bacteria in the reactor

Based on 16S rRNA gene targeted DGGE and sequencing, a distinct shift of the AOB composition was observed upon a decline in soluble reactor Cr(VI) concentrations (Fig. 3). During the prolonged period of the reactor operation which included Cr(VI) loading, the dominant AOB were closely related to *Nitrosococcus mobilis* (Band 2, Figs. 3 and 4, Table 2). However, as recovery of nitrification proceeded to yield rapid ammonia oxidation coincident with nitrite accumulation (days 107–139), the dominant AOB related to *N. mobilis* (Band 2 in Fig. 3) shifted to AOB related to *Nitrosomonas eutropha* (Band 4, Figs. 3 and 4, Table 2).

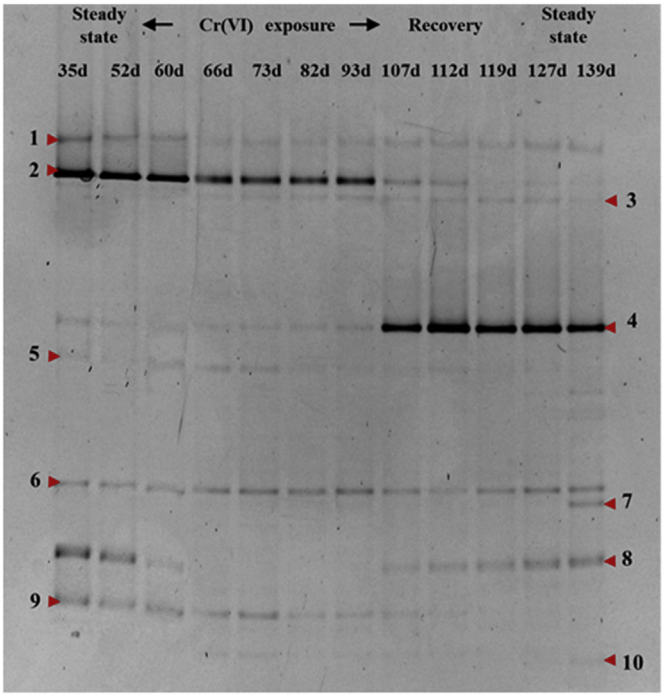

Fig. 3. DGGE profile of bacterial community in the test reactor based on 16S rRNA gene partial sequences.

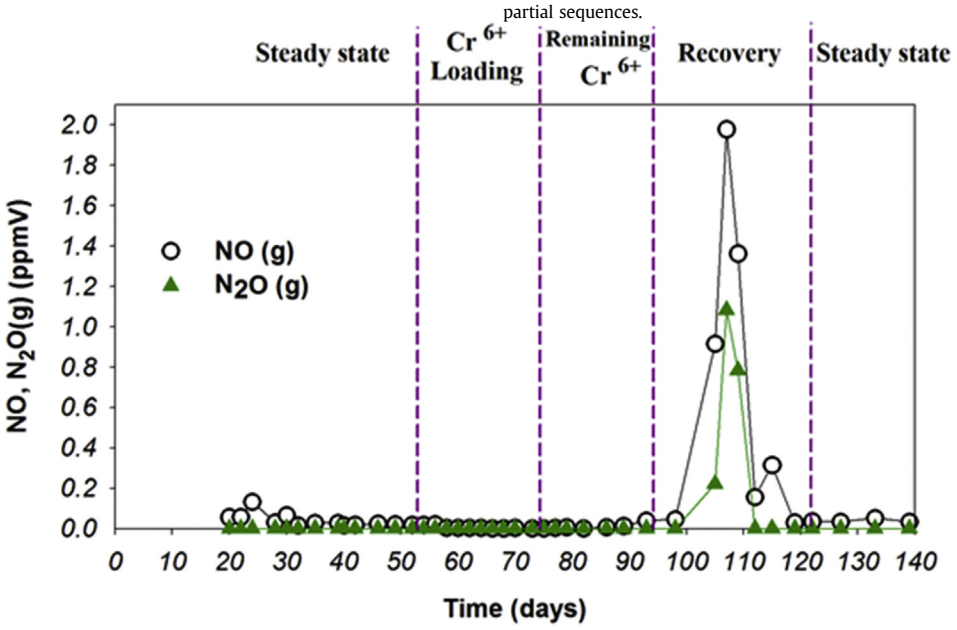

Fig. 2. N_2O and NO emissions from the test reactor.

