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Comparison of chromium III and VI toxicities in water using sulfur-oxidizing bacterial bioassays

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HIGHLIGHTS

- Toxicities of Cr III and Cr VI were compared on sulfur oxidizing bacterial bioassays in fed-batch and batch tests.
- Cr III was not toxic even at higher concentrations while Cr VI was toxic to SOB at lower concentrations.
- Cr (III) was non toxic due to the structural similarity with Fe^{3+} and absence of iron uptake pathway.
- The toxicity of Cr (VI) was mainly due to its structural similarity with SO_4^{2-}.
- An EC_{50} of 2.7 mg/L and 1.5 mg/L were measured for fed-batch and batch SOB tests.

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ABSTRACT

The toxicities of Cr (III) and Cr (VI) in water were evaluated using sulfur-oxidizing bacterial (SOB) bioassays both in batch and fed-batch conditions. Two days were enough for a quick buildup of SOB consortium in the master culture reactor (MCR). At concentrations up to 100 mg L^{-1}, Cr (III) was found to be nontoxic in both conditions, while Cr (VI) at very low concentrations (0.1–2 mg L^{-1}) was very toxic to the SOB. Literature review suggested that the nontoxic nature of Cr (III) might be due to the absence of the iron uptake pathway in *Acidithiobacillus caldus* (the predominant bacteria in our reactors), which is required for Cr (III) uptake. The 2-h median effective concentration (EC_{50}) values obtained for Cr (VI) in the batch and fed-batch tests were 2.7 mg L^{-1} and 1.5 mg L^{-1}, respectively.

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1. Introduction

Chromium (Cr) is among the first transitional metal series of the periodic table and occurs in oxidation states ranging from −2 to +6. In the environment, chromium valence states can be found as the 0 (elemental metal), +2 (divalent), +3 (trivalent), and +6 (hexavalent) forms (EPA, 1998). Divalent chromium forms unstable compounds, and it is readily oxidized to the trivalent form. Only trivalent and hexavalent chromium are important for human health (Dayan and Paine, 2001). Mostly, the chemistry of trivalent chromium is dominated by different forms of stable complexes with inorganic and organic ligands, whereas hexavalent chromium forms potent oxidizing oxides such as CrO_3 and CrO_4^{2-} (EPA, 1998).

Several methods have been employed for the measurement of heavy metals using high-performance liquid chromatography, atomic absorption spectroscopy, gas chromatography, or bioassays (van Wezel et al., 2010). Chemical methods are quantitative and sophisticated, but they require high expertise. On the other hand, bioassays monitor changes in microbial activities (Farré and Barceló, 2003) such as photosynthesis (Ahmed and Häder, 2010), growth rate (Eilersen et al., 2004), respiration (Del Campo et al., 2007), and bioluminescence (Hassan and Oh, 2010a). Many researchers have reported the use of microbes for bioassays, such as *Vibrio fischeri* (Tencaliec et al., 2006), *Photobacterium phosphorium* (Hassan and Oh, 2010b), *Pseudomonas aeruginosa* (Cho et al., 2004), algae (Moreira-Santos et al., 2004), and higher organisms like Daphnia (Hernando et al., 2005), earthworms, and fish (Mishra et al., 2000).

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Recently, sulfur-oxidizing bacteria (SOB) have been used repeatedly for toxic chemical detection (Oh et al., 2011). SOB are chemoautotrophic bacteria that consume reduced sulfur compounds using molecular oxygen (O_2) and were first identified by Sergey Winogradsky in 1885. SOB of the genus *Acidithiobacillus* utilize elemental sulfur (S^o) as the electron donor, as indicated by the following equation (Hassan et al., 2010):

$$S^\circ + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+, \quad \Delta G^\circ = -587 \text{ kJ/reaction} \tag{1}$$

SOB oxidize S° in the presence of O_2 and produce sulfate with two protons (H^+), which is a characteristic reaction resulting in a decreased pH and an increased electrical conductivity (EC). These changes in pH and EC can be detected easily by commonly available pH and EC meters (Oh et al., 2011).

The introduction of toxic chemicals into SOB reactors results in a reduction of EC that depends on the concentration of the toxic chemical (Hassan et al., 2013). In this study, fed-batch and batch SOB biosensors were developed for comparing the effects of Cr (III) and Cr (VI) on SOB for the assessment of water. Various concentrations of Cr (III) and Cr (VI) were tested, and the median effective concentration (EC_{50}) values were compared.

