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Detecting Oxidized Contaminants in Water Using Sulfur-Oxidizing Bacteria

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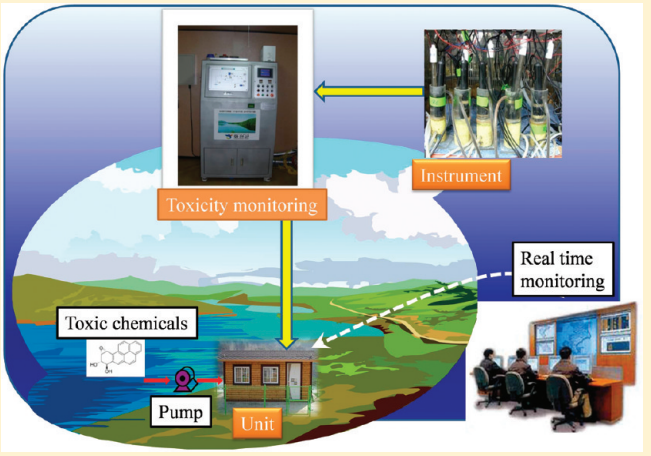
ABSTRACT: For the rapid and reliable detection of oxidized contaminants (i.e., nitrite, nitrate, perchlorate, dichromate) in water, a novel toxicity detection methodology based on sulfur-oxidizing bacteria (SOB) has been developed. The methodology exploits the ability of SOB to oxidize elemental sulfur to sulfuric acid in the presence of oxygen. The reaction results in an increase in electrical conductivity (EC) and a decrease in pH. When oxidized contaminants were added to the system, the effluent EC decreased and the pH increased due to the inhibition of the SOB. We found that the system can detect these contaminants in the 5−50 ppb range (in the case of NO_3^-, 10 ppm was detected), which is lower than many whole-cell biosensors to date. At low pH, the oxidized contaminants are mostly in their acid or nonpolar, protonated form which act as uncouplers and make the SOB biosensor more sensitive than other whole-cell biosensors which operate at higher pH values where the contaminants exist as dissociated anions. The SOB biosensor can detect toxicity on the order of minutes to hours which can serve as an early warning so as to not pollute the environment and affect public health.



INTRODUCTION

Release of nitrogen to surface and ground waters is one of the world's biggest unsolved water-quality problems. According to the U.S. Toxics Release Inventory, 51 million kg of inorganic nitrates were released as fertilizer from 1991 to 1993 in the U.S.A, and 23% reached the groundwater or surface water.[1] Since most nitrogenous materials tend to be oxidized to nitrates in the environment, all nitrogenous materials are potential sources of nitrates in surface and ground waters.

Perchlorate (ClO_4^-) is known to interfere with iodide uptake into the thyroid gland and the proper development of fetuses and children.[2] ClO_4^- is primarily used in ordnance and explosives, and as a rocket propellant in the defense and aerospace industries and has contaminated the water supplies of more than 20 million people, primarily in the southwest United States.[3] Because of its high solubility, nonreactivity, and poor absorption to soils, ClO_4^- persists and spreads rapidly throughout the environment.[4] As of 2006, an expected 164 million pounds of ClO_4^- will be disposed and will require treatment.[5]

Hexavalent chromium is often in its oxidized form as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$). CrO_4^{2-} is widely used in industries such as electroplating, leather tanning, metallurgy, petroleum refining, textile manufacturing, and pulp production. Its use has resulted in large quantities of chromium being discharged into the environment.[6] Chromates are also highly soluble and, therefore, mobile and bioavailable in aquatic systems. Acute toxicity includes gastric ulcers, and chronic toxicity includes mutagenic and carcinogenic effects.[7]

According to Hernando,[8] the assessment of contaminants emerging from contaminated sites or agricultural fields, or as effluents, based on the conventional, analytical detection of specific pollutants is not sufficient to assess environmental risk since toxicity is a biological response. The toxicity of pollutants is synergistic meaning the toxicity of multiple pollutants is greater than the sum of toxicity of the individual pollutants.[9−11] Therefore, a means to evaluate toxicity on living organisms is needed.

Many bioassay methods used for the detection of toxic chemicals are based on a change in physiological characteristics or motility such as in *Daphnia magna*[8,10] or a decrease in bioluminescence as in *Photobacterium phosphoreum*[12] or *Vibrio fischeri*.[10] The most common bacterial tool for toxicity measurements is Microtox.[13] Microtox can

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be used for assessing the toxicity of water samples by measuring a decrease in bioluminescence if the water samples contain toxic chemicals. Microtox correlates well with other bioassay methods using higher organisms.