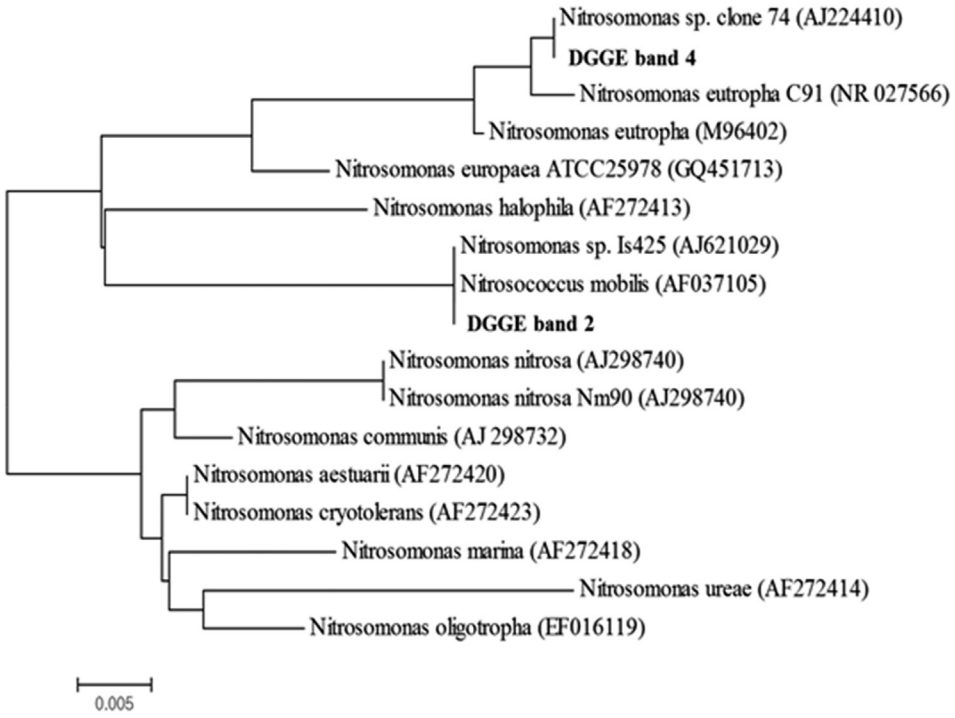

Fig. 4. Phylogeny of AOB inferred from sequencing of DGGE bands shown in Fig. 3.

Table 2
Phylogenetic affiliation of bacteria in the nitrification reactor inferred from sequencing of the DGGE bands shown in Fig. 3.

Band	Most closely related sequence	% Similarity	Accession no.
1	Uncultured bacterium 1498b PN-15	89	JQ766326
2	*Nitrosococcus mobilis*	99	AF037105
3	Uncultured bacterium 545	98	EU370835
4	*Nitrosomonas* sp. 74	98	AJ224410
5	Uncultured bacterium MABRDTU41	98	FJ529994
6	*Nitrobacter alkalicus* AN4	99	AF069958
7	Uncultured *Fluviicola* sp. JXS1-92	99	JN873202
8	Uncultured *Empedobacter* sp. YC4	99	GU062422
9	Uncultured bacterium HKT_60B4	98	JX170192
10	Uncultured bacterium NIT-EN-87	98	HQ843753

