**Effects of Cr(III) and Cr(VI) on nitrification inhibition as
determined by SOUR, function-specific gene expression and 16S
rRNA sequence analysis of wastewater nitrifying enrichments**

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Running Title: Nitrification inhibition by Cr(III) and Cr(VI)

Abstract

The effect of Cr(III) and Cr(VI) on nitrification was examined with samples from nitrifying enrichment cultures using three different approaches: by measuring substrate (ammonia) specific oxygen uptake rates (SOUR), by using RT-qPCR to quantify the transcripts of functional genes involved in nitrification, and by analysis of 16S rRNA sequences to determine changes in structure and activity of the microbial communities. The nitrifying bioreactor was operated as a continuous reactor with a 24 h hydraulic retention time. The samples were exposed in batch vessels to Cr(III) (10 – 300 mg/L) and Cr(VI) (1 – 30 mg/L) for a period of 12 h. There was considerable decrease in SOUR with increasing dosages for both Cr(III) and Cr(VI), however Cr(VI) was more inhibitory than Cr(III). Based on the RT-qPCR data, there was reduction in the transcript levels of *amoA* and *hao* for increasing Cr(III) dosage, which corresponded well with the ammonia oxidation activity measured via SOUR. For Cr(VI) exposure, there was comparatively little reduction in *amoA* expression while *hao* expression decreased for 1 to 3 mg/L Cr(VI) and increased at 30 mg/L Cr(VI). While *Nitrosomonas* spp. were the dominant bacteria in the bioreactor, based on 16S rRNA sequencing, there was a considerable reduction in *Nitrosomonas* activity upon exposure to 300 mg/L Cr(III). In contrast, a relatively small reduction in activity was observed at 30 mg/L Cr(VI) loading. Our data suggest that both Cr(III) and Cr(VI) were inhibitory to nitrification at concentrations near the high end of industrial effluent concentrations.

Keywords: Nitrification, SOUR, RT-qPCR, chromium, wastewater

1. Introduction

Chromium, a heavy metal that enters wastewater treatment plants (WWTPs) through industrial discharges, can be toxic to microorganisms carrying out important processes within biological wastewater treatment systems. Chromium usually occurs in the environment as trivalent (i.e., Cr(III)) or hexavalent (i.e., Cr(VI)) form. Cr(III) salts are used in textile dyeing, photography, and in the ceramic and glass industry (Çetin et al., 2008; Srivastava et al., 1986), while Cr(VI) salts are used extensively in industries linked to electroplating, manufacturing of dyes, paints, ceramics and explosives, and leather tanning (Cavaco et al., 2007; Jacobs and Testa, 2005). Generally, Cr(VI) is regarded as more toxic than the trivalent form. Cr(III) and Cr(VI) concentrations in the range of 1 - 30 mg/L (Chaudry et al., 1998; Moussavi and Barikbin, 2010) have been found for industrial wastewater effluents. Higher values of up to 500 mg/L have also been reported for Cr(III) (Petruzzelli et al., 2014; Patterson, 1985).

Nitrification, or the removal of nitrogen from wastewater, is a two-step process in which ammonia (NH_3), is aerobically oxidized to nitrate (NO_3^-), via ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), before being anaerobically reduced to nitrogen gas, via denitrifying bacteria. The effects of Cr(III) and Cr(VI) on nitrification have been studied using several methods such as respirometry (Çeçen et al., 2010), substrate removal (Cheng et al., 2011) and growth rate (Gikas and Romanos, 2006). Both Cr oxidation states have been known to be inhibitory towards AOB in activated sludge systems (Vaipoulou and Gikas, 2012). However, there is no consensus on chromium thresholds impacting nitrification performance. Indeed, data on the inhibition of nitrification by Cr(III) and Cr(VI) varies greatly among studies. The Cr(III) concentration at which 50% inhibition of nitrification occurs range from 37 mg/L (Cecen et al., 2010) to greater than 100 mg/L (Farabegoli et al., 2004). In general, Cr(III) concentrations above

