Water Research 102 (2016) 445–452

Contents lists available at ScienceDirect

Water Research

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

Cr(VI) reduction coupled with anaerobic oxidation of methane in a laboratory reactor

Yong-Ze Lu [a], Liang Fu [a], Jing Ding [b], Zhao-Wei Ding [a], Na Li [a], Raymond J. Zeng [a, b, *]

[a] CAS Key Laboratory for Urban Pollutant Conversion, Department of Chemistry, University of Science & Technology of China, Hefei 230026, China
[b] Advanced Laboratory for Environmental Research and Technology, USTC-CityU, Suzhou 215123, China

ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form
13 June 2016
Accepted 30 June 2016
Available online 1 July 2016

Keywords:
Anaerobic oxidation of methane
Chromium reduction
ANME-2d archaea
Coupling process
Microbial analysis
Biogeochemical cycles

ABSTRACT

The process of anaerobic oxidation of methane (AOM) is globally important because of its contribution to the carbon cycle in the environment. Besides, microorganisms play important roles in the environmental fate of chromium. However, there have been no studies to date on the interaction between methane and chromium in batch reactor systems. In this study, biological Cr(VI) reduction was investigated using methane as the sole electron donor. Isotopic $^{13}CH_4$ in the batch experiments and long-term performance in the reactor demonstrated that Cr(VI) reduction is coupled with methane oxidation. High-throughput sequencing of the 16S rRNA genes demonstrated that the microbial community had changed substantially after Cr(VI) reduction. The populations of ANME-2d archaea were enhanced, and they became the only predominant AOM-related microbe. Interestingly, other bacteria with significant increases in abundance were not reported as having the ability to reduce Cr(VI). According to these results, two mechanisms were proposed: 1) Cr(VI) is reduced by ANME-2d alone; 2) Cr(VI) is reduced by unknown Cr(VI)-reducing microbes coupled with ANME-2d. This study revealed the potential relationship between Cr(VI) reduction and CH_4 oxidation, and extended our knowledge of the relationship between the AOM process and biogeochemical cycles.

1. Introduction

Hexavalent chromium (Cr(VI)) is highly toxic to all living organisms and carcinogenic and mutagenic in humans (Kathiravan et al., 2011). It is an important raw material in the metallurgical, electroplating, printing, ceramics, wood preservation and leather manufacturing industries and commonly present in their effluent streams (Testa et al., 2004). Cr(VI) is toxic because of its high solubility and, as a result, it can be readily transported through groundwater (Faybishenko et al., 2008). The reduction of Cr(VI) to Cr(III) is a key process for the removal of Cr(VI) contamination in water and wastewater. When Cr(VI) is reduced to Cr(III), its solubility and mobility decrease (Pan et al., 2014). Chromium is also an environmental background element in soils on a global scale (Adriano, 2001). The two common oxidation states of Cr present in the environment, i.e. Cr(III) and Cr(VI), are interchangeable through oxidation and reduction reactions. Microbial reduction of Cr(VI) is seemingly ubiquitous in both Cr(VI)-contaminated and uncontaminated environments (Fuller et al., 2015; Katsaveli et al., 2012). Many genera of bacteria and archaea, common to different environments, are able to reduce Cr(VI) (He et al., 2015; Kashefi and Lovley, 2000; Miao et al., 2015). Under anaerobic conditions, there are two main chromate-reducing mechanisms: 1) indirect reduction through a non-enzymatic reduction pathway such that the products of microbial metabolism and/or decomposition, such as Fe^{2+} or H_2S, can mediate the reduction of Cr(VI) to Cr(III) (Kamaludeen et al., 2003); 2) direct reduction through an enzymatic reduction pathway (Ackerley et al., 2004).

A variety of organic compounds can be used as the electron donor for Cr(VI) reduction. Bartlett et al. reported that Cr(VI) was reduced by natural organic matter (NOM) in the soil and also humic substances in water (Bartlett, 1991). Similar to NOM, methane is widely distributed in the environment. However, the question is whether methane can serve as an electron donor for Cr(VI) reduction.

Among natural environments, wetland soils are the largest single source of greenhouse gas methane. The anaerobic oxidation

* Corresponding author. CAS Key Laboratory for Urban Pollutant Conversion, Department of Chemistry, University of Science & Technology of China, Hefei 230026, China.
E-mail address: rzeng@ustc.edu.cn (R.J. Zeng).

http://dx.doi.org/10.1016/j.watres.2016.06.065
0043-1354/

of methane (AOM), using electron acceptors such as sulfate, manganese/iron and nitrite/nitrate, has been identified as an important sink for methane in wetland soils (Katrin and Antje, 2009; Raghoebarsing et al., 2006; Siegert et al., 2011). Besides, in natural soils, such as aquatic sediments and soils, generally contain detectable levels of chromium (Richard and Bourg, 1991). Therefore, we can infer the coexistence of methane and chromium in environmental soils, but the relationship between them has seldom been studied. A characterized model methanotroph *Methylococcus capsulatus (Bath)* was reported to be able to reduce Cr(VI) with methane aerobically (Al Hasin et al., 2010). However, the connection between Cr(VI) and methane under anaerobic condition needs to be investigated. There are probable connections between the AOM process and chromium. By comparing the changes in Gibbs free energy (ΔG), it is evident that the process of methane oxidation coupled with Cr(VI) reduction ($\Delta G^{0\prime} = -878.8$ kJ mol^{-1} CH_4; Eq. (1)) is much more thermodynamically favorable than the process of methane oxidation coupled with nitrate reduction ($\Delta G^{0\prime} = -519.8$ kJ mol^{-1} CH_4; Eq. (2)). In theory, the bioreaction involving Cr(VI) (Eq. (1)) must exist, although scientists have not found any evidence for it thus far.

$$CH_4 + 4/3Cr_2O_7^{2-} + 32/3H^+ \rightarrow 8/3Cr^{3+} + CO_2 + 22/3H_2O \quad (\Delta G^{0\prime} = -878.8 \text{ kJ mol}^{-1} CH_4) \quad (1)$$

$$CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^- + 2H_2O \ (\Delta G^{0\prime} = -519.8 \text{ kJ mol}^{-1} CH_4) \quad (2)$$

Experimental evidence for denitrifying anaerobic methane oxidation (DAMO) has been obtained using sludge from bioreactors, in which the DAMO process involves both DAMO archaea and DAMO bacteria (Islas-Lima et al., 2004) or solely DAMO bacteria (Haroon et al., 2013). A follow-up study found that the DAMO archaea belong to the ANME-2d family and are related to the NC10 phylum (Shi et al., 2013). Although the enriched DAMO cultures have been documented and characterized thoroughly, the impact of Cr(VI) on them have never been studied seriously. It has been reported that Cr(VI) intervention was able to affect the denitrification performance and alter the microbial community structure and function in an activated sludge system (Miao et al., 2015). But how the community structure of DAMO organisms shifts under Cr(VI) stress remains unknown.