2. Materials and methods

2.1. Sulfur master culture reactor (MCR)

An aerobic MCR was operated with a total volume of 1 L to provide a consistent supply of sulfur and sulfur-oxidizing microorganisms. The MCR was packed with granular sulfur particles (750 g) of 2.0–4.0 mm in diameter. Return aerobic sludge was used to inoculate the MCR. Tap water diffused with air was held in a holding tank (100 L) for at least 1 day to remove residual chlorine. Granular activated carbon was also added to the tap water (100 mg L^{-1}) to remove chlorine and chloramine. Selected characteristics of the tap water used are summarized in Table 1. Air was introduced into the MCR using stone diffusers, with a total flow rate of 2.6 L/min. The MCR was operated in fed-batch mode at 40 ± 1 °C. The reactor was fed with tap water for 10 min (25 mL/min), and the batch reaction time was 1 h 50 min. An increase in the EC indicated SOB growth. The MCR became active within a few hours of inoculation and reached stable conditions within 2 days before use in the batch experiments (Fig. 1).

2.2. SOB reactor construction and operation in fed-batch mode

The SOB reactors were constructed as described elsewhere (Oh et al., 2011). The schematic diagram for the fed-batch acrylic reactor is illustrated in Fig. 2 A. The reactor has two chambers: a reaction chamber (H: 9.2 cm, W: 3.5 cm, L: 3.5 cm) and a measurement chamber (H: 5.2 cm, W: 2.5 cm, L: 2.5 cm). The total volume of the reactor is 145 mL (112 mL + 33 mL), whereas the working volume is 81 mL.

Each SOB reactor was packed with 50 mL (82 g) of sulfur particles (2–4 mm) taken from the MCR. Air was provided at a flow rate of 1.5 L/min at the bottom of the reactor. The fed-batch reactor was fed with tap water using a peristaltic pump (pp150D, Techno Poong Lim Co.) equipped with a pump head (77200-62, Masterflex Easyload II). Rapid tap water feeding was done for 30 s, and then the reactor was set for the 30-min reaction. At each feeding, 35 mL of water was supplied into the reactor. Upon reaching stable conditions, the reactor was exposed to Cr (VI) (0, 0.1, 0.5, 1.0, and 2.0 mg L^{-1}) or Cr (III) (0, 2.0, 10.0, 50.0, and 100.0 mg L^{-1}). The reactor, air pumps, and influent container were all kept at 45 °C.

Table 1
Characteristics of the tap water used in this study (mean and standard deviation, calculated from three replicates).

Parameter	Mean ± SD
pH	7.06 ± 0.04
Conductivity (μS/cm)	57.4 ± 3.8
Alkalinity (mg L^{-1} as $CaCO_3$)	17.5 ± 0.0
Cl^- (mg L^{-1})	6.2 ± 0.5
NO_2^--N (mg L^{-1})	*ND
NO_3^--N (mg L^{-1})	1.7 ± 0.2
PO_4^{2-} (mg L^{-1})	*ND
SO_4^{2-} (mg L^{-1})	9.5 ± 0.7
Cr (VI)	*ND
Cr (III)	*ND
Fe^{2+}	*ND
Fe^{3+}	*ND

*ND, not detected.

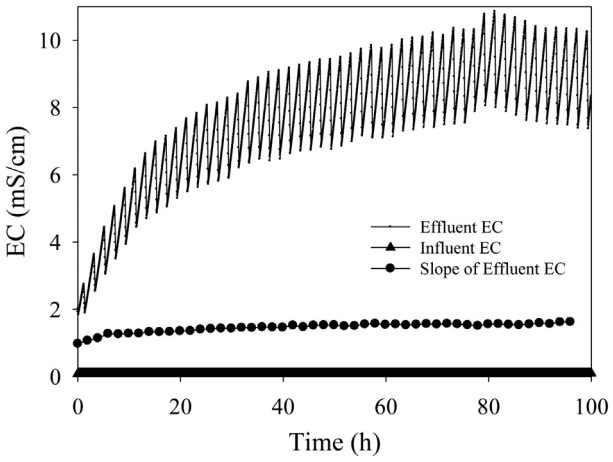

Fig. 1. MCR consortium buildup to obtain a stable EC.