From an ecological perspective, AOB related to the *N. mobilis* lineage, are moderately halophilic and possess a higher affinity for ammonia-N (Gieseke et al., 2003). Indeed, AOB related to *N. mobilis* have been found to be the co-dominant AOB species with other AOB such as *Nitrosomonas* sp. in the nitrifying activated sludge of an industrial WWTP receiving sewage with high ammonia concentrations (Juretschko et al., 1998; Wagner et al., 1998; Rowan et al., 2003). Therefore, the dominance of AOB related to the *N. mobilis* lineage in environments with low ammonia concentrations as observed during full-nitrification is in keeping with documented literature. On the other hand, *N. eutropha* related AOB have a high half-saturation coefficient for ammonia (Martens-Habbena et al., 2009), tending to be better adapted to locations with very high N-loads and reactor N-concentrations such as partial nitrification reactors (Ahn et al., 2011) or full-nitrification reactors with transient periods of high NH_3–N accumulation (such as the system herein). Given that the reactor was operated with high N accumulation (after days 105), *N. eutropha* can be enriched during extended periods of high extant ammonia-N concentrations in nitrification reactors.

Throughout the entire operation of the reactor, NOB related only to *Nitrobacter* sp (Band 6, Fig. 3, Table 2). were detected based on DGGE profiling, and NOB related to *Nitrospira* sp. were not detected. In general, *Nitrobacter* sp. are believed to have a selective advantage over *Nitrospira* sp. in environments with high extant nitrite concentrations (Kim and Kim, 2006; Ahn et al., 2008). However, the continuous lack of detection of *Nitrospira* sp. under low nitrite concentrations during full-nitrification, when the reactor NO_2^-–N concentrations were low was unexpected. Possibly, this result reflects the competitive ability of a well-established culture of *Nitrobacter* sp. (which was indeed present in the initial inoculum (Ahn et al., 2011)) capable of growing at low nitrite concentrations.

Cr(VI) exposure had a negative impact on the overall microbial concentrations in the reactor (as measured using EUB targeted qPCR, Fig. 5a). Prior to Cr(VI) input, the reactor fractions of AOB and NOB relative to EUB were 24.6% ± 2.8% and 6.15 ± 0.9% (Fig. 5b), cells/cells), respectively. These fractions are in good correspondence with relative thermodynamic yields (0.33 mg VSS/mg NH_4^+–N and 0.083 mg VSS/mg NO_2^-–N for AOB and NOB, respectively) (Rittmann and McCarty, 2012). Within the NOB, concentrations of *Nitrospira* sp. (0.01% of EUB, $5.0 \times 10^5 \pm 5.5 \times 10^4$ copies/mL) were consistently much lower than those of *Nitrobacter* sp (6.27% of EUB, $1.9 \times 10^8 \pm 1.4 \times 10^7$ copies/mL, Fig. 5a), as also indicated qualitatively using DGGE (Fig. 3).

Upon Cr(VI) exposure, a maximum 8.5-fold decrease was observed in the EUB and AOB concentrations (1.2×10^{10} to 1.98×10^9 and 2.3×10^9 to 2.79×10^8 copies/mL, respectively) representing approximately 18% of AOB/EUB. Upon a decrease in soluble reactor Cr(VI) concentrations to non-detectable levels, EUB and AOB concentrations increased to 8.67×10^9 and 3.0×10^9 copies/mL and eventually recovered to pre-shock EUB and AOB concentrations of $6.1 \times 10^9 \pm 1.0 \times 10^9$ and $1.2 \times 10^9 \pm 2.0 \times 10^8$ copies/mL, respectively. The impact of Cr(VI) exposure on NOB concentrations was not as pronounced as on AOB and EUB leading to a transient increase in the NOB/EUB fraction (days 60–98, Fig. 5b). These trends were subsequently reversed during the recovery period (Fig. 5a and b). Ultimately, this overall impact on