The main aim of this study was to investigate the effects of Cr(VI) intervention on an AMO process inoculated with DAMO cultures. ^{13}C-labeled CH_4 was used to investigate the possible involvement of methane oxidation in Cr reduction. Meanwhile, high-throughput sequencing, metagenomic analysis and quantitative PCR (qPCR) were used to evaluate the changes in microbial consortia structure after Cr reduction. Finally, a mechanism for Cr(VI) reduction and methane oxidation was proposed. It is anticipated that these findings may extend our knowledge of the AOM process in biogeochemical element cycles.

2. Materials and methods

2.1. Inocula and cultivation conditions

A parent reactor culture containing DAMO archaea and DAMO bacteria was enriched with methanogenic sludge (1.18 mmol dissolved CH_4/L and 0.06 μmol Cr(VI)/L) and activated sludge from a wastewater treatment plant (1.36 μmol dissolved CH_4/L and 0.39 μmol Cr(VI)/L) in a 3-L glass reactor with a working volume of 2 L that had been running for more than 2 years. The temperature was controlled at 35 °C and the pH was controlled between 7.0 and 8.5. Nitrate was added by injection of a concentrated stock solution to maintain the concentration of nitrate between 0.7 and 14 mmol/L. A gas mixture (95% CH_4, 5% CO_2) was used to flush the reactor to provide methane. Each month, the reactor contents were allowed to settle and 500 mL of supernatant were exchanged for fresh medium in which the dissolved oxygen concentration was below the detection limit. After enrichment over a long time period, the consumption rate of NO_3^-–N was stable at around 10.0 mg/L per day.

2.2. Mineral salts medium

The mineral salts medium contained (per L) $KHCO_3$ 0.5 g, KH_2PO_4 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.2265 g, NH_4Cl 0.12 g (not present in the medium of the parent reactor), acidic trace element solution 0.2 mL and alkaline trace element solution 0.5 mL. The acidic trace element solution contained (per L) $FeSO_4 \cdot 7H_2O$ 2.085 g, $ZnSO_4 \cdot 7H_2O$ 0.068 g, $CoCl_2 \cdot 6H_2O$ 0.12 g, $MnCl_2 \cdot 4H_2O$ 0.5 g, $CuSO_4$ 0.32 g, $NiCl_2 \cdot 6H_2O$ 0.095 g, H_3BO_3 0.014 g and HCl 100 mmol. The alkaline trace element contained (per L) $Na_2WO_4 \cdot 2H_2O$ 0.05 g, Na_2MoO_4 0.242 g and NaOH 0.4 g. The medium was sparged with N_2–CO_2 (95:5, v/v) for more than 30 min to obtain anaerobic conditions, and the pH was adjusted to 7.3–7.6 by manual addition of 1 M HCl or 1 M NaOH.

2.3. Isotope tracer experiments

Potential methane oxidation in the experimental vials was evaluated using ^{13}C stable isotope labelling. Two identical 100-mL glass vials with 50-mL working volumes were used: one as the control without CH_4, named the control vial, and the other for CH_4 addition, named the methane vial. Both vials contained 40 mL of N_2-purged mineral salt medium and 10 mL of nitrate-free inocula from the parent reactor. The methane vial was continuously flushed with CH_4–CO_2 (95:5, v/v) and the control vial was continuously flushed with N_2–CO_2 (95:5, v/v). After about 30 min of flushing, the vials were covered with butyl rubber stoppers. Subsequently, Cr(VI) stock solution was injected into each vial, resulting in a final concentration of 0.01 mM Cr in each vial. From the methane vial, 10 mL of headspace gas was removed and replaced with an equal volume of $^{13}CH_4$ (>99%, Sigma Aldrich), resulting in a final concentration of 20% $^{13}CH_4$ (by volume). The vials were stirred at 180 rpm in an incubator shaker, and the temperature was maintained at 35 °C. All experiments were performed in duplicate.

2.4. Long-term Cr(VI) reduction with methane oxidation in the bioreactor

A 450-mL bottle with 300 mL of working volume was used to determine the long-term performance of Cr(VI) reduction. The inocula were obtained from the parent reactor after NO_3^-–N was exhausted (the consumption rate of NO_3^-–N was 10.15 mg/L/day). The inocula were diluted with mineral salts medium to a biomass concentration of approximately 0.11 g volatile suspended solids (VSS)/L. Every 5–10 days, the bioreactor was sparged with CH_4–CO_2 (95:5, v/v) and injected with Cr(VI) stock solution to a final concentration of approximately 10 mg Cr/L. After all the Cr(VI) was reduced, the reactor contents were allowed to settle and 100 mL of the supernatant were removed and replaced with fresh mineral salts medium and sparged with CH_4–CO_2 (95:5 v/v) for 15 min. Cr(VI) was supplied via the Cr(VI) stock solution.

2.5. Valence state analysis of reduced chromium

After reacting with Cr(VI), Cr-loaded microbes were centrifuged at 5000 g for 10 min and collected. They were then washed three

times with deionized-distilled water and dried at 60 °C for 6 h. The valence state of the chromium bound to the microbes was analyzed by X-ray photoelectron spectroscopy (XPS) recorded on a VG ESCALAB MKII X-ray photoelectron spectrometer, using non-monochromatized Mg K-alpha X-rays as the excitation source.

2.6. DNA extraction and quantitative PCR

DNA in the inoculum and Cr(VI) reduction microbes was extracted from 10 mL of liquid using the PowerSoil DNA isolation Kit (MoBio Laboratories) according to the instruction manual (Hu et al., 2014). Quantitative (q) PCR was performed on a Light-Cycler480 Software Setup (Roche) with SybrGreen PCR Master Mix (ABI) to estimate the abundance of DAMO archaea and DAMO bacteria. The sequences of primers used are shown in Table S1. Standard curves were obtained with serial dilutions of plasmid DNA containing the target genes, and copy numbers were calculated based on the threshold cycle values (Fu et al., 2015).

2.7. PCR amplification of 16S rRNA genes and sequencing using Illumina MiSeq PE300

A pair of universal 16S rRNA gene primers (341b4_F – 806_R) reported by Lu et al. (2015) were used to amplify V4 region of the 16S rRNA gene with the barcode. The 50 μL reaction solution consisted of 10 ng of the extracted DNA template, 2.5 U of Platinum® Taq DNA polymerase (Invitrogen, USA), 5 μL of the supplied 10× TAP buffer (Takara, China), 0.5 μL of dNTPs (10 mM) and 0.5 μL of the combined forward and reverse primers. PCR was conducted under the following conditions: 94 °C for 3 min; 5 cycles of 94 °C for 30 s, 45 °C for 20 s and 65 °C for 30 s; 20 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. The PCR products were purified using a SanPrep Column DNA Gel Extraction Kit (Sangon, China) and quantified with a Qubit 2.0 fluorometer (Invitrogen, USA). The amplicons from different samples were mixed in equimolar amounts and sequenced on the Illumina MiSeq platform with the MiSeq Reagent Kit v3 (Illumina, USA). Details of the method of sequencing data analysis were presented in Supporting information. This part of work was conducted by Novogene Institute (Beijing, China).