100 mg/L are known to affect wastewater nitrification performance, and some studies have reported concentrations between 160 mg/L to 320 mg/L as lethal (Gikas and Romanos, 2006). Conversely, other studies have reported that Cr(VI) is inhibitory to nitrification at concentrations as low as 5 mg/L (Cheng et al., 2011; Stasinakis et al., 2003), while others report growth stimulation of nitrifiers at concentrations up to 25 mg/L (Gikas and Romanos, 2006). Cecen et al. (2010) reported that 50% inhibition of nitrification occurred at around 39 mg/L of Cr(VI), while Samaras et al. (2009) found no effect on nitrification within 1 to 50 mg/L Cr(VI).

Respirometry based assays such as specific oxygen uptake rate (SOUR) is the most commonly used approach to determine the level of nitrification inhibition (Li et al., 2015; Çeçen et al., 2010; Madoni et al., 1999; Chandran and Love, 2008). In general, respirometry methods measure total inhibition, but cannot determine which AOB groups remain active or are significantly inhibited after toxicant exposure. This is important as different AOB groups may react differently to different inhibitors, plus the metabolic response in a given system to the presence of inhibitory compounds may be largely dependent on characteristics of the microbial community (Wittebolle et al., 2008). Moreover, most of the respirometry studies have used pure cultures (i.e., *Nitrosomonas europaea*), and therefore limited data is available on how complex nitrifying microbial communities respond to the presence of inhibitors (Chandran and Love, 2008; Radniecki et al., 2009). Therefore, alternate methods are needed to better understand how the composition and physiology of the microbial community can be influenced upon exposure to nitrification inhibitors.

Investigations using multiple physiological and genetic approaches to measure inhibition of nitrifying wastewater microbial communities exposed to chromium are limited. Moreover, there is minimal data on the changes in functional gene expression of AOB subjected to

chromium inhibition. In this study, we applied reverse transcriptase – qPCR (RT-qPCR) assays targeting function-specific genes linked to nitrifying bacteria to measure the relative expression of AOB in response to acute exposure (12 h) of nitrifying enrichments to varying concentrations of Cr(III) and Cr(VI). The most sensitive step in nitrification, the oxidation of ammonia to nitrite (NO_2^-) by AOB, is linked to the activity of ammonia monooxygenase (*amoA*) and hydroxylamine oxidoreductase (*hao*) (Hooper et al., 1997). The oxidation of ammonia to hydroxylamine (NH_2OH) is carried out by *amoA* and NH_2OH oxidation to NO_2^- involves *hao*. Since the expression of these genes corresponds with the activity of enzymes thereby encoded, it is reasonable to assume that exposure of nitrifying bacteria to chromium can induce stress that would impact the transcription of genes coding for these enzymes (Park and Ely, 2008). Therefore, the transcriptional responses of these functional genes in AOB were examined for concentrations of Cr(III) and Cr(VI) relevant to wastewater treatment plants. We compared the transcriptional responses to SOUR based measurements for the same set of samples to evaluate the efficacy of using functional-gene transcript levels as indicators of nitrification inhibition. Furthermore, we studied the shift in microbial activity of the nitrifying enrichments exposed to chromium using next generation sequencing of 16S rRNA libraries.

2. Materials and Methods

2.1 Nitrifying bioreactor

The bioreactor was established using return activated sludge taken from a municipal wastewater treatment plant (Metropolitan Sewer District of Greater Cincinnati) in Cincinnati, OH. The sludge was fed in a 10 L continuous stirred tank reactor (CSTR) as described earlier (Hu et al., 2002). The CSTR was operated with a solids retention time (SRT) of 35 d and a hydraulic retention time (HRT) of 1 d. To enrich for nitrifying microbial communities the reactor was fed a