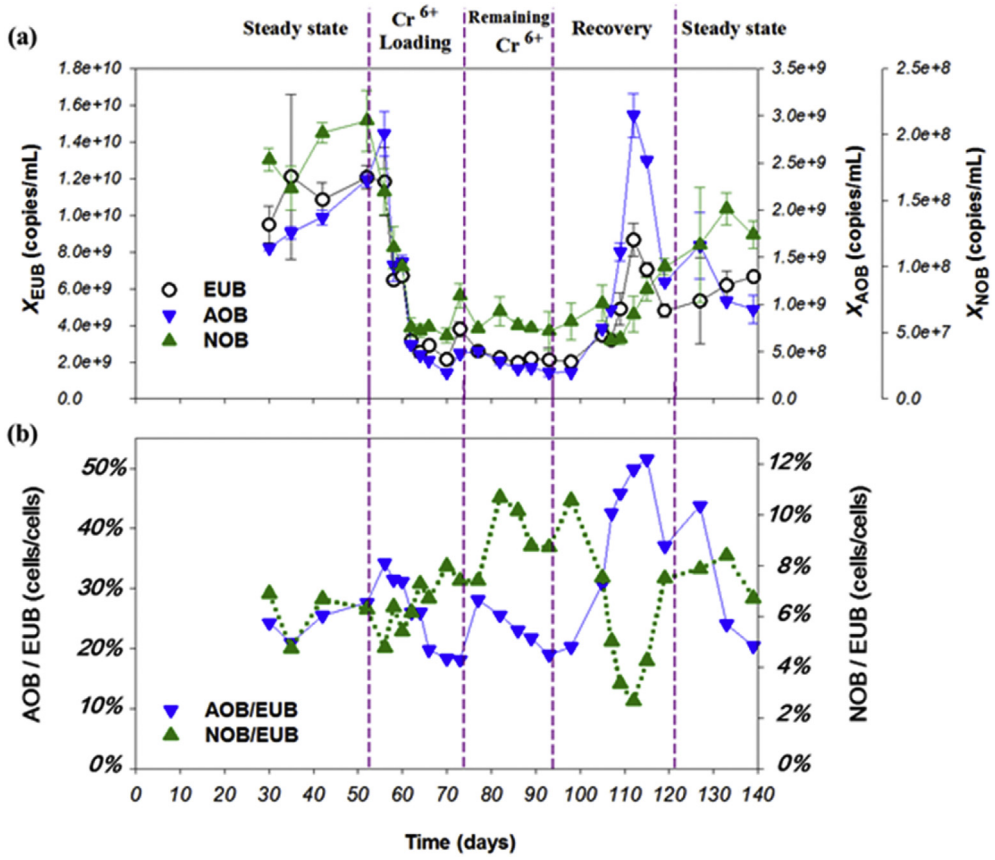

Fig. 5. Effect of Cr(VI) on (a) concentrations and (b) fractions of AOB and NOB in the test reactor. Symbols represent average and standard deviation of triplicate measurements.

microbial concentrations on AOB, NOB and EUB could be a result of an impact on microbial activity or biomass yield or both. In this study, we did not directly look at the impact of Cr(VI) on the thermodynamics of growth (and biomass yields) of AOB and NOB. However, possible impacts on expression of key genes in AOB and NOB catabolism, which could act as measures of activity were indeed explored (described in the following section).

3.4. Gene expression responses in nitrifying bacteria to Cr(VI) exposure

In terms of functional gene expression, a rapid decrease in the mRNA concentrations of the *amo*A gene (coding for ammonia oxidation, Fig. 6) was observed and corresponded well with the observed severe ammonia accumulation (Fig. 6). In contrast, mRNA concentrations of the *nxr*A gene (coding for nitrite oxidation of *Nitrobacter* sp.) were relatively more consistent throughout the study period (Fig. 6). These results indicate that Cr(VI) exposure indeed selectively and *directly* inhibited AOB activity while not influencing the NOB activity. Therefore, decreases in reactor nitrate concentrations during Cr(VI) loading were not due to *direct* inhibition of NOB activity, but rather by *indirect* substrate (nitrite) limitation of NOB through the inhibition of AOB activity (Fig. 1). The selective inhibition of AOB activity and resulting decrease in ammonia turnover by AOB also contributed to a transient reduction in AOB fractions relative to NOB fractions (Fig. 5b).