2.3. Batch experiments

For the batch experiments, a 7-chamber reactor was constructed using acrylic (Fig. 2 B). Each chamber (H: 9 cm, W: 3 cm, L: 3 cm) has a separate air diffuser with a separate air pump to provide proper aeration (300 mL/min). From the MCR, 30 g of SOB-attached sulfur particles was placed in each chamber, followed by 40 mL of tap water. At the start of the experiment, Cr (VI) (0, 0.1, 0.5, 1.0, 2.0, 10.0, and 50.0 mg L^{-1}) or Cr (III) (0, 2.0, 10.0, 50.0, and 100.0 mg L^{-1}) was spiked in each chamber at defined concentrations. At the appropriate times (0, 0.5, 1.0, and 2.0 h) the pH and EC were measured manually using pH and EC meters. All tests were performed in duplicates and the average values were reported.

2.4. Chemicals and analyses

All chemicals including trivalent chromium (as $CrCl_3$) and hexavalent chromium (as $K_2Cr_2O_7$) used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pH and EC were measured manually over time using pH (Orion, 410A, Boston, MA, USA) and EC (Inlab 737 Mettler Toledo, Schwerzenbach, Switzerland) meters. Anions were analyzed by filtering the tap water samples with a 0.45-μm membrane filter (Fisher Scientific) prior to analysis using ion chromatography (ICS-900, Dionex Corp AS40 Column, Sunnyvale, CA, USA). The toxicity of trivalent and hexavalent chromium was calculated according to the following equations (Zhang et al., 2015):

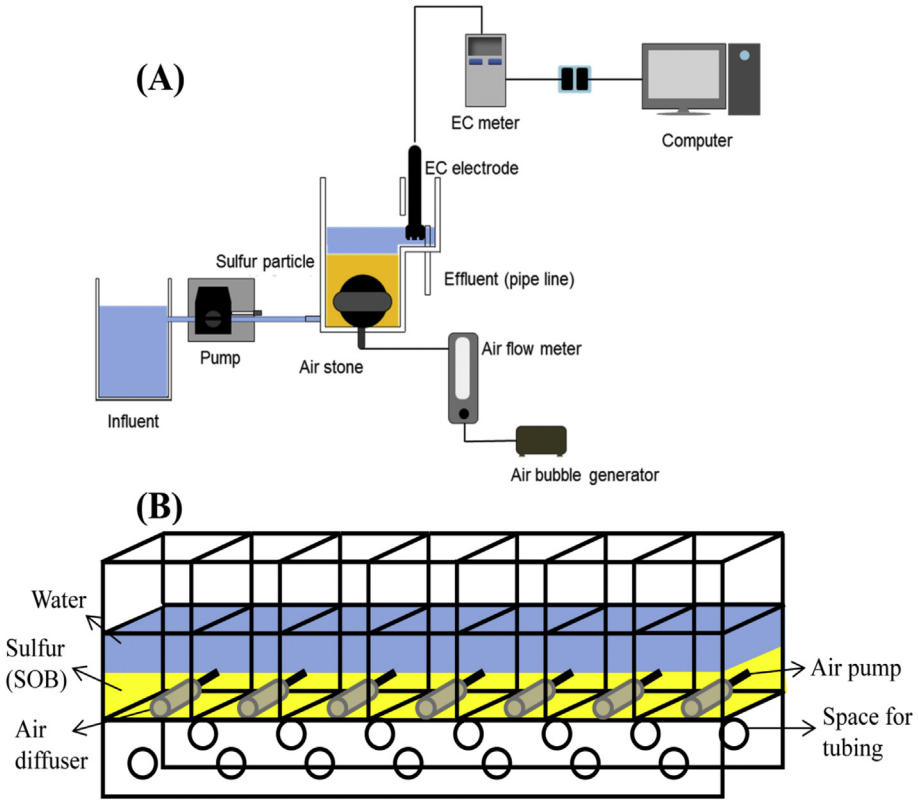

Fig. 2. Schematic diagrams of the SOB toxicity monitoring system using fed-batch **(A)** and batch test **(B)** conditions.

For the fed-batch test:

$$\text{Inhibition\%} = \left[1 - \left(\frac{\text{5 min slope of Cr EC}}{\text{5 min slope of control EC}}\right) \times 100\right] \quad (2)$$

For the batch test:

$$\text{Inhibition \%} = \left[1 - \left(\frac{\text{Slope of Cr EC}}{\text{Slope of control EC}}\right) \times 100\right] \quad (3)$$

3. Results

3.1. MCR operation

The total amount of sulfur used in the MCR was 800 g. The initial pH and EC values of the tap water used were 7.06 ± 0.04 and 0.06 ± 0.003 mS/cm, respectively. Initially, the EC varied between 1.9 mS/cm and 3.6 mS/cm, with a slope of 0.98 mS/cm/min. Stable conditions were reached in 2 days, and the EC change was between 7.4 mS/cm and 10.3 mS/cm, with a slope of 1.6 mS/cm/min (Fig. 1). When the MCR attained steady-state conditions, some SOB particles were removed for further experiments.