basic nutrient medium containing 500 mg/L $\text{NH}_4^+\text{-N}$, $(\text{NH}_4)_2\text{SO}_4$ and essential macro- and micro-nutrients (Table S1). The feed did not contain any organic matter in order to minimize heterotrophic activity. The pH of the reactor was automatically maintained at 7.5 ± 0.1 with 1 M sodium bicarbonate. Aeration was provided using filtered laboratory air at 1 L/min. Mixing was provided by magnetic stirring at 200 rpm. Regularly, the ammonium removal capacity of sludge was monitored by the measurement of ammonium ($\text{NH}_4^+\text{-N}$) concentrations in the effluent and the total chemical oxygen demand (tCOD) of the reactor (Fig. S1). Once steady-state was achieved within the bioreactor (effluent $\text{NH}_4^+\text{-N}$ concentration less than 5 mg/L), sludge samples were taken from this reactor and used in inhibition studies.

2.2 Batch experiments

Inhibition studies were performed in batch vessels incubated with samples from the nitrifying bioreactor with and without (control) exposure to Cr(III) or Cr(VI). Chromium(III) chloride (CrCl_3) and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) (ACS grade certified, Fisher Scientific, Fair Lawn, NJ) were used to prepare stock solutions for Cr(III) and Cr(VI) respectively. All reagents and their dilutions were prepared using autoclaved Milli-Q water. The concentrations of Cr(III) used in the batch assays were 10, 30, 100 and 300 mg/L, and the concentrations of Cr(VI) were 1, 3, 10 and 30 mg/L. These Cr concentrations were chosen because many Cr-contaminated industrial wastewaters had 10 - 300 mg /L of Cr(III) (Petruzzelli et al., 2014; Patterson, 1985) and 1 - 30 mg/L of Cr(VI) (Chaudry et al., 1998; Moussavi and Barikbin, 2010) and they likely represented worst-case scenario for municipal WWTPs.

For batch assays, samples (50 mL) collected from the reactor were diluted with deionized autoclaved water (50 mL). MOPS (3-(N-morpholino) propanesulfonic acid) was added as a buffer (20 mM final concentration) (Hu et al., 2002). For every experimental run, the

concentration of Cr(III) and Cr(VI) in each vessel was adjusted by the addition of calculated amounts of standard aqueous solutions of CrCl₃ and K₂Cr₂O₇, respectively. The pH was adjusted to 7.5 and then samples were placed on a magnetic stirrer and constantly mixed at 100 rpm and 25 ± 1 °C for 12 h. Subsamples (1 mL) were collected from both the control and treatment vessels before and after the 12 h exposure period and centrifuged at 21000 x g for 5 min. The supernatant was removed and the pellets were stored at -80 °C until nucleic acid extraction. All experiments were performed in duplicates for each concentration. A control with no added Cr(III) or Cr(VI) was used for every experiment.

2.3 SOUR

SOUR was determined in duplicate using an extant batch respirometry assay as previously described (Hu et al., 2002). At the end of the 12 h exposure period, nitrifying samples were transferred to 100 mL water-jacketed glass vessels, maintained at 25 ± 0.5 °C. Biomass suspensions were then aerated with pure oxygen gas followed by injection of a substrate aliquot (20 mg/L NH₄⁺-N) to yield substrate-independent maximal oxygen uptake rates. The decrease in the dissolved oxygen (DO) level in the vessel due to ammonia oxidation was continuously measured in real-time via a DO probe (YSI 5300A, Yellow Springs Instruments, OH) and recorded by a personal computer interfaced to a DO monitor. The inhibition of nitrification activity was expressed as the difference between the measured SOUR with and without metal exposure. The respirograms were used to express inhibition by measuring the differences between the maximum specific oxygen uptake rates in the absence (SOUR_{control}) and presence (SOUR_{sample}) of metal exposure as follows:

$$\% \text{ inhibition} = (\text{SOUR}_{\text{control}} - \text{SOUR}_{\text{sample}}) / \text{SOUR}_{\text{control}} \times 100\% \quad (1)$$