Notably, the decrease (Fig. 6, days 53–73) and recovery in *amoA* profiles (Fig. 6, days 80–100) was more rapid than the deterioration and recovery in reactor nitrogen conversion performance (Fig. 1), microbial speciation (Fig. 3) or biomass concentrations (Fig. 5). This trend is in keeping with a recent study, which also reported that expression of select catabolic genes could 'precede' trends in reactor performance (Park et al., 2010b). Therefore, the expression of genes such as *amoA* could be useful as predictive biomarkers of process performance in engineered nitrification processes. Interestingly, after recovery from the Cr(VI) shock, the *amo*A expression reached levels higher than those at pre-shock and did not decrease to pre-shock levels. These results suggest that AOB possibly retain higher transcript concentrations of genes coding for key metabolic pathways (*in casu*, *amoA* for ammonia oxidation) well after overcoming transient shocks. Such over-retention potentially results in better capacity to address subsequent transients.

From a fundamental perspective, this study represents one of the few concurrent and complementary interrogations of process performance, kinetics, microbial ecology, gene expression and gaseous signatures in response to both *inhibition* and *recovery*. From a practical engineering perspective, it is shown than even transient inhibition episodes could have irreversible changes in the microbial structure, although full recovery of reactor performance is achieved. The potential of *amoA* expression as a predictive biomarker and off-gas measurements of N_2O as a chemical marker of nitrification performance is also presented.

4. Conclusions

In sum, we present herein the multi-level responses of a full nitrification reactor to a transient 20 d Cr(VI) shock imposed directly into the reactor. Essentially Cr(VI) exposure led to selective inhibition of AOB more than NOB, as inferred from a combination of reactor performance, relative AOB and NOB concentrations and

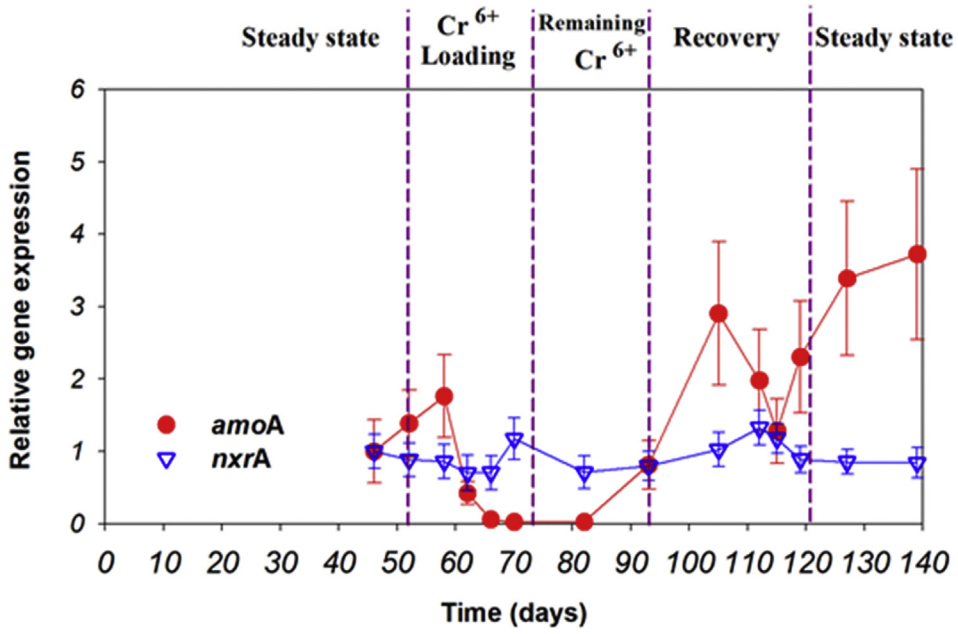

Fig. 6. Effect of Cr(VI) on the expression of *amo*A and *nxr*A genes in the test reactor. Symbols represent average and standard deviation of triplicate measurements.

