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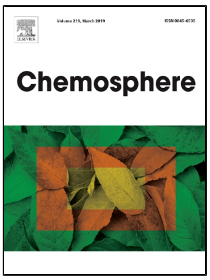

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Real-time monitoring of water quality of stream water using sulfur-oxidizing bacteria as bio-indicator

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Abstract

In aquatic ecosystems, real-time water-quality (WQ) biomonitoring has become the most effective technology for monitoring toxic events by using living organisms as a biosensor. In this study, an online WQ monitoring system using sulfur oxidizing bacteria (SOB) was tested to monitor WQ changes in real-time in natural stream water. The WQ monitoring system consisted of three SOB reactors (one continuous and two semi-continuous mode reactors). The SOB system did not detect any toxicity in relatively-unpolluted, natural stream water when operated for more than six months. When diluted swine wastewater (50:1) was added to the influent of the reactors, the system detected toxic conditions in both the continuous and semi-continuous operational modes, showing 90% inhibition of SOB activity within 1 h of operation. The addition of 30 mg/L NO_2^--N or 2 mg/L of Cr^{6+} to the influents of SOB reactors resulted in the complete inhibition of the SOB activity within 1-2 hours. The results demonstrated the successful application of an SOB bioassay as an online toxicity monitoring system for detecting pollutants from stream or river waters.

Keywords: Nitrite ion (NO_2^--N), Biomonitoring, Chromium, Swine wastewater, Toxicity

Introduction

Contaminated water represents a source of disease in humans and other organisms (Bae et al., 2012; Hassan et al., 2012) making efficient monitoring of water resources is necessary for reliably managing water quality (WQ) in aquatic ecosystems. Chemical-specific sensors or chemical instruments; such as gas chromatography, high-performance liquid chromatography and atomic absorption spectroscopy can be used to identify water pollutants

(e.g., heavy metals and other oxidants) in water (van Wezel et al., 2010; Hassan et al., 2012). Although these chemical-specific sensors are sensitive and quantitative (Woutersen et al., 2011), they cannot provide comprehensive, real-time information on water samples containing complex chemical compounds, each of which differs in its effects on aquatic ecosystems (van der Schalie et al., 2001). Therefore, it is very crucial to develop and improve existing technologies methods and tools to proactively detect hazardous substances in aquatic ecosystems (Kholodkevich et al., 2008).

In recent years, biomonitoring technologies have become viewed as the most appropriate methods for collecting real-time data of instantaneously ecologically relevant heavy metals in aquatic ecosystems (Hansen, 2008; Zhou et al., 2008). In particular, biomonitoring methods to continuously monitor water quality have been based on variations in the response of organisms to the presence of pollutants (based on changes in the physiology and/or behavior of organisms) (Chen et al., 2012; Hassan et al., 2012). Many types of organisms, such as fish (Mishra et al., 2000), algae (Zhang et al., 2012), living cells (Wolf et al., 2013), *Daphnia* spp. (Guilhermino et al., 2000) and various invertebrates (Beyene et al., 2009) have been used as bioindicators because they respond to the toxic effects of heavy metals and other contaminants in aquatic environments.

Recently, sulfur-oxidizing bacteria (SOB) have been used as bioindicators for detecting heavy metals and other toxic chemicals in water (Van Ginkel et al., 2011; Hassan et al., 2016). In unpolluted water, SOB oxidizes elemental sulfur (S^o) to sulfuric acid (H_2SO_4) under aerobic conditions (Madigan et al., 2009). The production of hydrogen ions (H^+) from this reaction decrease the water pH, while SO_4^{2-} increases the electrical conductivity (EC) of the water (Hassan et al., 2012). However, activity of SOB is inhibited in the presence of toxic chemicals, which results an increase in pH and a decrease in EC (Oh et al., 2011). Thus,

changes in EC (in response to changes in the physiological activity of SOB) can be used to provide an ecologically-relevant indicator of toxicity that can support standard toxicity tests using online biomonitors (Van Ginkel et al., 2010; Hassan et al., 2012, 2013).