3.2. Effects of Cr (VI) and Cr (III) on the SOB in fed-batch mode

The SOB biosensor in fed-batch mode (Fig. 3) was used to detect Cr (VI) and Cr (III). For Cr (VI), four different concentrations were considered (0.1, 0.5, 1.0, and 2.0 mg L^{-1}). At the lowest concentration (0.1 mg L^{-1}), hexavalent chromium did not have any effect on the SOB activity over 4 h. However, when 0.5, 1.0, or 2.0 mg L^{-1} Cr (VI) was injected into the reactor, there was a 30%, 60%, and 98% decrease in the SOB activity, respectively. Immediately after injecting Cr (VI) into the reactor, the slope started to decrease, reaching a maximum after Cr exposure for 4 h. A 2-h EC_{50} of 1.5 mg L^{-1} was obtained for Cr (VI) in the fed-batch tests (Fig. 7). In the case of Cr (III), there was no noteworthy inhibition found (Fig. 4) during either the fed-batch or batch reaction, even at a very high concentration (100 mg L^{-1}). Due to the increase of the Cr (III) salt concentration, the influent EC was increased. In addition, there was an increasing pattern of SOB activity in the presence of Cr (III). The EC changes in the fed-batch test increased with exposure to Cr (III). For 2.0, 10.0, 50.0, and 100.0 mg L^{-1} Cr (III), the total increases in EC were 6.3%, 7.7%, 18.2%, and 3.0%, respectively, after exposure for 1 h; while they were 6.9%, 5.5%, 24.6%, and 12.6%, respectively, after exposure for 2 h.

3.3. Effects of Cr (VI) and Cr (III) on the SOB in batch mode

In the batch test, the pH and EC values were measured as indicators of SOB activity. For 0, 0.1, 0.5, 1.0, 2.0, 10.0, and 50.0 mg L^{-1} Cr (VI), the initial pH values were 3.0 ± 0.04, 3.2 ± 0.04, 3.1 ± 0.01, 3.2 ± 0.04, 3.3 ± 0.04, 3.3 ± 0.04, and 3.2 ± 0.02, and the final pH values were 2.5 ± 0.05, 2.6 ± 0.02, 2.6 ± 0.02, 2.6 ± 0.05, 2.7 ± 0.04, 3.1 ± 0.03, and 3.1 ± 0.02, respectively (Fig. 5). These data suggest that there was a gradual decrease in pH. The initial EC of the reactors (0.58 mS cm^{-1}) was adjusted using KCl according to the initial EC of the control reactor so that the EC changes would be comparable. For 0, 0.1, 0.5, 1.0, 2.0, 10.0, and 50.0 mg L^{-1} Cr (VI), the final EC values of the reactor were 1.3 ± 0.00, 1.2 ± 0.00, 1.2 ± 0.00, 1.1 ± 0.01, 1.1 ± 0.00, 0.7 ± 0.00, and 0.66 ± 0.00 mS cm^{-1}, respectively. Thus, the EC was decreased by 10.9%, 12.4%, 26.6%, 29.0%, 84.6%, and 90.6% for 0.1, 0.5, 1.0, 2.0, 10.0, and 50.0 mg L^{-1} Cr (VI), respectively. The 2-h EC_{50} for Cr (VI) in the batch test was