A non-competitive inhibition model was used to estimate the half-inhibition coefficient, K_i by minimizing the sum of squared errors between the measured and predicted inhibition using Solver (MS Excel, 2013). The predicted inhibition based on the metal concentration (I) was calculated as follows:

$$\% \text{ inhibition} = 100 / (1 + K_i / I) \quad (2)$$

2.4 RT-qPCR assays

The transcript levels of *amoA* and *hao* in the tested samples were determined using RT-qPCR assays. RNA and DNA were extracted from the stored cell pellets using AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) as described previously (Kapoor et al., 2015b). cDNA was synthesized from the purified total RNA extracts using the Superscript III, following the manufacturer's instructions (Life Technologies, San Francisco, CA). RT-qPCR was performed on QuantStudio 6 Flex Real-Time PCR system (Life Technologies, San Francisco, CA) with Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using published primers (Table S2). AOB relative abundance was determined by qPCR using AOB 16S rRNA gene primers (Table S2). The transcript levels of each functional gene were normalized against AOB 16S rRNA gene copies as quantified by qPCR.

2.5 16S rRNA and 16S rRNA gene sequencing

The bacterial composition of nitrifying bioreactor was determined via the analysis of high-throughput sequencing of 16S rRNA and 16S rRNA gene (16S rDNA) libraries generated with barcoded primers 515F/806R (Caporaso et al., 2011) using as PCR templates RNA (cDNA) and DNA, respectively. The relative changes in bacterial groups in the nitrifying bioreactor samples exposed to different chromium concentrations were inferred by 16S rRNA sequence abundance

(i.e., using cDNA as sequencing templates). The cDNA-based sequences were used to infer potentially active bacterial populations. Gene libraries developed using DNA as the template were used to determine total bacterial composition within each sample (i.e., regardless of the level of activity). PCR reactions used to generate the sequencing libraries were performed in 25 μ L volumes using the Ex *Taq* kit (Takara) with 200 nM each of the forward and reverse primer and 2 μ L of template nucleic acid. Cycling conditions involved an initial 5 min denaturing step at 94 °C, followed by 30 cycles of 45 s at 94 °C, 60 s at 50 °C, and 90 s at 72 °C and a final elongation step of 10 min at 72 °C. PCR products were visualized on an agarose gel to confirm product sizes. PCR products were then pooled and cleaned prior to multiplex sequencing on an Illumina Miseq benchtop sequencer using pair-end 250 bp kits at the Cincinnati Children's Hospital DNA Core facility. Sequence reads were processed using MOTHUR v1.25.1 (Schloss et al., 2009) as described earlier (Gomez-Alvarez et al., 2012).

3. Results and discussion

3.1 Effects of Cr(III) and Cr(VI) on SOUR

There was as an increase in nitrification inhibition as determined by SOUR as the applied metal dose to nitrifying biomass increased for both Cr(III) and Cr(VI) exposure (Fig. 1). A linear relationship was observed between Cr concentration and SOUR based inhibition (Cr(III), $R^2 = 0.97$; Cr(VI), $R^2 = 0.89$). For Cr(III) exposure, nitrification inhibition increased by 25% for 300 mg/L Cr(III) (Fig. 1a), whereas there was around 35% increase in inhibition after exposure to 30 mg/L Cr(VI) (Fig. 1b). Based on K_i estimates Cr(VI) had a greater inhibitory effect toward ammonium oxidation than Cr(III). K_i values derived from the non-competitive inhibition model were 50 mg/L and 909 mg/L for Cr(VI) and Cr(III), respectively.