Sulfur-oxidizing bacteria (SOB) can be used for the continuous monitoring of toxicity, and their use circumvents many of the obstacles associated with conventional assays. SOB, first identified by Sergey Winogradsky in 1885, are chemoautotrophic bacteria that oxidize reduced sulfur compounds to sulfuric acid in the presence of oxygen (O_2). Some SOB, such as genus *Acidithiobacillus* use elemental sulfur (S^0) particles as the electron donor, as shown by eq 1:[14,15]

$$S^0 + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+$$
$$\Delta G^{\circ\prime} = -587 \text{ kJ/reaction} \quad (1)$$

Oxidation of S^0 results in the formation of sulfate (SO_4^{2-}) and two protons (H^+), which lowers the medium pH to ~2. The production of SO_4^{2-} also increases the electrical conductivity (EC) of the medium. Generally, EC is proportional to the concentration of ions in the medium and is a measurement of the ability of a medium to carry an electric current and varies both with the number and type of ions the medium contains. Thus, the decrease in pH and the increase in EC reflect the formation of sulfuric acid as the result of sulfur oxidation by SOB and can easily be detected using simple EC and pH meters.[16] In the presence of oxidized contaminants, the activity of SOB will be inhibited, which will cause an increase in pH and a decrease in EC. Furthermore, the low pH of the SOB biosensor puts the oxidized contaminants mostly into their free, protonated form which may act as an uncoupler and reduce the growth of the SOB.[17−19]

Traditional potentiometric microbial biosensors measure the difference between a working electrode and a reference electrode, and the signal is correlated to the concentration of the analyte. This method requires a very stable reference electrode and may be a limitation of these transducers.[20] However, the SOB biosensor does not have a reference electrode, and recalibration is not needed since only changes in pH are needed to detect the inhibition of SOB.

Rogers and Williams[21] note that simplicity, ruggedness (insensitivity to external conditions), and cost-effectiveness are key components of biosensors. Biosensors should be extremely versatile both in the range of compounds and matrices for which they can be adapted. Biosensors able to detect a variety of toxic chemicals will be most competitive since the need for detecting any single pollutant is small. Here, we report our findings using batch and continuous systems with inexpensive sulfur particles and SOB which demonstrate a simple, rugged, continuous, and cost-effective means to detect oxidized contaminants in water.

MATERIALS AND METHODS

Culture and Medium. Aerobic return activated sludge was used as the inoculum to a sulfur master-culture reactor (SMCR) containing S^0. The sludge was taken from the Chuncheon Wastewater Treatment plant in Chuncheon City, Kangwon-do, Republic of Korea. Synthetic streamwater was prepared by diluting the following nutrient mineral buffer (NMB) solution 100 times: $NaHCO_3$ (3.13 g/L), NH_4Cl (0.31 g/L), KCl (0.13 g/L), NaH_2PO_4 (4.22 g/L), Na_2HPO_4 (2.75 g/L). Trace metal and vitamin solutions, previously described by Oh et al.,[22] were also added. The EC, pH, and alkalinity of the synthetic water were 0.12 mS/cm, 6.9−7.2, and 17 mg/L as $CaCO_3$, respectively.

Reactor Construction and Experiments. *Batch Tests.* S^0 (600 g, 2−4 mm in diameter) was placed in a 1 L beaker (Diamond, Korea) and filled with 600 mL diluted NMB solution. Air was introduced to the SMCR using a stone diffuser with a flow rate of 150−250 mL/min. The SMCR was inoculated with sludge and incubated at 30 °C in a semicontinuous mode. From the SMCR, 200 mL was wasted and refilled with 200 mL diluted NMB solution at 4−7 day intervals. The SMCR was operated for 9−10 days to reach steady-state conditions (i.e., pH = 1−2 and EC 18 mS/cm) and then used for batch testing.

Experiments were carried out in 100 mL media bottles (Horex, Germany) that contained 25 mL (38 g) of S^0 particles with attached SOB from the SMCR and 50 mL of 100, 5, or 1 times diluted NMB containing different concentration of NO_2^- to study the effect of pH, free nitrous acid (HNO_2), and alkalinity on SOB. The test bottles were capped with aluminum foil to prevent evaporation, and the tests were conducted at 30 °C and agitated at 150 rpm to supply air in a shaking incubator (Figure 1) for 12 h. The pH and EC were measured at 0, 6, and 12 h.