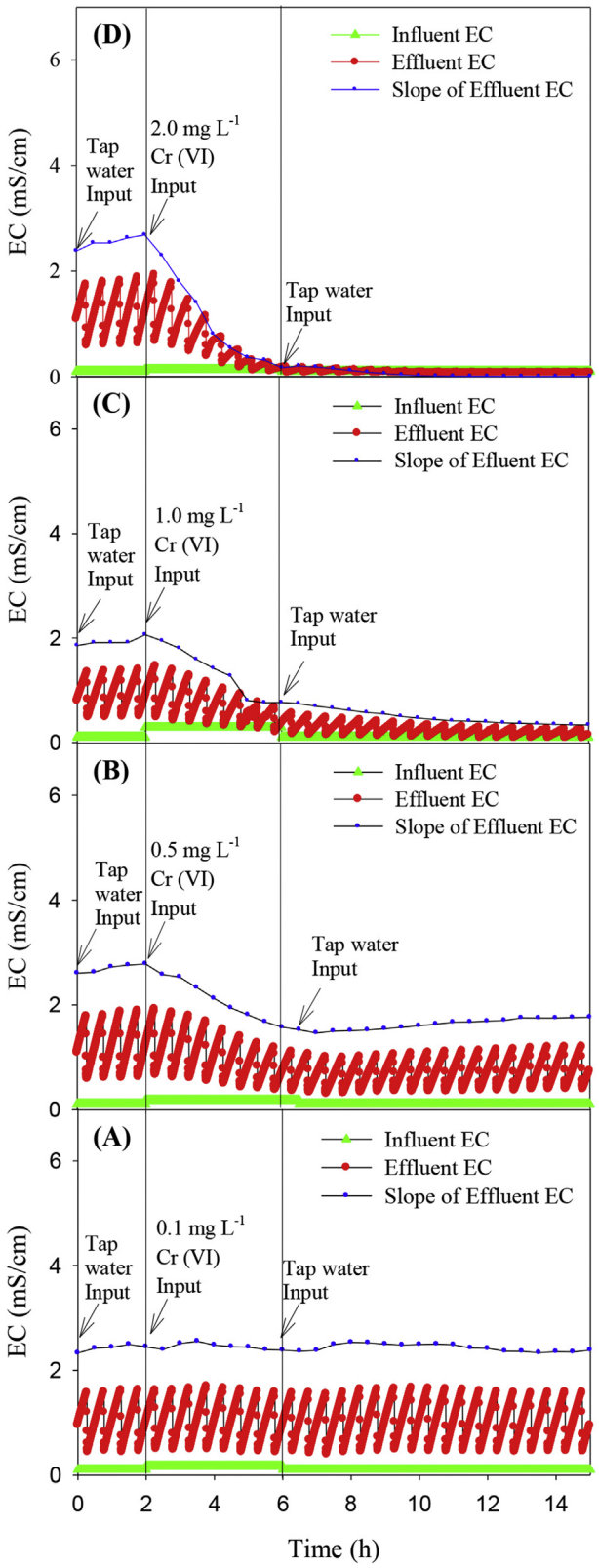

Fig. 3. Effect of Cr (VI) on fed-batch SOB operation for various concentrations (A) 0.1, (B) 0.5, (C) 1.0, and (D) 2.0 mg L^{-1}.