2.8. Microscopic investigation

Samples for transmission electron microscopy coupled with energy dispersive X-ray spectroscopy (TEM-EDX) were centrifuged at 5000 g. They were chemically fixed and then thin-sectioned at a thickness of 70 nm prior to analysis. The details of sample preparation and TEM-EDX analysis were described in Zhou et al. (2014).

2.9. Chemical analysis

Dissolved methane was measured using the method of Alberto et al. (2000). The methane content in headspace was measured with gas chromatography (FULI, China) (Fu et al., 2015), and the $^{13}CO_2$ produced from $^{13}CH_4$ was measured with gas chromatography-mass spectroscopy (Aglient7890A/5975C, USA) (Ettwig et al., 2009). Soluble Cr(VI) concentrations were measured using the 1,5-diphenylcarbazide method (Urone, 1955). Nitrate was measured with a discrete photometric analyzer Aquakem 200 (Thermo Fisher Scientific, Finland) according to the standard methods (Federation and Association, 2005).

3. Results

3.1. Cr(VI) reduction coupled with methane oxidation

The reducing capacities of Cr(VI) from the incubations under methane and control conditions are shown in Fig. 1. Cr(VI) reduction commenced after inoculation and was depleted at almost 32 days in the methane vials, while the concentrations of Cr(VI) in the control vials remained almost stable. Meanwhile, the isotopic study in the methane vials showed that the ratio of $^{13}CO_2$ to $^{12}CO_2$ increased, indicating that methane oxidation was involved in Cr(VI) reduction.

Cr(VI) reductions in the presence of methane under different medium conditions (control, ferric-free, sulfate-free) are evaluated in Fig. S1. There was little diversity in results among the different medium conditions. These experiments demonstrated that Cr(VI) reduction had nothing to do with ferric or sulfate ions. Meanwhile, the lower potential Gibbs free energy provided by AOM coupled with Cr(VI) (Eq. (1)) compared to that with NO_3^- (Eq. (2)) indicates that AOM occurs more easily with Cr(VI), as is actually proved in Fig. S2. As shown in Fig. S2, even though Cr(VI) or NO_3^- could be reduced independently in the presence of CH_4, only Cr(VI) was reduced when Cr(VI) and NO_3^- co-existed in the system.

3.2. Long-term performance of Cr(VI) reduction

Fig. 2 presents the long-term performance of Cr(VI) reduction in the presence of methane in the reactor. Apparently, Cr(VI) reduction was accompanied by methane oxidation. The 210-day operating period was divided into four parts (marked by segments in Fig. 2) according to the Cr(VI) consumption. Through linear regression analysis, the reduction rate of Cr(VI) was 1.735 μmol day^{-1} in part I, 1.300 μmol day^{-1} in part II and 1.138 μmol day^{-1} in part III. In part IV, the reduction rate gradually dropped and reached a lag phase. The amount of methane consumed corresponded to the amount of Cr(VI) reduced. The consumption rate of methane was 0.565, 0.387 and 0.387 μmol day^{-1} in parts I, II and III, respectively. The methane content continued to drop in part IV and gradually increased at the end (Fig. 2).

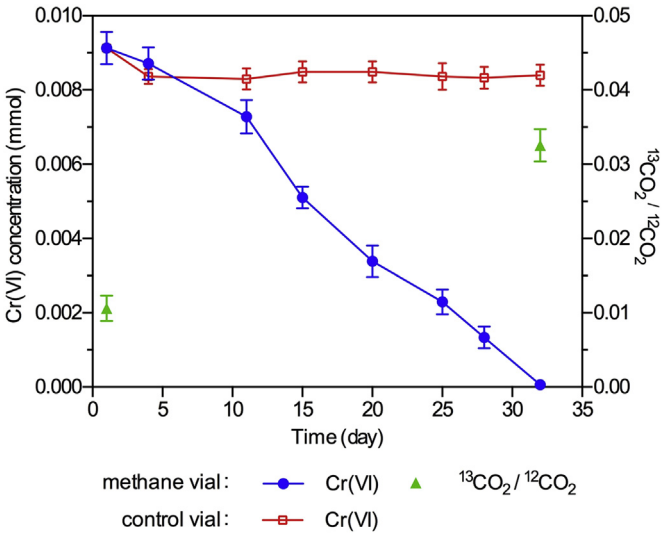

Fig. 1. Cr(VI) reduction combined with methane oxidation in experimental vials.

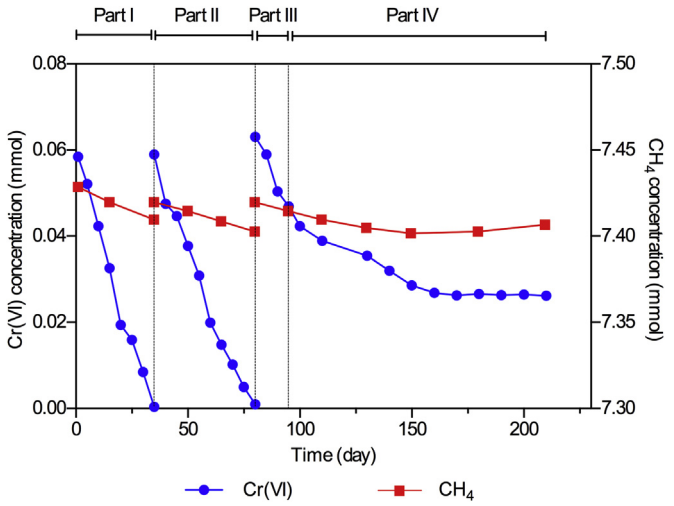

Fig. 2. Long-term performance of Cr(VI) reduction in a reactor.

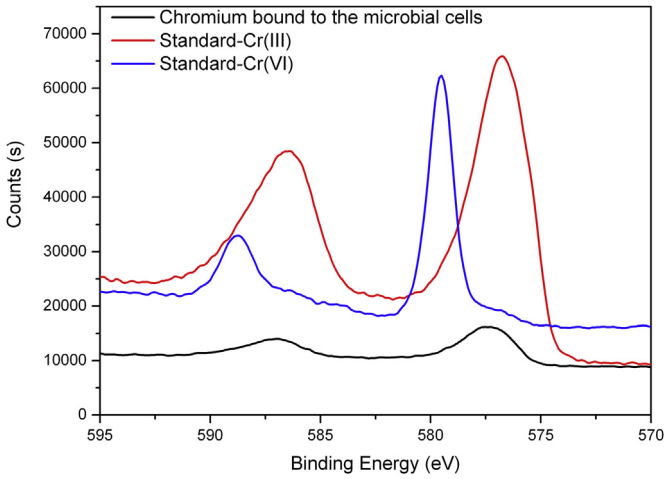

Fig. 4. XPS spectrum of microorganisms in the Cr(VI) reduction reactor.