The greater inhibitory effect to nitrifiers of Cr(VI) compared to Cr(III) has also been observed in previous studies for activated sludge mixed cultures in either a continuous-flow system or batch cultures (Stasinakis et al., 2003; Gikas and Romanos, 2006; Lamb and Tollefson, 1973; Novotnik et al., 2014). While these studies concluded that Cr(VI) is more toxic than Cr(III) to the nitrifying bacteria, they are not in agreement with other findings showing that hexavalent chromium was less inhibitory than trivalent chromium for activated sludge mixed cultures in batch tests using oxygen uptake rates as a measure of inhibition (Mowat, 1976). The differences in the SRT of the reactor may impact the outcome of the inhibition tests. In the current study, the nitrifying reactor was operated at SRT of 35 d which is longer than the aforementioned studies. When the tested biomass or sludge is cultivated under different SRT conditions, the nitrifying process could be different since the physiological state of the cells may impact the uptake of inhibitory compounds including metals (Li et al., 2015). For instance, if exposure to a given inhibitor leads only to a reduction in specific activity (as captured in a reduction in growth rate), it might still be possible to modify the operation of the nitrifying bioreactor by increasing the SRT and still achieve the desired degree of nitrification. Another source of variation may be due to the wide range of exposure times (8h to 14d) used to study inhibition (i.e., 8-9 h in Lamb and Tollefson, 1973; 12 h in our study; 24 h in Novotnik et al. 2014; 5 or 14 d in Mowat, 1976). These differences in the duration of metal exposure may cause alterations in the metal sorption kinetics onto the sludge and intracellular transport (internalization) into the bacterial cells. It should be noted that there are limited studies on metal-microbe interaction for chromium to allow a deep understanding of toxicity in vivo, especially for nitrifying bacteria (Megharaj et al., 2003; Fulladosa et al., 2005). However, it is generally assumed that the toxicity of chromium is associated with its ability to penetrate the cell

membrane. Within the cell, Cr(VI) is reduced to Cr(III), which forms substitution-inert complexes with a number of cell components thereby inhibiting activity (Megharaj et al., 2003; Wong and Trevors, 1988). This has important implications to plant managers as it addresses the importance to establish Cr(III) and Cr(VI) concentration thresholds that may be used to prevent inhibition in treatment plants.

Although the present study is based on the effects of total dosed chromium in solution, it should be noted that part of Cr(III) added may precipitate in the near neutral pH range, so that the actual concentration of Cr(III) in solution was slightly lower. To determine the solubility of Cr(III) at neutral pH, dosages of 10, 30, 100 and 300 mg/L Cr(III) respectively were added to biomass samples followed by pH adjustment and the concentrations of dissolved Cr(III) determined after 12 h by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (Table S3). Even at high Cr(III) dosage (300 mg/L), the final dissolved concentrations for Cr(III) were measured very low (< 5mg/L). The precipitation of Cr(III) after pH adjustment has been reported in other studies (Çeçen et al., 2010; Novotnik et al., 2014; Vaňková et al., 1999). However, the inhibitory effect of Cr(III) increased with increasing dose suggesting that particulate Cr(III) also impacted nitrification. In addition, Cr(III) forms hydroxide complexes of low solubility that may be sorbed onto biomass (Çeçen et al., 2010). All together, these data suggest that additional studies are needed to better establish acceptable thresholds that are applicable to a range of treatment plants receiving different types of waste.

3.2 Effects of Cr(III) and Cr(VI) on gene expression

The relative changes in transcript levels of *amoA* and *hao* (normalized against AOB 16S rRNA gene copies) were measured via RT-qPCR for the same samples tested via SOUR. In nitrifying cultures exposed to Cr(III), there was a decrease in *amoA* and *hao* expression with increasing

metal concentrations (Fig. 2a). The transcript levels of *amoA* and *hao* were severely reduced during exposure to 300 mg/L Cr(III). However, in cultures exposed to Cr(VI), there was comparatively little reduction in *amoA* expression (Fig. 2b). There was a notable decrease in *hao* expression from 1 to 3 mg/L Cr(VI), yet the expression was slightly stimulated upon exposure to higher concentrations of Cr(VI) suggesting that the intracellular RNA content of these functional genes may not strictly correspond with the activity of enzymes under metal exposure. Similar results have been shown in previous studies (Kapoor et al., 2015a; Radniecki et al., 2009). Thus, a critical research need is the ability to identify and quantify inhibition in different steps of nitrification. Such distinctions are needed when faced with selective inhibitors of nitrification steps. For instance, while certain heavy metals could inhibit ammonia to hydroxylamine oxidation, they may not have an impact on hydroxylamine oxidation or nitrite reduction. Such inferences can only be made by targeting functional genes implicated in different steps of nitrification.