Continuous Tests. For continuous tests, the procedures are described elsewhere.[22,23] A schematic of the continuous SOB biosensors are shown in Figure 1E. Briefly, the biosensors contain 50 mL (82 g) of S^0 particles (2−4 mm) and were inoculated with aerobic sludge and incubated at 30 °C for three days in a batch mode. Air was introduced from the bottom (150−250 mL/min) to supply O_2 as an electron acceptor. The biosensors were then fed synthetic streamwater continuously in up-flow mode using adjustable peristaltic pumps. The biosensors were operated at a hydraulic retention time (HRT) of 30 min for approximately 2−3 days each to reach steady-state conditions (i.e., stable EC and pH values). Then several oxidant contaminants (NO_3^-, NO_2^-, $Cr_2O_7^{2-}$, ClO_4^-, and a mixture of NO_3^- and NO_2^-) at different concentrations were spiked to the influents of the biosensors, and the effluent EC and pH values were monitored. The EC and pH meters were connected to a computer, and all data were automatically recorded every 10 min.

Chemicals and Analyses. All oxidized contaminants were of analytical grade and were used without further purification. The oxidized contaminants tested were NO_3^-, NO_2^-, ClO_4^-, and $Cr_2O_7^{2-}$. All the chemicals used in this study were purchased from Sigma Aldrich Chemical. Toxicity was monitored by measuring changes in electrical conductivity, EC (LUTRON meter model YK 22 CT), and pH (LUTRON meter model pH 208c) before and after injection. The inhibitory effect (I) of NO_2^- on SOB growth in batch mode was calculated using the following equation

$$I(\%) = \frac{EC_{control} - EC_{sample}}{EC_{control}} - 100 \quad (2)$$

where I is percentage of inhibition after 12 h, and $EC_{control}$ and EC_{sample} are the electrical conductivity for the control and the sample after 12 h, respectively. The effective concentration (EC50) responsible for 50% growth inhibition of SOB was calculated by using TOXCALC (v5.0.32, Tidepool Scientific Software). These values were calculated by the Maximum Likelihood-Probit analysis test.

RESULTS AND DISCUSSION

Continuous Tests of Oxidized Contaminants. When the oxidized contaminants were injected into the SOB biosensors in continuous mode, a decrease in EC occurred within minutes to a few hours depending on the concentration and type of toxicant