3.3. Distribution and valence state of chromium in the system

Upon Cr(VI) reduction, no soluble chromium products were detected in the medium except Cr(VI). To investigate the distribution of chromium, conventional thin-section TEM of samples together with EDS was analyzed. As demonstrated in Fig. 3a, if Cr(VI) was not introduced into the system, the microbial cells cut by transverse section were of the elliptical type, and the cell structures could be seen clearly. After Cr(VI) reduction, the microbial cells appeared ill-defined and non-transparent sediment adhered to them, indicating that the microbial cells were greatly affected during the Cr(VI) reduction and immobilization processes (Fig. 3b). EDS analysis of the material in the red circle in Fig. 3b showed that the external surfaces of the microbial cell membranes contained bound chromium due to bioaccumulation. The amount of chromium inside the microbial cells was less than that external to the cells and no obvious precipitates had formed inside the cells. The white areas in both Fig. 3a and b were resin caused by ultrathin sectioning.

To investigate the possible valence state of chromium bound to the microbial cells after Cr(VI) reduction, XPS analysis was conducted. Fig. 4 shows the XPS results for the microbial cells in the reactor. The Cr-loaded microbial cells gave two distinct peaks: 585.0–588.0 eV (Cr $2p_{1/2}$) and 576.0–578.0 eV (Cr $2p_{3/2}$). The spectrum of the microbial cells indicated that chromium bound to them was mostly in the Cr(III) oxidation state, which suggests the complete reduction of chromium to Cr(III) on the surface of the cells with no absorption of Cr(VI). The reduction of Cr(VI) and its immobilization in the form of Cr(III) on the cells has been demonstrated in both pure cultures and mixed species of activated sludge (Mary Mangaiyarkarasi et al., 2011; Wang et al., 2013).

3.4. Microbial community changes induced by Cr(VI) reduction

In order to investigate the structure of the microbial community and its shifts, the 16S rRNA genes of both the inoculum and reactor culture were subjected to high-throughput sequencing on the Illumina Miseq platform. The results showed that biodiversity tended to increase slightly after Cr(VI) reduction in the reactor, as the Shannon indices increased from 3.81 to 4.06 (Table 1). This result was also confirmed by the rarefaction curves (Fig. S3). A total of 20,650 raw sequences and 18,805 effective sequences of average length 416 bp were obtained from the inoculum and sample from the Cr(VI) reduction reactor, respectively (Table 1). The effective sequences represented 97% of the microbes in the inoculum and

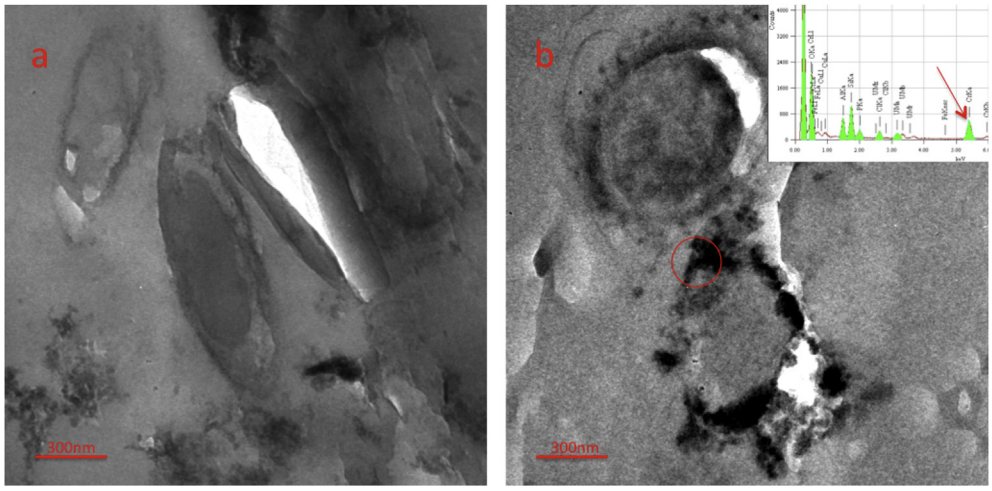

Fig. 3. Microscopic investigations of TEM of the inoculum (a) and microorganisms in the Cr(VI) reduction reactor (b). Insert: TEM-EDS of red circular portion.

Table 1
Summary of Illumina Miseq high-throughput sequencing of 16S rRNA genes.

Sample	Raw tags	Effective tags	Mean length	OTU number	Shannon	ACE	Chao1	Coverage
Inoculum	9910	9571	419 bp	568	3.81	2051	1276	0.97
Cr(VI) reduction	10,740	9234	413 bp	714	4.06	2734	1667	0.96

96% of the microbes in the reactor culture after Cr(VI) reduction, respectively. The OTU number of 714 for the reactor culture was a little higher than that of 568 for the inoculum. ACE and Chao1 values of the reactor culture were also higher than those of the inoculum except for the Alpha diversity indexes of Shannon.

The distributions of bacteria and archaea at the phylum level are shown in Fig. 5a. In the inoculum, bacteria and archaea accounted for 94.8% and 5.2% of the microbes, respectively. The dominant phyla included Chlorobi, Proteobacteria, NC10, Chloroflexi and GN04 with relative abundances of 40.3%, 10.4%, 10.3, 8.7% and 6.5%, respectively. Compared with the reactor culture, the bacterial abundance was lower at 82.5% and the archaea abundance was higher at 17.5% in the inoculum. The dominant phyla altered to Proteobacteria, Euryarchaeota, Planctomycetes, Armatimonadetes and Chloroflexi with abundances of 43.5%, 17.5%, 12.8%, 8.8% and 4.7%, respectively, in the reactor culture. The relative abundances of Chlorobi, NC10 and GN04 decreased to 4.0%, 0.3% and 2.8%, respectively.