There was a strong correlation between *amoA* expression and SOUR-based inhibition for samples exposed to varying concentrations of Cr(VI) (pearson correlation coefficient, $r = -0.896$, $p < 0.05$) suggesting a direct correspondence between functional gene expression and physiological activity in these samples. Although not as strong as Cr(VI), there was a good correlation between *amoA* expression and SOUR-based inhibition for samples exposed to Cr(III) ($r = -0.708$, $p < 0.05$). These results suggest that chromium directly inhibited the ammonia oxidation activity of AOB at the gene expression level.

Several studies have employed function-specific genes to monitor inhibition of nitrification by heavy metals such as copper, zinc and cadmium. For instance, Park and Ely (2008) identified *amoA* as a candidate stress gene for monitoring inhibition of nitrification by

Cu²⁺, Cd²⁺, and Hg²⁺. In another study, the expression of *amoA* and *hao* was monitored in continuously cultured *N. europaea* cells exposed to zinc chloride (Radniecki et al., 2009). The relationship between SOUR and functional gene expression of nitrifying bacteria has been studied for several heavy metals (Ouyang et al., 2016; Kapoor et al., 2015a) and other inhibitors such as dimethyl sulfide (Fukushima et al., 2014). However, there are few studies that have examined functional genes to study the effect of chromium on nitrification in wastewater systems (Kim et al., 2013). Here we applied RNA-based RT-qPCR assays to measure transcriptional responses of *amoA* and *hao* in conjunction with SOUR in nitrifying enrichment exposed to different concentrations of Cr(III) and Cr(VI). While overall the results show that the differences in the relative transcript levels of *amoA* and *hao* were to some extent in agreement with SOUR during exposure to chromium, additional data is needed to further demonstrate the responses at the protein synthesis (i.e., translation) and enzyme activity level.

3.3 Effects of Cr(III) and Cr(VI) on 16S rRNA sequences

Community sequence analysis of the 16S rRNA gene was used to elucidate the abundance and relative activity of bacterial populations present in the nitrifying bioreactor. An average of approximately 450,000 16S rDNA and 16S rRNA sequences representing 19,359 to 58,215 OTUs per sample were analyzed from samples obtained on different dates (n = 7) (Fig. 3). The sequencing libraries obtained on different dates displayed high degrees of similarity, based on a comparison of taxonomic groups identified and their abundance proportions (correlation coefficients, $r > 0.9$) indicating that the microbial composition of the bioreactor was similar over the experimental period. At the 16S rDNA level, the nitrifying enrichments hosted a diverse bacterial community with over 40% of sequences associated with the phylum Proteobacteria. The majority of the Proteobacteria-like sequences belong to members of the Nitrosomonadales

order (Fig. 3a). Most of the remaining sequences (20–35%) are closely related to members of the phylum Bacteroidetes, the vast majority within the Cytophagales order. Nitrite oxidizing bacteria (NOB) sequences related to *Nitrobacter* spp. were also detected (1-5 % of total sequences). At the 16S rRNA level, the most active bacterial group was identified as Nitrosomonadales (> 70%) (Fig. 3b). Although Cytophagales were abundant at the 16S rDNA level, they were comparatively less abundant (hence, arguably less active) than the Nitrosomonadales. Some other groups which were detected at the 16S rDNA level but were not active at 16S rRNA level (i.e., < 2% total sequences) included Xanthomonadales, Rhizobiales, Burkholderiales, Saprospirales, and Caulobacterales among others. Due to its role in protein synthesis the abundance of rRNA molecules often correlates with cell bacterial growth and overall physiological state (Hunt et al., 2013). Thus, differences observed in our study in the bacterial composition by 16S rRNA and 16S rDNA derived sequences can be explained by the fact that the rRNA-based sequences provide information on the active members of the community while DNA-derived sequences represent the bacterial populations present in the sample regardless of their physiological status (Kapoor et al., 2015b).