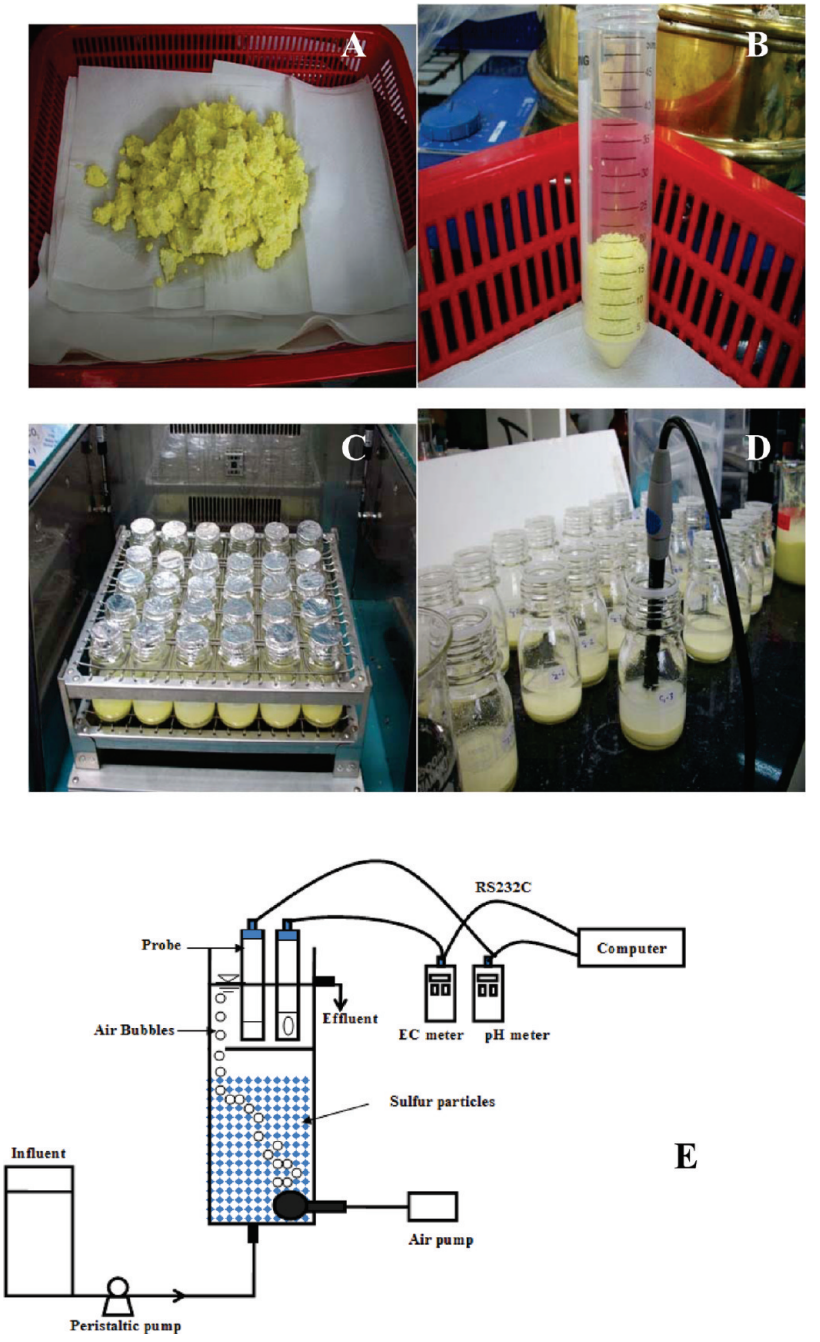

Figure 1. Sulfur particles from the SMCR and the batch configuration of the SOB biosensor. (A) Sulfur particles with SOB from SMCR. (B) Approximately 25 mL of sulfur particles to be used in media bottles for batch test. (C) Incubation at 30 °C and 150 rpm. (D) Checking pH and EC at different time intervals. (E) Continuous SOB biosensor with automatic pH and EC measurements.[22].

(Figure 2). Generally, the percentage of inhibition was increased by increasing the exposure time or the concentration of the toxic chemical in the influent. The EC of the influents averaged 0.12 mS/cm. The initial effluent EC values of the different biosensors shown in Figure 2 (ranging from 1.1 ± 0.15 to 1.35 ± 0.16 mS/cm) were higher than influent values, but slightly different. This is likely due to different sulfur particle size surface areas. Even though 50 mL (82 g) of sulfur was added to each biosensor, the total mass and surface area of sulfur were different which led to different production rates of sulfuric acid, EC, and pH values.[22] This phenomenon is not significant since changes in in-reactor EC and pH are what is important for detection. Furthermore, changes in EC are more important than changes in pH since there is low buffering capacity in the influent, and thus even incomplete inhibition will lead to some sulfuric acid and drop the pH into this range.

NO_3^- was less toxic compared to NO_2^-. While the EC of the SOB biosensor reached the influent EC when 0.5−3 ppm NO_2^- was injected, the SOB biosensor did not detect 5 ppm of NO_3^-. The EC decreased rapidly after injection of 20 ppm of NO_3^- and slightly decreased when 10 ppm of NO_3^- was injected (Figure 2b). At 10 and 20 ppm NO_3^-, the EC stabilized at a value higher than the influent EC which suggests the SOB acclimated to NO_3^-.