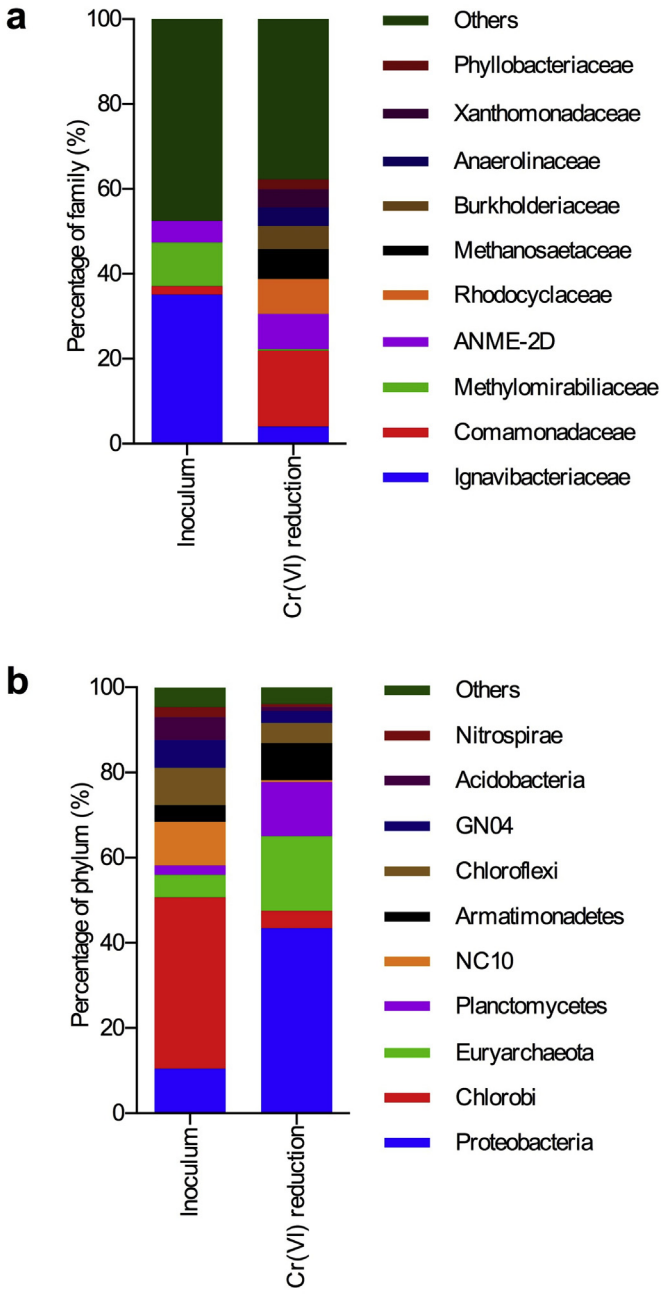

Fig. 5. Relative abundances of microorganisms in the inoculum and Cr(VI) reduction reactor. a, Family; b, Phylum.

On the family level, the dominant microorganisms in the inoculum included 'Others', Ignavibacteriaceae, Methylomirabiliaceae, ANME-2d and Comamonadaceae with relative abundances of 47.5%, 35.1%, 10.3%, 5.1% and 2.0%, respectively (Fig. 5b). Compared with the inoculum, the percentages of Others, Comamonadaceae, ANME-2d, Rhodocyclaceae and Methanosaetaceae in the reactor culture were 37.7%, 17.9%, 8.4%, 8.1% and 7.1%, respectively, while Ignavibacteriaceae and Methylomirabiliaceae decreased to 4.0% and 0.3%, respectively.

In this study, the NC10 phylum and ANME-2d family were the only potential AOM microorganisms detectable in the inoculum and reactor culture. All of the sequences in the NC10 phylum were affiliated with DAMO bacteria of the genus *Candidatus Methylomirabilis*. All of the sequences in the ANME-2d family were unclassified and grouped into the same 714 OTUs. These OTUs were similar to those reported for DAMO archaea with identity of >98%. NC10 abundance decreased substantially from 10.3% to 0.3% in response to Cr(VI) reduction, while ANME-2d growth was stimulated (abundance changed from 5.1% to 8.4%) slightly. This variation was also confirmed by quantifying their 16S ribosomal RNA (rRNA) genes. The copy number of the DAMO bacterial 16S rRNA gene was 1.51×10^6 copies/L in the inoculum and decreased sharply to 1.39×10^4 copies/L in the reactor culture. In contrast, the copy number of the DAMO archaeal 16S rRNA gene was 1.36×10^4 copies/L in the inoculum and increased slightly to 1.56×10^4 copies/L in the reactor culture (Table 2).

Different from the relative abundances of AOM microorganisms, the content of reported Cr(VI) reduction microorganisms was low (Table S2). The *Pseudomonas* genus belonging to the Pseudomonadaceae family and the Enterobacteriales order belonging to the Proteobacteria phylum have a tendency to reduce Cr(VI). However, the abundances of microbes of the *Pseudomonas* genus and Enterobacteriales order in the reactor culture were only 0.4% and 0.6%, respectively.

4. Discussion

Although increasing interest has been paid to AOM processes recently (Segarra et al., 2015), so far little attention has been focused on the potential association between Cr(VI) reduction and methane oxidation. In this study, an enriched culture of an AOM process based on environmental sediment was used to confirm that Cr(VI) reduction was coupled to methane oxidation. Nevertheless, the Cr(VI) reducing ability during the AOM process was obviously weaker than the activity of most types of microbes that are capable of Cr(VI) remediation (Yan et al., 2014). *Methylococcus*

Table 2
Abundances of DAMO archaea (16S rRNA genes) and DAMO bacteria (16S rRNA genes) estimated from qPCR assays for microorganisms in the inoculum and Cr(VI) reduction reactor.

		DAMO archaea	DAMO bacteria
Abundance (copies/L)	Inoculum	1.36×10^4	1.51×10^6
	Cr(VI) reduction	1.56×10^4	1.39×10^4
Percentage change (%)		+14.71	−99.08

capsulatus (Bath), which is a model methanotroph in the environment, also demonstrated a low reducing rate when bioremediating Cr(VI) with methane aerobically (Al Hasin et al., 2010). That might have been because of the low solubility of methane as the sole carbon and electron donor. On the other hand, microbial communities obtained from environmental samples generally present relatively low Cr(VI) reduction rates (Martins et al., 2010). Moreover, in natural soil, Cr(VI) reduction may be extremely slow, requiring years (Bartlett, 1991). The mole ratio between Cr(VI) reduction and methane oxidation was calculated as shown in Table 3. The measured ratio was about 0.326 in part I and increased to 0.352 after deducting the background according to the control vial data of Fig. 1. This measured ratio was comparable to the theoretical value of 0.375 (Eq. (1)). It indicates the existence of AOM-related Cr(VI) reduction. The Cr(VI) reduction rate decreased from part I to part III until it ceased in part IV. More than the toxicity of Cr(VI), the growth rate of the AOM consortium was so low that the Cr(III) continuously accumulating around them would easily cause a negative impact on them (Caldwell et al., 2008). What was worse, some extracellular components, such as peptidoglycan, present in the cell walls of the bacteria, were reported to be potent binder of Cr(III) (Thatoi et al., 2014). The binding Cr(III) was hard to be removed from the cells. Besides, the methane oxidation rate also decreased from part I to part III until the increase in methane content in part IV. Meanwhile, methanogens such as Methanosaeta increased substantially (from 0 to 7.1%); this indicates that there is methane production via methanogenesis (Mori et al., 2012). Microbes that could not adapt to the toxic effects would have been degraded and released some dissolved organic matter (DOM). The released DOM was used for methane production via methanogenesis (Kampas et al., 2007). Although both the methane oxidation rate and the Cr(VI) reduction rate decreased from part I to part III, the methane oxidation rate decreased more than the Cr(VI) reduction rate because of methanogenesis. That is why the equivalence ratios in part II and part III were smaller than in part I.