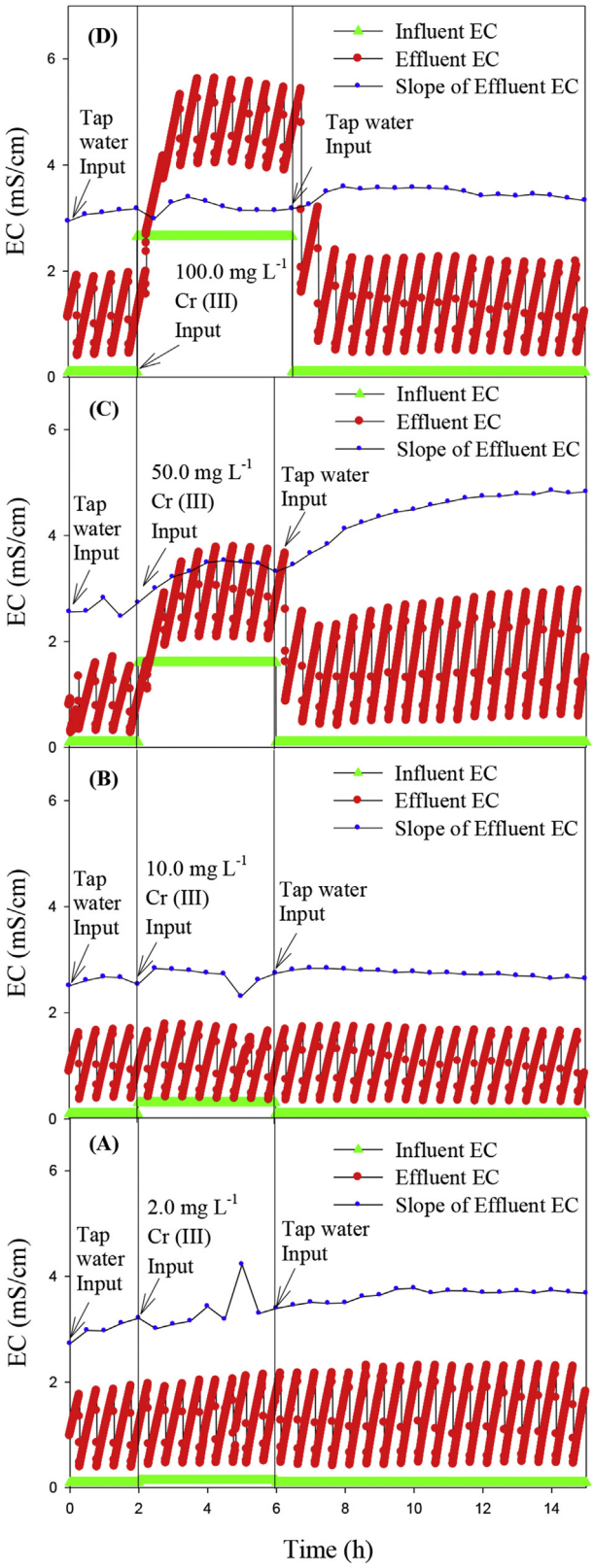

Fig. 4. Effect of Cr (III) on fed-batch SOB operation for various concentrations (A) 2.0, (B) 10.0, (C) 50.0, and (D) 100.0 mg L^{-1}.

calculated to be 2.7 mg L^{-1} (Fig. 7). As in the fed-batch tests, trivalent chromium showed no toxicity (Fig. 6). The final EC values of the reactors having 2 and 10 mg L^{-1} Cr (III) were similar to the EC values of the control reactor, whereas the final EC values were slightly higher in the reactors with 50 and 100 mg L^{-1} Cr (III).

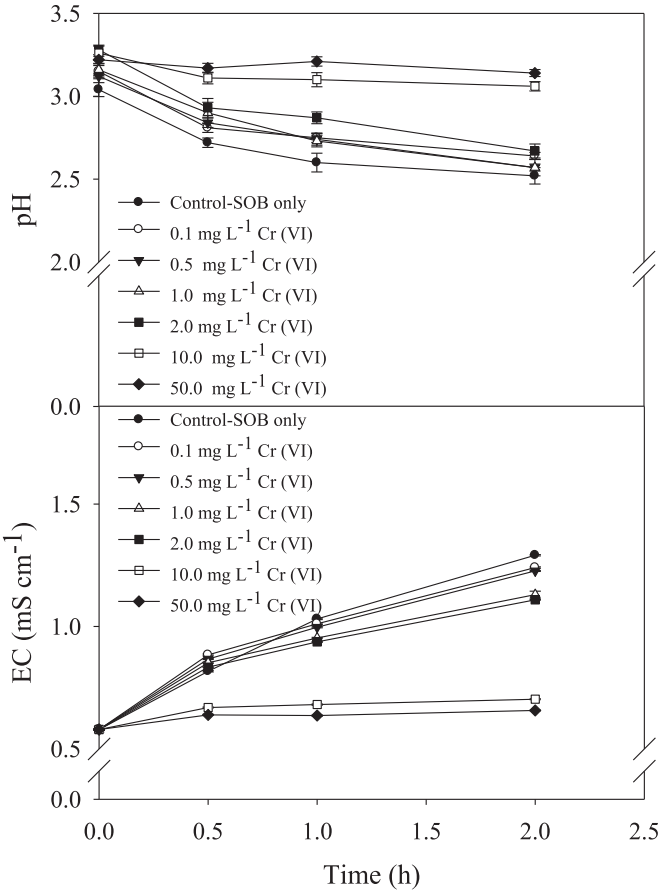

Fig. 5. Batch test results of the pH and EC for Cr (VI).