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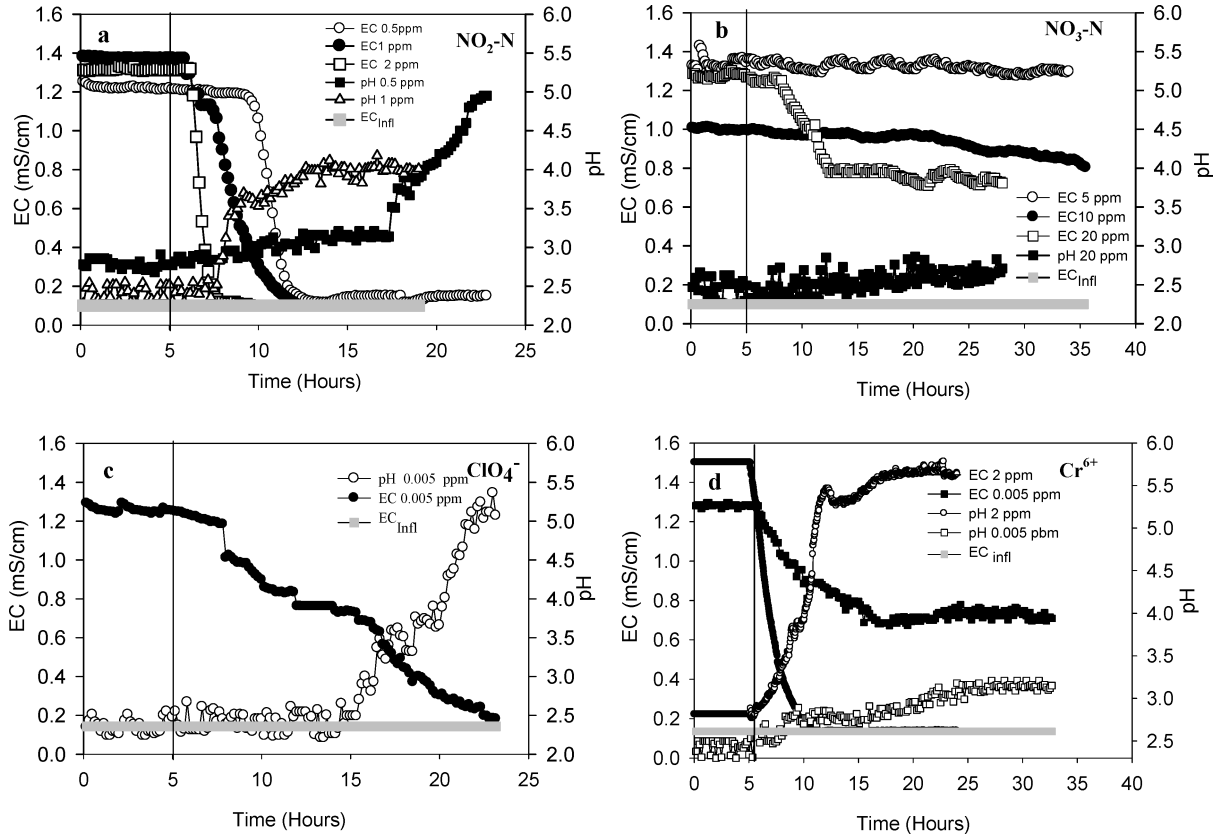

Figure 2. Detection of different oxidized contaminants using the SOB biosensor in continuous mode.

The injection of ClO_4^- (5 ppb) decreased the EC in a few hours, and complete inhibition of SOB was observed after 23 h of injection (Figure 2c). The injection of 5 ppb Cr^{6+} caused an immediate and gradual decrease in EC which stabilized after about 15 h (Figure 2d). The injection of 2 ppm Cr^{6+} caused an immediate and steep decrease in EC and increase in pH, and the in-reactor EC reached that of the influent which indicates near complete inhibition of the SOB (Figure 2d).

In addition to being strong oxidizers, these oxidized contaminants are also acids. Hence, part of the toxic effect could be due to the free, protonated, undissociated form (HNO_3, HNO_2, $HClO_4$, $H_2Cr_2O_7$). The pK_a values of HNO_3, HNO_2, and $HClO_4$ are −1.4, 3.1, and −7, respectively. $H_2Cr_2O_7$ has two pK_a's: −0.2 and 6.51. When pH < pK_a, these acids are predominantly protonated and are able to penetrate the cell and deprotonate at the higher pH inside the cell. This raises the H^+ concentration inside the cell, acts as an uncoupler, and weakens the proton motive force for energy conservation.[17−19] Compared to other bioassays, the results here show that the SOB biosensor was highly sensitive to detect these oxidized contaminants at very low concentrations compared to other biosensors, and this may be due to the low operating pH of the SOB biosensor. For example, Cho et al.[24] measured a 30-min EC_{50} for Cr^{6+} at 17 500 ± 1900 ppb using *Vibrio fischeri* in the Microtox assay and 6300 ± 1500 ppb using bioluminescent *Janthinobacterium lividum* YH9-RC which is about an order of magnitude higher than observed here.

Batch Tests with NO_2^-. The detection of different concentrations of NO_2^- using SOB in batch mode is shown in Figure 3. In all controls, EC increased and pH decreased as there was no inhibition from NO_2^-. Of all the contaminants tested, NO_2^- has the highest pK_a (3.1), and thus at the SOB biosensor pH of 2, the free HNO_2 form should exert the most toxicity due to uncoupling. In addition, under acidic conditions, NO^+ or NO radicals form spontaneously from NO_2^-. Many bacterial species are susceptible to NO_2^- toxicity because of the formation of metal−nitrosyl complexes that occurs when NO^+ or NO radicals interact with bacterial enzymes.[25] In SOB, these radicals likely react with FeS centers to form FeS−nitrosyl complexes which are much more toxic than NO_2^- itself.[26] It is unknown if the other oxidized contaminants form radicals under acidic conditions.