Nitrosomonas sp. were the major detected AOB in the nitrifying enrichment culture. 16S rRNA gene sequences showed that the relative activity of Nitrosomonadales tended to decrease along with the increase of Cr(III) and Cr(VI) dosage (Table 1). There was a severe reduction in *Nitrosomonas* 16S rRNA sequences after exposure to 300 mg/L Cr(III). Lower levels of Cr(VI) (i.e., 1 and 3 mg/L) caused relatively few changes in the activity of nitrifying bacteria at the 16S rRNA level. At 30 mg/L, Cr(VI) reduced the activity of *Nitrosomonas* bacteria and caused changes in the overall microbial community structure as determined by 16S rRNA sequencing analysis. As previously suggested, Cr(VI) could be effectively up-taken by bacterial cells and

reduced to Cr(III), which may subsequently reacted with intracellular metabolites to generate Cr(III) complexes (such as ascorbate-Cr(III) species, organo-Cr(III) species and NAD⁺-Cr(III) complex) that bind to protein and nucleic acids (RNA, DNA) causing detrimental effects (Puzon et al., 2005).

The results of this study compare favorably with recently documented reductions in specific ammonia oxidation activity in response to chromium exposure (Su et al., 2015; Schaerer, 2009). Su et al. (2015) investigated the response of nitrifying bacteria to Cr(VI) exposure in sequencing batch reactors. They observed that a Cr(VI) loading of 5–8 mg/L reduced the nitrification efficiencies and respiration activities of AOB, but caused little variation in the relative abundance of nitrifying bacteria as determined by 16S rDNA gene profiling, while higher Cr(VI) loading of 10–20 mg/L caused reductions in abundance of some of the dominant nitrifying bacterial populations. Su et al. (2015) used 16S rDNA fingerprinting to deduce the changes in population of nitrifying bacteria. In contrast, our study used 16S rRNA sequencing because the rDNA levels were more or less constant (data not shown) in the batch cultures exposed to chromium for relative short exposure time (12 h). Both the studies reached the same conclusions, nonetheless the rRNA-based approach also provide information on the general metabolic status of the targeted bacterial populations since the abundance of rRNA molecules is the product of cell growth and its physiological state (Poulsen et al., 1993; Kapoor et al., 2015b).

4. Conclusions

In this study, the comparison of Cr(III) and Cr(VI) exposure on nitrification inhibition was evaluated using molecular methods such as functional gene expression and 16S rRNA sequencing combined with conventional nitrification inhibition assays (i.e. SOUR) to better understand the response of wastewater nitrifying systems to the presence of chromium. Both

Cr(III) and Cr(VI) significantly inhibited the nitrification process, and their inhibitory effect increased with increasing concentrations. Using the non-competitive inhibition model, we estimated that at least 50 mg/L of Cr(VI) and 900 mg/L of Cr(III) would cause a 50% nitrification inhibition, respectively. It should be noted that most of Cr(VI) can be reduced to Cr(III), which may be precipitated in the form of hydroxides at neutral pH (Novotnik et al., 2014). It has been speculated that Cr(III) enter cells through passive diffusion (Cohen et al., 1993), so the concentration inside the cell increases slowly requiring higher concentrations and exposure time to cause nitrification inhibition. Thus, the inflow of higher chromium concentrations to WWTP could have harmful effects on nitrifying bacteria that can inhibit nitrogen removal. However, both of these values are higher than it would be expected in industrial wastewater discharges, which range from 1 mg/L to 30 mg/L (Chaudry et al., 1998; Moussavi and Barikbin, 2010). Thus, chromium, especially Cr(VI), is not likely to produce acute nitrification inhibition in wastewater systems under normal environmental exposures. In fact, under similar environmentally relevant concentrations chromium was proved to be the least toxic among the metals $Ag > Hg > Cd > Cr(III) = Cr(VI)$ (Çeçen et al., 2010).