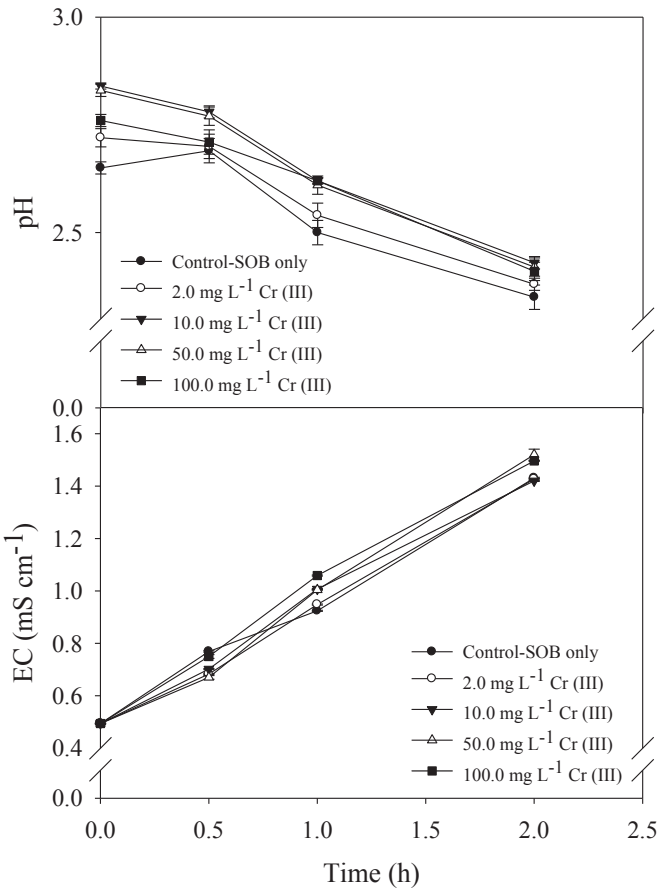

Fig. 6. Batch test results of the pH and EC for Cr (III).

4. Discussion

In this study, the effects of Cr (III) and Cr (VI) on SOB were compared. Cr (III), at concentrations of up to 100 mg L^{-1}, was found to have no effect on the SOB activity. This finding can be explained by the octahedral structure of Cr (III), which makes it quite difficult to enter into cells. There are numerous other mechanisms by which Cr (III) can enter into cells, such as passive diffusion and pinocytosis. Even if Cr (III) enters into cells and binds to DNA, it does not cause any DNA damage (Eastmond et al., 2008). It has been shown that Cr (III) toxicity occurs through the Fe^{3+} uptake pathway (Venkata Ramana and Sivarama Sastry, 1994) because Cr (III) has a structural similarity with Fe^{3+} ion (octahedral). This structural similarity causes Cr (III) to enter into cells. According to Hallberg and Lindström *Acidithiobacillus caldus* (the predominant bacteria in our reactors) can neither oxidize ferrous iron nor sulfidic ores (Hallberg and Lindström, 1994). Since, *A. caldus* can not uptake and metabolize ferrous iron, there is no chance of Cr (III) toxicity. Therefore, the main reason for Cr (III) being non-toxic in SOB reactors is the absence of the Fe^{2+} uptake.

Chromate is the main form of Cr (VI), which has a tetrahedral anionic structure that is similar to that of sulfate and phosphate. The structural similarity allows it to enter cells easily through anion channels (Zhitkovich, 2004). The reduction of Cr (VI) to Cr (III) occurs inside the cell by normal cellular reducing agents like glutathione, cysteine, thiols, and ascorbic acid, resulting in stable complexes with cellular proteins and DNA (O'Brien et al., 2003). There are three proposed mechanisms for Cr (VI) DNA damage. The first model describes the direct action of reactive oxygen species such as hydroxyl and other free radicals on DNA, thus causing damage. These free radicals are formed during the reduction of Cr (VI) to Cr (III). The second model suggests that Cr (V) and Cr (IV) intermediates bind with DNA, resulting in DNA damage. In the third model, Cr (III) directly binds and forms DNA-chromium adducts and cross-links after reduction from Cr (VI) to Cr (III) (O'Brien et al., 2003; Zhitkovich, 2004; Sedman et al., 2006). The contributions of these three pathways are still unknown, but it is evident that the binding of Cr (III) complexes (ascorbate, glutathione, cysteine, etc.) with DNA exerts major damage (Zhitkovich, 2004).

Moreover, Cr (III) binds with hydroxides, making strong complexes. It exists as hexa-aquachromium (+3) and other hydrolyzed forms in diluted solutions when there are no complexing agents present except for H_2O or OH. $Cr(H_2O)^{3+}$ and its deprotonated forms $CrOH^{2+}$, $Cr(OH)_2^+$, and $Cr(OH)_3$ occur in the range of pH 3.8 to 11.5. $CrOH^{2+}$, which is soluble and predominant in the environment, exists in the range of pH 2 to 6.3. Cr (III) complexes more favorably with OH^- ions, compared to SO_4^{2-}, NO_3^-, and CO_3^{2-} (Rai et al., 1989). In addition, Cr (VI) exists in hydrochromate, chromate, and dichromate forms. The hydrochromate form predominates in low-pH aqueous solutions. Therefore, the increased toxicity of Cr (VI) could be due to the presence of hydrochromate ($HCrO_4^-$) (Sivakumar and Subbhuraam, 2005).