In order to test if NO_2^- is more toxic under acidic conditions, the NMB media was diluted 0, 5, and 100 times in order to decrease the alkalinity and allow the pH to drop (Figure 3). The top two figures show that there was little difference in EC and pH when the media was not diluted. This is likely due to the initial pH of 7.0 which is higher than the optimal range for SOB (pH 2−3). The media was then diluted five times, the initial pH was 6.0, and the medium initial alkalinity was reduced from 1567 to 293 mg/L as $CaCO_3$ (Table 1). Since the in-reactor pH dropped to as low as ∼3, the toxic effect at concentrations from 0.05 to 0.5 ppm was observed. When the media was diluted 100 times and the initial pH was ∼3, the in-reactor pH was lower and the toxic effect was higher (Figure 4). Table 1 shows the EC_{50}'s, and Figure 4 shows the percent inhibition for each of the media. Overall, the data shows that toxicity increases with a decrease in pH and alkalinity and an increase in NO_2^- (i.e., HNO_2).

In-reactor SO_4^{2-} concentrations parallel EC concentrations (data not shown). SO_4^{2-} production was highest in the media diluted 100 times since the pH was in the optimal range for SOB.

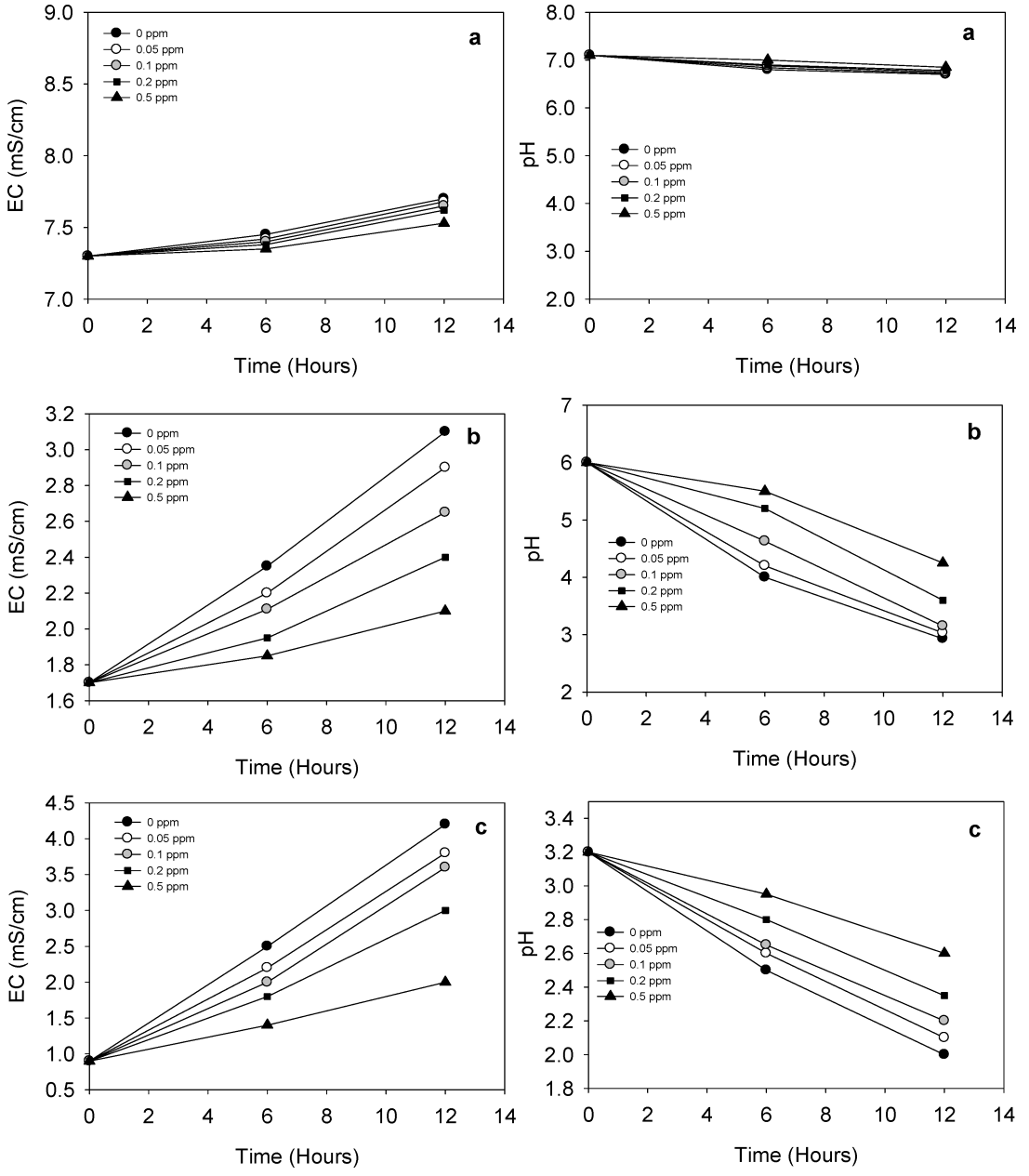

Figure 3. EC and pH values of media containing alkalinities of (a) 1567, (b) 293, and (c) 17.5 mg/L as $CaCO_3$ and NO_2^- concentrations of 0−0.5 ppm.

However, the difference in SO_4^{2-} production at NO_2^- concentrations from 0.05 and 0.5 ppm was greatest using the media diluted 100 times which shows that the lower pH, i.e., the higher HNO_2 concentration, exerts a toxic effect.