This study also showed that Cr(VI) addition altered the microbial community structure, which agrees with Miao et al. (2015). The variation in microbes that are capable of methane oxidation was remarkable. Within archaea, the unclassified genus belonging to the ANME-2d family that was detected in both the inoculum and the Cr(VI) reduction reactor appeared to out-compete the others after Cr(VI) reduction. The ANME-2d family reportedly contains microbes that are capable of methane oxidation (Haroon et al., 2013), and their dominance in the Cr(VI) reduction reactor corresponded to the coupling of methane oxidation with Cr(VI) reduction. Moreover, AOM coupled to iron, manganese, sulfate and nitrate reduction has been demonstrated in consortia containing anaerobic methanotrophic (ANME) archaea (Beal et al., 2009; Boetius et al., 2000; Haroon et al., 2013). Most of the previous microbiological investigations of AOM involved a high biomass of ANME (Lloyd et al., 2006). The various oxidizing materials coupled with AOM and the presence of a mosaic of ANME phylotypes demonstrate their generality in different methane oxidation fields. That might explain why ANME-2d plays a large role in the Cr(VI)-AOM process. Although it is another microorganism in the inoculum that is related to methane oxidation, the population of the NC10 phylum declined dramatically in the Cr(VI) reduction reactor. This indicated that NC10 was negligibly involved in the Cr(VI) reduction process. NC10 was previously shown to be involved in a unique anaerobic methane oxidation pathway called the “Intra-Aerobic” (Ettwig et al., 2010). O_2 was produced intracellularly and used as a co-substrate for methane mono-oxygenation, which means that NC10 can only carry out methane oxidation in the presence of substances that can produce O_2 by dismutation, such as NO_2^- and ClO_4^- (Luo et al., 2015). However, the Cr(VI) reduction pathway was never reported to involve dismutation to produce O_2. That is why NC10 microbes do not survive during the Cr(VI)-AOM process. Beside the methane oxidation-related microbes, the populations of Cr(VI) reduction-related microbes also changed. The *Pseudomonas* genus (which increased from 0 to 0.4%) and the Enterobacteriales order (which stabilized around 0.6%) that were detected in the Cr(VI) reduction reactor were reported previously to have a tendency to reduce Cr(VI) (Katsaveli et al., 2012; Xu et al., 2009). However, in this study their abundances were too low to play a major role in Cr(VI) reduction in the reactor. Interestingly, the microbial communities that had substantially increased included the Comamonadaceae family (from 0.2% to 16.0%), Phycisphaerae class (from 1.7% to 11.3%) and *Azospira* genus (from 0 to 8.1%), which have never been reported to be Cr(VI)-resistant and/or Cr(VI)-reducing strains. Consequently, there may be unreported species among these enhanced microbial communities that are capable of Cr(VI) reduction, especially ones present at low concentration in the inoculum.

With no involvement of ferric or sulfate reduction (Figs. S1 & S2), and based on the existing knowledge of the AOM process and ANME-2d, two mechanisms are proposed for the coupling process between methane oxidation and Cr reduction. First, Cr(VI) is reduced by ANME-2d alone (Fig. 6a), and CH_4 is oxidized by

Table 3
The mole ratios of methane oxidation to Cr(VI) reduction based on theoretical calculations and practical measurements.

	Theoretical value	Measured value		
		Part I	Part II	Part III
Methane oxidation rate (μmol day^{-1})		0.565	0.387	0.339
Cr(VI) reduction rate (μmol day^{-1})		1.735 (1.604*)	1.300	1.138
Methane oxidation/Cr(VI) reduction	0.375	0.326 (0.352*)	0.298	0.298

Note: “*” indicates the value after deducting the background according to the control vial of Fig. 1.

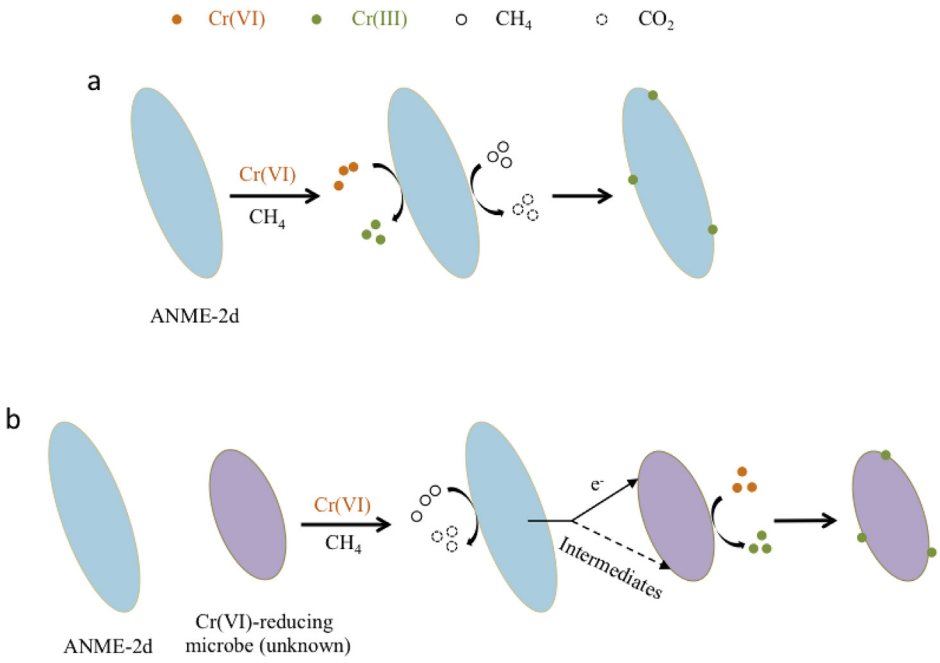

Fig. 6. Proposed mechanism of Cr(VI) reduction coupled with CH_4 oxidation.

ANME-2d to generate the electrons for reducing Cr(VI). The reduced Cr(III) is bound to ANME-2d. Haroon et al. reported that ANME-2d was capable of independent AOM through reverse methanogenesis using nitrate as the terminal electron acceptor (Haroon et al., 2013). Therefore, Cr(VI) may be reduced by ANME-2d through the same pathway as nitrate. Second, Cr(VI) is reduced by an unknown Cr(VI)-reducing microbe coupled with ANME-2d (Fig. 6b). CH_4 is oxidized by ANME-2d to generate an intermediate or electron, which is then utilized by an unknown Cr(VI)-reducing microbe to reduce Cr(VI). The reduced Cr(III) is bound to the Cr(VI)-reducing microbe. The reported archaeal groups responsible for AOM (ANME-1, ANME-2 and ANME-3) commonly have sulfate/manganese/iron-reducing bacterial partners (Beal et al., 2009). Thus, ANME-2d is also probably associated with an unknown microbe during Cr(VI) reduction. Although there was no clear identification of genera that belong to the ANME-2d family or other specific genera responsible for Cr(VI) reduction, as the communities remained relatively diverse, future identification of specific bacterial species could help improve our understanding of the Cr(VI) reduction mechanism combined with methane oxidation.