expression of key catabolic genes in AOB (*amoA*) and NOB (*nxrA*). A precedence in *amoA* expression relative to reactor performance was observed and warrants further exploration of using gene expression measures as predictive biomarkers of engineered bioreactor performance. A related but *indirect* impact on NOB was also inferred, as reflected in reduced substrate (NO_2^-–N) availability due to reduced AOB activity. Transient increases in NO and N_2O emissions were also measured and essentially reflected the recovery of AOB from the inhibitory impacts of Cr(VI). Our results therefore highlight the utility of complementary application of conventional process, microbial ecological, gene expression and off-gas measurements for detailed elucidation of the underlying *direct* and *indirect* manifestations of nitrification inhibition.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Science, ICT & Future Planning (2014R1A1A1002622) to YMK, the Water Environment Research Foundation Nutrient Challenge and a National Science Foundation CAREER award (NSF CBET 0846650) to KC.

References

Ahn, J.H., Yu, R., Chandran, K., 2008. Distinctive microbial ecology and biokinetics of autotrophic ammonia and nitrite oxidation in a partial nitrification bioreactor. Biotechnol. Bioeng. 100 (6), 1078–1087.

Ahn, J.H., Kwan, T., Chandran, K., 2011. Comparison of partial and full nitrification processes applied for treating high-strength nitrogen wastewaters: microbial ecology through nitrous oxide production. Environ. Sci. Technol. 45, 2734–2740.

American Public Health Association (APHA), 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, DC. USA.

Chandran, K., Stein, L.Y., Klotz, M.G., van Loosdrecht, M.C.M., 2011. Nitrous oxide production by lithotrophic ammonia-oxidizing bacteria and implications for engineered nitrogen-removal systems. Biochem. Soc. Trans. 39 (6), 1832–1837.

Chen, J.L., Ortiz, R., Steele, T.W., Stuckey, D.C., 2014. Toxicants inhibiting anaerobic digestion: a review. Biotechnol. Adv. 32 (8), 1523–1534.

Cheng, L., Li, X., Jiang, R., Wang, C., Yin, H., 2011. Effects of Cr(VI) on the performance and kinetics of the activated sludge. Bioresour. Technol. 102, 797–804.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68, 245–253.

Ferris, M.J., Muyzer, G., Ward, D.M., 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62 (2), 340–346.

Gieseke, A., Bjerrum, L., Wager, M., Amann, R., 2003. Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. Environ. Microbiol. 5, 355–369.

Graham, D.W., Knapp, C.W., Van Vleck, E.S., Bloor, K., Lane, T., Graham, C.E., 2007. Experimental demonstration of chaotic instability in biological nitrification. ISME J. 1, 385–394.

Hermansson, A., Lindgren, P.E., 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real time PCR. Appl. Environ. Microbiol. 67, 972–976.

Jiang, D., Khunjar, W.O., Wett, B., Murthy, S.N., Chandran, K., 2015. Characterizing the metabolic trade-off in *Nitrosomonas europaea* in response to changes in inorganic carbon supply. Environ. Sci. Technol. 49, 2523–2531.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Röser, A., Koops, H.P., Wagner, M., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64 (8), 3042–3051.

Kim, D.-J., Kim, S.-H., 2006. Effect of nitrite concentration on the distribution and competition of nitrite-oxidizing bacteria in nitratation reactor systems and their kinetic characteristics. Water Res. 40 (5), 887–894.

Kim, Y.M., Park, D., Lee, D.S., Park, J.M., 2008. Inhibitory effects of toxic compounds on nitrification process for cokes wastewater treatment. J. Hazard. Mater. 152, 915–921.