In conclusion, for concentrations of chromium used in this study, neither hexavalent nor trivalent chromium caused more than 35% inhibition of nitrification, although the hexavalent chromium exerted greater inhibition than the trivalent chromium based on ammonia dependent SOUR during 12 h batch exposure. Higher Cr(VI) loadings led to higher inhibitory effects based on SOUR, although there were no considerable changes in *amoA* expression of nitrifying populations for concentrations less than 10 mg/L. A loading of 1 - 10 mg/L Cr(VI) inhibited the respiration activities of AOB, but did not considerably change the nitrifying community structure, while 30 mg/L Cr(VI) inhibited nitrification by more than 30% and reduced the

relative activity of the nitrifying bacteria. Comparatively, there was a relative decrease in SOUR, *amoA* expression and 16S rRNA based activity of AOB with an increase in Cr(III) loadings. The correspondence between the relative expression of functional genes, SOUR and microbial activity shown in this study demonstrates the value of RNA based function-specific assays as complement to SOUR assays for better understanding of the effect of chromium on nitrification activity in wastewater.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Fig. 1. Comparison of nitrification inhibition by Cr(III) and Cr(VI) measured by SOUR as a function of total metal concentration after a 12-h exposure of (a) Cr(III) (10 – 300 mg/L), and (b) Cr(VI) (1 – 30 mg/L). Results are the average of two independent experiments for each concentration (experimental duplicates) and error bars show the range of the duplicates.

Fig. 2. Comparison of nitrification inhibition by Cr(III) and Cr(VI) measured by gene expression as a function of total metal concentration after a 12-h exposure of (a) Cr(III) (10 – 300 mg/L), and (b) Cr(VI) (1 – 30 mg/L). The transcript levels of functional genes were normalized against AOB 16S rRNA gene copies. Controls are plotted on the y-axis (i.e. 0 mg/L Cr(III) or Cr(VI)). Note that both *amoA* and *hao* are approaching zero copies at 300 mg/L of Cr(III). Results are the

521 average of two independent experiments for each concentration (experimental duplicates) and
522 error bars show the range of the duplicates.

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Fig. 3. Bacterial community composition of the nitrifying bioreactor based on (a) 16S rRNA and (b) 16S rRNA gene sequencing of cDNA and DNA templates respectively, obtained on multiple dates (n=7).

Table 1. Changes in 16S rRNA-based microbial activity of nitrifying enrichments exposed to chromium.

Bacterial order	Cr(III) (mg/L)				Cr(VI) (mg/L)			
	10	30	100	300	1	3	10	30
Nitrosomonadales	-22.21^a	-28.90	-34.26	-65.86	-0.50	-6.35	-5.13	-10.88
Cytophagales	+10.11	-13.07	-23.06	-22.56	-0.01	+4.90	+3.74	+8.06
Xanthomonadales	+0.92	+8.91	+12.4	-1.51	+0.29	+0.38	+0.28	+0.26
Rhizobiales	+5.51	+12.72	+14.63	+82.00	+0.57	+0.57	+0.71	+2.01

^a The changes in activity were calculated by measuring the relative difference in the percentage of 16S rRNA sequences of major bacterial populations. The sequences were obtained from high-throughput sequencing of 16S rRNA using cDNA (as template) from nitrifying biomass after 12 h exposure in absence (control) and presence of Cr(III) (10 – 300 mg/L) and Cr(VI) (1 – 30 mg/L) respectively. Significant changes (> 5%) are highlighted in bold.