According to estimations, the AOM process consumes 5–20% of total global methane flux (Valentine and Reeburgh, 2000) and is an important part of the global carbon cycle today. Among the AOM processes, electron acceptors found so far include sulfate, manganese, iron and nitrate (nitrite), and all of the AOM processes have been linked to ANME (Beal et al., 2009; Boetius et al., 2000; Raghoebarsing et al., 2006). Due to the widespread use of chromium, large quantities of Cr compounds are discharged into the environment and can ultimately have significant biological and ecological effects (Kotaś and Stasicka, 2000). But the connection between the AOM process and chromium in the environment has never before been examined. Our study confirmed that the AOM process occurs when coupled with Cr(VI) reduction, which supports the idea that AOM is directly linked to metal reduction. Moreover, ANEM-2d have been shown to play a leading role in methane oxidation when coupled with Cr(VI) reduction, which enhances the reducing capacity of ANME. Despite the slow reduction rate of Cr(VI) coupled with AOM, it is likely an important part of biogeochemical methane cycling.

5. Conclusions

An enriched AOM culture was used to study the potential relationship between methane oxidation and Cr(VI) reduction. Based on the results of this study, the following conclusions were drawn:

- Cr(VI) was reduced with enriched AOM culture, the control experiment with N_2 and $^{13}CH_4$ analysis revealed that AOM was involved in Cr(VI) reduction.
- Microbial community changed significantly after several cycles of Cr(VI) reduction. The populations of ANME-2d archaea became the only predominant AOM-related microbe with their both numbers and proportions rising. However, other obviously increased bacteria were not reported as having the ability to reduce Cr(VI).
- Two possible mechanisms were proposed according to the results we found: 1) Cr(VI) is reduced by ANME-2d alone; 2) Cr(VI) is reduced by unknown Cr(VI)-reducing microbes coupled with ANME-2d.

Acknowledgments

The authors would like to acknowledge the financial support of the National Natural Science Foundation of China (51178444), the National Hi-Technology Development 863 Program of China (2011AA060901), the Hundred-Talent Program of Chinese Academy of Science, the Collaborative Innovation Center of Suzhou Nano Science and Technology, the Program for Changjiang Scholars and Innovative Research Team in University, and the Fundamental Research Funds for the Central Universities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.06.065.

References

Ackerley, D.F., Gonzalez, C.F., Park, C.H., Blake, R., Keyhan, M., Matin, A., 2004. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli*. Appl. Environ. Microbiol. 70 (2), 873–882.

Adriano, D., 2001. Trace Elements in Terrestrial Environments. Springer, New York, pp. 1–27.

Al Hasin, A., Gurman, S.J., Murphy, L.M., Perry, A., Smith, T.J., Gardiner, P.H.E., 2010. Remediation of chromium(VI) by a methane-oxidizing bacterium. Environ. Sci. Technol. 44 (1), 400–405.

Alberto, M.C.R., Arah, J.R.M., Neue, H.U., Wassmann, R., Lantin, R.S., Aduna, J.B., Bronson, K.F., 2000. A sampling technique for the determination of dissolved methane in soil solution. Chemosphere Glob. Change Sci. 2 (1), 57–63.

Bartlett, R.J., 1991. Chromium cycling in soils and water: links, gaps, and methods. Environ. Health Perspect. 92, 17–24.

Beal, E.J., House, C.H., Orphan, V.J., 2009. Manganese- and iron-dependent marine methane oxidation. Science 325 (5937), 184–187.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jorgensen, B.B., Witte, U., Pfannkuche, O., 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407 (6804), 623–626.

Caldwell, S.L., Laidler, J.R., Brewer, E.A., Eberly, J.O., Sandborgh, S.C., Colwell, F.S., 2008. Anaerobic oxidation of methane: mechanisms, bioenergetics, and the ecology of associated microorganisms. Environ. Sci. Technol. 42 (18), 6791–6799.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H., van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., den Camp, H., Janssen-Megens, E.M., Francoijs, K.J., Stunnenberg, H., Weissenbach, J., Jetten, M.S.M., Strous, M., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464 (7288), 543.

Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S.M., Strous, M., 2009. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl. Environ. Microbiol. 75 (11), 3656–3662.

Faybishenko, B., Hazen, T.C., Long, P.E., Brodie, E.L., Conrad, M.E., Hubbard, S.S., Christensen, J.N., Joyner, D., Borglin, S.E., Chakraborty, R., Williams, K.H., Peterson, J.E., Chen, J., Brown, S.T., Tokunaga, T.K., Wan, J., Firestone, M., Newcomer, D.R., Resch, C.T., Cantrell, K.J., Willett, A., Koenigsberg, S., 2008. Situ long-term reductive bioimmobilization of Cr(VI) in groundwater using hydrogen release compound. Environ. Sci. Technol. 42 (22), 8478–8485.

Federation, W.E., Association, A.P.H., 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association (APHA), Washington, DC, USA.

Fu, L., Ding, Z.-W., Ding, J., Zhang, F., Zeng, R., 2015. The role of paraffin oil on the interaction between denitrifying anaerobic methane oxidation and anammox processes. Appl. Microbiol. Biotechnol. 1–12.

Fuller, S., Burke, I., McMillan, D.G., Ding, W., Stewart, D., 2015. Population changes in a community of alkaliphilic iron-reducing bacteria due to changes in the electron acceptor: implications for bioremediation at alkaline Cr(VI)-contaminated sites. Water Air Soil Pollut. 226 (6), 1–15.

Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z., Tyson, G.W., 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500 (7464), 567–570.

He, D., Zheng, M., Ma, T., Li, C., Ni, J., 2015. Interaction of Cr(VI) reduction and denitrification by strain Pseudomonas aeruginosa PCN-2 under aerobic conditions. Bioresour. Technol. 185 (0), 346–352.

Hu, B., He, Z., Geng, S., Cai, C., Lou, L., Zheng, P., Xu, X., 2014. Cultivation of nitrite-dependent anaerobic methane-oxidizing bacteria: impact of reactor configuration. Appl. Microbiol. Biotechnol. 98 (18), 7983–7991.

Islas-Lima, S., Thalasso, F., Gómez-Hernandez, J., 2004. Evidence of anoxic methane oxidation coupled to denitrification. Water Res. 38 (1), 13–16.