Continuous tests were then conducted using NO_2^- concentrations ranging from 0.3 to 2 ppm (Figure 5). Since different SOB biosensors were used in these tests, the initial stable EC values were different as noted above. The initial pH values of all SOB biosensors averaged 2.42 ± 0.36 (data not shown). After 5 h of steady conditions, NO_2^- was injected at an HRT of 30 min, and a decrease in EC was observed. The decrease was nearly immediate when 1.5 and 2 ppm was injected while there was a short lag when lesser concentrations were injected. The inset to Figure 5 plots the rate of decrease in EC versus each NO_2^- concentration, and shows that the rate of decrease in EC was greater as the NO_2^- concentration increased.

Figure 6 compares the toxic effects of NO_2^- and NO_3^- while in continuous mode. After 5 h of steady conditions, NO_2^- was injected (0.4 ppm), and there was an observed decrease in EC and an increase in pH in about one hour. As the pH increased to ∼3.0, the EC stabilized and NO_3^- was injected (10 ppm) which caused a minor decrease in EC and increase in pH. When the EC stabilized at ∼19 h, both NO_2^-−N (0.4 ppm) and NO_3^-−N (10 ppm) were injected which caused a greater decrease in EC and increase in pH. When NO_2^- was injected for the second time, the pH was ∼3.7 and higher than when NO_2^- was injected initially (pH = 2). At the higher pH, the toxicity was less as noted by the slopes in the EC curves which gives further evidence that HNO_2 may be exerting the toxicity. These results also show that NO_2^- was more toxic than NO_3^- likely due to the higher pK_a of NO_2^-. Furthermore, the concentrations of NO_2^- and NO_3^- were detected below regulatory

Table 1. EC_{50}'s Calculated Using the TOXICALC Program

NMB (times diluted)	alkalinity (mg/L as $CaCO_3$)	EC_{50} (ppm)
1	1567	0.079
5	293	0.055
100	17.5	0.038

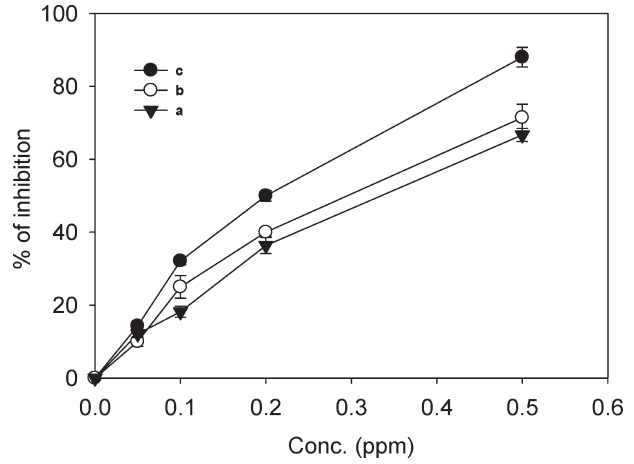

Figure 4. Percent inhibition as a function of alkalinity [(a) 1567, (b) 293, and (c) 17.5 mg/L as $CaCO_3$] and NO_2^- concentration (0−0.5 ppm).