In this study, the 2-h EC_{50} values for Cr (VI) in the batch and fed-batch tests were 2.7 mg L^{-1} and 1.5 mg L^{-1}, respectively. For example, a 2-h EC_{50} of 35.6 mg L^{-1} was obtained for nitrifying bacteria, a 3-h EC_{50} of 36.2 mg L^{-1} was obtained for activated sludge inhibition, and a 30-min EC_{50} of 22.5 mg L^{-1} was obtained for *V. fischeri* (Dalzell et al., 2002). In addition, for Cr (III), a median

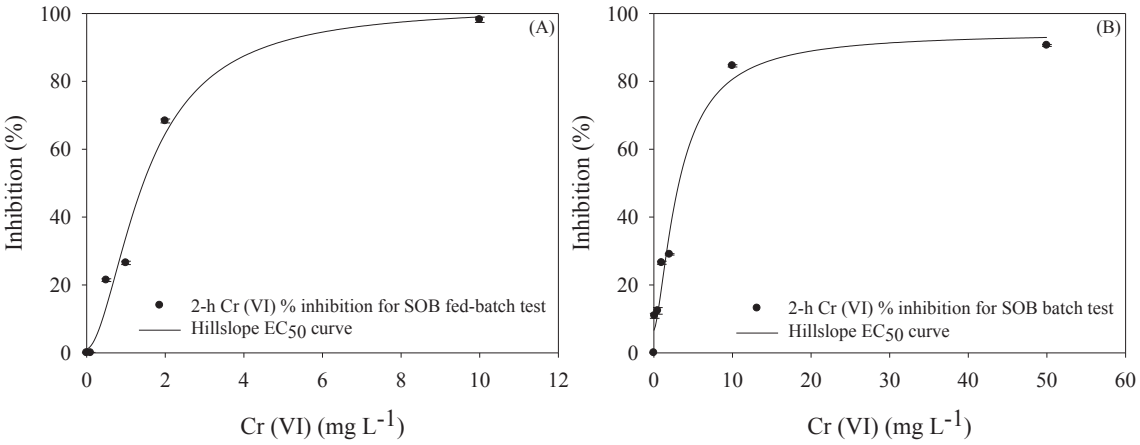

Fig. 7. EC_{50} value determination of Cr (VI) for the fed-batch **(A)** and batch tests **(B)** using SOB.

lethal concentration of 2.55 mg L^{-1} was obtained for the earthworm *Eisenia fetida* (Sivakumar and Subbhuraam, 2005), the 72-h EC_{50} for the green algae *Pseudokirchneriella subcapitata* was 17.4 μg/L (Vignati et al., 2010), and the 48-h EC_{50} for *Daphnia magna* was 0.6–111 mg L^{-1} (Ponti et al., 2014).

Moreover, the EC_{50} obtained in this study for the batch test was higher than that of the fed-batch test. The batch test receives toxicant at the beginning of the test, and there is no further addition of toxicant. In the case of the fed-batch test, the influent is feeding new feed containing toxicant every 30 min. In the batch test, it is assumed that the toxicant binds to all of the receptors to cause bacterial damage and that no more toxicant can enter the reactor. However, the fed-batch reactors have new feed with a similar concentration, so if there are any binding sites remaining for the toxicant, the toxicant will bind with the binding site and cause more damage. Since in the fed-batch tests the SOB were exposed to Cr (VI) every 30 min, more damage was caused to the SOB in fed-batch mode compared to batch mode, where the exposure time for the toxicant was different.

Of note, the slope of EC production was increased when the SOB were exposed to Cr (III). The increase in slope may be due to the unstable reactor conditions at the beginning of the experiment, thus resulting in an increased EC.

Comparing both the batch and fed-batch tests, we can conclude that both of these tests have different applications and that SOB water assessment can easily be done simply by checking the EC. The batch test is very suitable for portable use in remote areas and can be used by less technical people. In contrast, the fed-batch test can be used for large bodies of water in which a large portion of water can be covered. The high sensitivity of the fed-batch test as well as the portability, ease of use, and quick results of the batch test make both tests applicable in different settings.

5. Conclusion

The present study demonstrated that Cr (III) is non-toxic and that Cr (VI) is very toxic to SOB. The doses at which Cr (VI) was toxic were higher in the fed-batch test, compared to the batch test. Hexavalent chromium showed 2-h EC_{50} values of 2.7 and 1.5 mg L^{-1} in the batch and fed-batch tests, respectively. The non-toxicity of Cr (III) and toxicity of Cr (VI) were mainly due to their structural similarities with Fe^{3+} and SO_4^{2-}, respectively. Since there is no Fe^{2+} uptake pathway in *A. caldus*, Cr (III) was nontoxic even at high concentrations. In contrast, the SOB were severely affected by Cr (VI) and found to be very sensitive to it. Cr (VI) toxicity was based on the sulfate and phosphate transport pathways.

Acknowledgments

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