Kamaludeen, S.B., Megharaj, M., Juhasz, A., Sethunathan, N., Naidu, R., 2003. In: Ware, G. (Ed.), Reviews of Environmental Contamination and Toxicology. Springer, New York, pp. 93–164.

Kampas, P., Parsons, S.A., Pearce, P., Ledoux, S., Vale, P., Churchley, J., Cartmell, E., 2007. Mechanical sludge disintegration for the production of carbon source for biological nutrient removal. Water Res. 41 (8), 1734–1742.

Kashefi, K., Lovley, D.R., 2000. Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by *Pyrobaculum islandicum*. Appl. Environ. Microbiol. 66 (3), 1050–1056.

Kathiravan, M.N., Karthick, R., Muthukumar, K., 2011. Ex situ bioremediation of Cr(VI) contaminated soil by Bacillus sp.: batch and continuous studies. Chem. Eng. J. 169 (1–3), 107–115.

Katrin, K., Antje, B., 2009. Anaerobic oxidation of methane: progress with an unknown process. Annu. Rev. Microbiol. 63 (1), 311–334.

Katsaveli, K., Vayenas, D., Tsiamis, G., Bourtzis, K., 2012. Bacterial diversity in Cr(VI) and Cr(III)-contaminated industrial wastewaters. Extremophiles 16 (2), 285–296.

Kotaś, J., Stasicka, Z., 2000. Chromium occurrence in the environment and methods of its speciation. Environ. Pollut. 107 (3), 263–283.

Lloyd, K.G., Lapham, L., Teske, A., 2006. An anaerobic methane-oxidizing community of ANME-1b archaea in hypersaline Gulf of Mexico sediments. Appl. Environ. Microbiol. 72 (11), 7218–7230.

Lu, Y.-Z., Ding, Z.-W., Ding, J., Fu, L., Zeng, R.J., 2015. Design and evaluation of universal 16S rRNA gene primers for high-throughput sequencing to simultaneously detect DAMO microbes and anammox bacteria. Water Res. 87, 385–394.

Luo, Y.-H., Chen, R., Wen, L.-L., Meng, F., Zhang, Y., Lai, C.-Y., Rittmann, B.E., Zhao, H.-P., Zheng, P., 2015. Complete perchlorate reduction using methane as the sole electron donor and carbon source. Environ. Sci. Technol. 49 (4), 2341–2349.

Martins, M., Faleiro, M.L., Chaves, S., Tenreiro, R., Santos, E., Costa, M.C., 2010. Anaerobic bio-removal of uranium (VI) and chromium (VI): comparison of microbial community structure. J. Hazard. Mater. 176 (1–3), 1065–1072.

Mary Mangaiyarkarasi, M.S., Vincent, S., Janarthanan, S., Subba Rao, T., Tata, B.V.R., 2011. Bioreduction of Cr(VI) by alkaliphilic *Bacillus subtilis* and interaction of the membrane groups. Saudi J. Biol. Sci. 18 (2), 157–167.

Miao, Y., Liao, R., Zhang, X.-X., Wang, Y., Wang, Z., Shi, P., Liu, B., Li, A., 2015. Metagenomic insights into Cr(VI) effect on microbial communities and functional genes of an expanded granular sludge bed reactor treating high-nitrate wastewater. Water Res. 76 (0), 43–52.

Mori, K., Iino, T., Suzuki, K.-I., Yamaguchi, K., Kamagata, Y., 2012. Aceticlastic and NaCl-requiring methanogen "*Methanosaeta pelagica*" sp. nov., isolated from marine tidal flat sediment. Appl. Environ. Microbiol. 78 (9), 3416–3423.

Pan, X., Liu, Z., Chen, Z., Cheng, Y., Pan, D., Shao, J., Lin, Z., Guan, X., 2014. Investigation of Cr(VI) reduction and Cr(III) immobilization mechanism by planktonic cells and biofilms of *Bacillus subtilis* ATCC-6633. Water Res. 55 (0), 21–29.

Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Damste, J.S.S., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440 (7086), 918–921.

Richard, F.C., Bourg, A.C.M., 1991. Aqueous geochemistry of chromium: a review. Water Res. 25 (7), 807–816.

Segarra, K.E.A., Schubotz, F., Samarkin, V., Yoshinaga, M.Y., Hinrichs, K.U., Joye, S.B., 2015. High rates of anaerobic methane oxidation in freshwater wetlands reduce potential atmospheric methane emissions. Nat. Commun. 6.

Shi, Y., Hu, S., Lou, J., Lu, P., Keller, J., Yuan, Z., 2013. Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor. Environ. Sci. Technol. 47 (20), 11577–11583.

Siegert, M., Krüger, M., Teichert, B., Wiedicke, M., Schippers, A., 2011. Anaerobic oxidation of methane at a marine methane seep in a forearc sediment basin off Sumatra, Indian Ocean. Front. Microbiol. 2, 249.

Testa, S.M., Guertin, J., Jacobs, J.A., Avakian, C.P., 2004. Sources of Chromium Contamination in Soil and Groundwater. CRC Press, Boca Raton, FL.

Thatoi, H., Das, S., Mishra, J., Rath, B.P., Das, N., 2014. Bacterial chromate reductase, a potential enzyme for bioremediation of hexavalent chromium: a review. J. Environ. Manag. 146, 383–399.

Urone, P.F., 1955. Stability of colorimetric reagent for chromium, s-diphenylcarbazide, in various solvents. Anal. Chem. 27 (8), 1354–1355.

Valentine, D.L., Reeburgh, W.S., 2000. New perspectives on anaerobic methane oxidation. Environ. Microbiol. 2 (5), 477–484.

Wang, Y.M., Sevinc, P.C., Belchik, S.M., Fredrickson, J., Shi, L., Lu, H.P., 2013. Single-cell imaging and spectroscopic analyses of Cr(VI) reduction on the surface of bacterial cells. Langmuir 29 (3), 950–956.

Xu, W.-h., Liu, Y.-g., Zeng, G.-m., Li, X., Song, H.-x., Peng, Q.-q., 2009. Characterization of Cr(VI) resistance and reduction by *Pseudomonas aeruginosa*. Trans. Nonferrous Metals Soc. China 19 (5), 1336–1341.

Yan, F.-F., He, Y.-R., Wu, C., Cheng, Y.-Y., Li, W.-W., Yu, H.-Q., 2014. Carbon nanotubes alter the electron flow route and enhance nitrobenzene reduction by *Shewanella oneidensis* MR-1. Environ. Sci. Technol. Lett. 1 (1), 128–132.

Zhou, C., Ontiveros-Valencia, A., Cornette de Saint Cyr, L., Zevin, A.S., Carey, S.E., Krajmalnik-Brown, R., Rittmann, B.E., 2014. Uranium removal and microbial community in a H2-based membrane biofilm reactor. Water Res. 64 (0), 255–264.