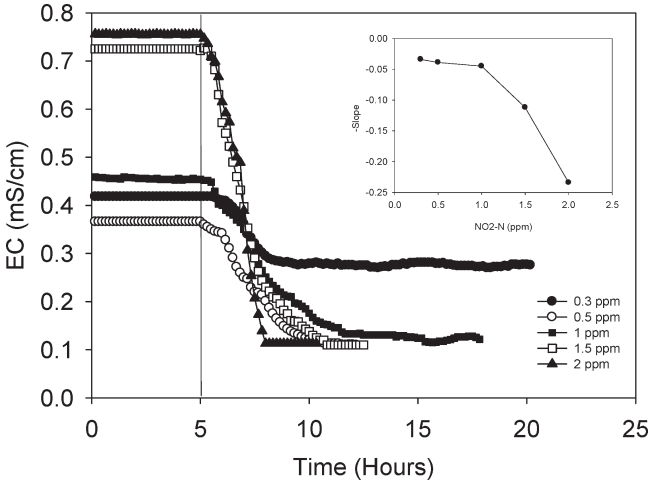

Figure 5. NO_2^- toxicity while in continuous mode. The decrease in EC after spiking NO_2^- increased with increasing NO_2^- concentration (inset).

levels. Even though the pH during the second injection of both NO_2^- and NO_3^- (pH ~ 4) was higher than the first injection of NO_2^- (pH ~ 2), the effects of NO_2^- and NO_3^- are likely additive. Thus, the detection of toxicity via a biological response is likely better information than chemical measurements which only ensure that the water meets regulatory requirements.

The results presented here regarding the toxicity of NO_2^-, particular HNO_2, agree with that of other studies. Using *Nitrosomonas europaea*, Stein and Arp[27] showed that ammonia oxidizing activity was lost when the HNO_2 concentration reached 2.47 mg/L at pH 5.7. Using *Pseudomonas putida* at pH 6.9, Bringman and Kuhn[28] showed that an HNO_2 concentration of 0.02 mg/L

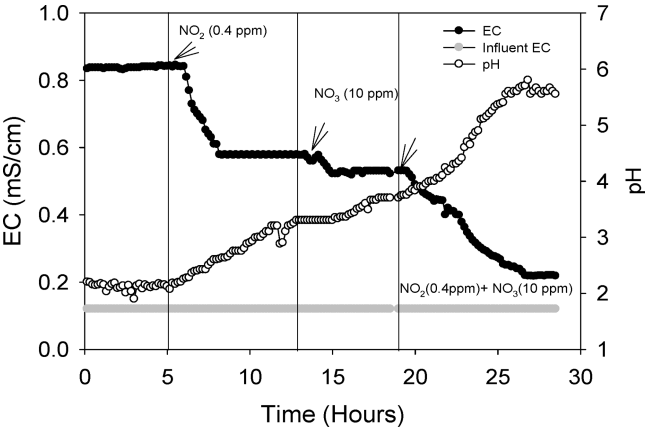

Figure 6. NO_2^- and NO_3^- toxicity while in continuous mode.

was toxic. While using a *Pseudomonas sp.* at pH 6.9, Bollag and Henninger[29] showed that a HNO_2 concentration of 0.05 mg/L was toxic. Weon et al.,[30] using a *Acinetobacter sp.*, observed an IC_{50} at 0.1 mg/L HNO_2. As a comparison, the highest HNO_2 concentration in the present study at pH 2 was 0.47 mg/L.

A total of four oxidized contaminants were detected on the order of minutes to hours in the 5−50 ppb range (in the case of NO_3^-, 10 ppm was detected) which is lower than many whole-cell biosensors to date. The increased sensitivity is due to the low operating pH of the SOB biosensor. Under the acidic conditions of the SOB biosensor, nearly all of the oxidized contaminants are in their free, protonated form and can act as uncouplers. In regard to NO_2^-, NO^+ or NO radicals are generated which inhibit bacterial enzymes which may be more inhibitory than HNO_2 itself. The use of the SOB-based biosensor circumvents many of the obstacles associated with biosensors. First, it is simple, uses little energy, and can be operated for at least several months. The air sparging of sulfur particles laden with SOB in a continuous flow reactor while measuring the pH and EC is sufficient for detecting toxicity. Second, periodic calibration of pH and EC meters are not needed because EC or pH changes over time are more important than exact values. Third, sulfur particles are very cheap and no toxic byproducts are produced: the total mass of sulfate produced is low. Since the SOB biosensor gives a biological response to toxicity, the response to the additive effect of multiple contaminants is likely a better predictor of overall toxicity than the measurement of individual chemicals.

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