Kindaichi, T., Kawano, Y., Ito, T., Satoh, H., Okabe, S., 2006. Population dynamics and in situ kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. Biotechnol. Bioeng. 94 (6), 1111–1121.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. Rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184.

Law, Y., Ni, B.-J., Lant, P., Yuan, Z., 2012. N_2O production rate of an enriched ammonia-oxidising bacteria culture exponentially correlates to its ammonia oxidation rate. Water Res. 46 (10), 3409–3419.

Ma, Y., Sundar, S., Park, H., Chandran, K., 2015. The effect of inorganic carbon on microbial interactions in a biofilm nitritation-anammox process. Water Res. 70, 246–254.

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461, 976–979.

Navarro, E., Fernandez, M.P., Grimont, F., Clays-Josserand, A., Bardin, R., 1992. Genomic heterogeneity of the genus *Nitrobacter*. Int. J. Syst. Bacteriol. 42, 554–560.

Norton, J.M., Alzerreca, J.J., Suwa, Y., Klotz, M.G., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Arch. Microbiol. 177, 139–149.

Novotnik, B., Zuliani, T., Ščančar, J., Milačič, R., 2014. Inhibition of the nitrification

process in activated sludge by trivalent and hexavalent chromium, and partitioning of hexavalent chromium between sludge compartments. Chemosphere 105, 87–94.
Painter, H.A., Loveless, J.E., 1983. Effect of temperature and pH value on the growth-rate constants of nitrifying bacteria in the activated-sludge process. Water Res. 17 (3), 237–248.
Park, D., Yun, Y.-S., Park, J.M., 2005. Studies on hexavalent chromium biosorption by chemically-treated biomass of *Ecklonia* sp. Chemosphere 60, 1356–1364.
Park, H., Rosenthal, A., Jezek, R., Ramalingam, K., Fillos, J., Chandran, K., 2010a. Impact of inocula and growth mode on the molecular microbial ecology of anaerobic ammonia oxidation (anammox) bioreactor communities. Water Res. 44, 5005–5013.
Park, H., Rosenthal, A., Ramalingam, K., Fillos, J., Chandran, K., 2010b. Linking community profiles, gene expression and N-removal in anammox bioreactors treating municipal anaerobic digestion reject water. Environ. Sci. Technol. 44 (16), 6110–6116.
Poly, F., Wertz, S., Brothier, E., Degrange, V., 2008. First exploration of *Nitrobacter* diversity in soils by a PCR cloning-sequencing approach targeting functional gene nxrA. FEMS Microbiol. Ecol. 63, 132–140.
Rittmann, B.E., McCarty, P.L., 2012. Environmental Biotechnology: Principles and Applications. Tata McGraw-Hill Education, p. 472.
Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoa as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63 (12), 4704–4712.
Rowan, A.K., Moser, G., Gray, N., Snape, J.R., Fearnside, D., Curtis, T.P., Barer, M.R., Head, I.M., 2003. A comparitive study of ammonia-oxidizing bacteria in lab-scale industrial wastewater treatment reactors. Water Sci. Technol. 48 (3), 17–24.
Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Papanikolaou, E.C., Tsakon, A., Lekkas, T.D., 2003. Effects of chromium (VI) addition on the activated sludge process. Water Res. 37 (9), 2140–2148.
Vaiopoulou, E., Gikas, P., 2012. Effects of chromium on activated sludge and on the performance of wastewater treatment plants: a review. Water Res. 46, 549–570.
Wagner, M., Noguera, D.R., Juretschko, S., Rath, G., Koops, H.P., Schleife, K.H., 1998. Combining fluorescent *in situ* hybridization (FISH) with cultivation and mathematical modeling to study population structure and function of ammonia-oxidizing bacteria in activated sludge. Water Sci. Technol. 37 (4–5), 441–449.
Yu, R., Kampschreur, M.J., van Loosdrecht, M.C.M., Chandran, K., 2010. Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. Environ. Sci. Technol. 44, 1313–1319.