SOBs are important bioindicator species because they can sense a wide range of pollutants, from heavy metals to oxidized contaminants (Gurung et al., 2011). In this study, an SOB-based online toxicity-monitoring system (using SOB as a bioindicator) was developed and installed near a stream to test and evaluate an SOB-monitoring system by monitoring the natural stream water for over six months. Also, the system was tested by spiking swine wastewater and other pollutants such as NO_2^--N, and Cr^{6+}

Materials and Methods

Sulfur master culture reactor (SMCR)

By operating the SMCR in a fed-batch mode, the SMCR had a consistent supply of SOB-attached sulfur particles. Aerobic, return-activated sludge was used as an inoculum, which had been collected from the Chuncheon Wastewater Treatment plant in Chuncheon City (Gangwondo, Korea). The SMCR contained 500 g of Sº (about 1-4 mm diameter) placed in a 1-L beaker and filled with 500 mL of tap water. Chlorine and chloramines in tap water were removed by air sparging and adding activated carbon (200 mg/L) before being added to the SMCR, and incubated at 30°C for four days in batch mode. After incubating for four days, the SMCR was operated in fed-batch mode (all the tap water was wasted and 500 mL of tap water was fed every two days). The SMCR was operated under aerobic conditions, using a stone diffuser with a flow rate of 1.5 L/min and incubated. A drop in pH and an increase in EC indicated SOB growth in the SMCR. The SMCR was operated for more than 30 days in fed-batch mode before being used in the online toxicity monitoring system.

Monitoring system construction and operation

The monitoring system was installed in a monitoring station near the Bukhan River in Chuncheon, Korea. Water was pumped from the stream to an influent tank at the station until it overflowed (water in the tank was the influent for the monitoring system). Three identical rectangular reactors (each reactor working vol.: 70 mL) were constructed from acrylic. The reactors were packed with 50 mL (82 g) of sulfur particles from the SMCR, and air was introduced from the bottom of the reactor at a flow rate of 200 mL/min. The reactors were placed inside an incubator maintained at 40 ºC. The SOB biosensors were operated in continuous and semi-continuous modes (described in detail below). A schematic diagram of the online toxicity-monitoring system, with its working module is shown in **Fig.1**. The pumps, valves, heaters, and EC meters were monitored or controlled using a programmable logic controller (PLC) and HMI software (SCADA, KDTSYSTEMS, Sungnam, Korea). The front panel of the PLC setup was designed with a touch screen, which allowed us to control the operation of the monitoring system.

Fig 1

One SOB reactor (R1) was operated in continuous mode, as previously described by (Van Ginkel et al., 2011). In this mode, stream water was continuously pumped to the SOB reactor using peristaltic pumps, which provided a hydraulic retention time of 30 min. The other two SOB reactors (R2 and R3) were operated in semi-continuous mode, as described by Gurung et al., (Gurung et al., 2012). In this mode, water was pumped to the SOB reactors at 60 mL/min. Reactor R2 received 0.5 min of rapid feeding with 30 min of reaction time with the SOB, while reactor R3 received 0.5 min of rapid feeding (begun 15 min after the reaction of R2 had begun) followed by 30 min of reaction time with the SOB. All three reactors were

operated and fed with relatively, unpolluted river water for more than six months to test the system. The biosensors were then fed with diluted swine wastewater (diluted 50:1) as a toxicant. In later experiments, the SOB biosensors were fed with either 30 mg/L NO_2^--N or 2 mg/L Cr^{6+} as toxicants. The reactions of the SOB (indicated by the decrease in EC) were calculated according to equation 1 (continuous mode) and equation 2 (semi-continuous mode):

In continuous mode:

$$\text{Toxicity} = [1 - \frac{EC_{out,t} - EC_{out}}{EC_{ss} - EC_{in}}] \times 100 \quad (1)$$

where, EC_{ss}= steady state EC value, EC_{in}= influent EC value, $EC_{out,t}$= EC at time 't' after injection of toxicant, EC_{out}= EC of effluent after injection of toxicant.

In semi-continuous mode:

$$\text{Toxicity} = \left[1 - \frac{EC_{slope,t}}{EC_{slope,\ no\ inhibition}}\right] \times 100 \quad (2)$$

where, if toxicity value < 0%, then toxicity= 0%.

Chemicals and analyses

All chemicals were of analytical grade. NO_2-N (in the form of $NaNO_2$, Sigma-Aldrich) and Cr^{6+} (in the form of $K_2Cr_2O_7$, Sigma-Aldrich) were used as toxic chemicals in the experiments. When NO_2^--N and Cr^{6+} were spiked to the influent, toxicity was monitored by measuring changes in EC. The reactors were also fed with diluted swine wastewater and the resulting effluent EC values were monitored by an EC meter (M-Cubic Cooperation, Daejeon, Korea). EC values and toxicities were automatically recorded at 2 min intervals. Chemical oxygen demand (COD) and biochemical oxygen demand (BOD), total nitrogen (TN), total phosphate (TP), total soluble salts (TSS), and volatile soluble salts (VSS), ammonia (NH_4^+-N)

were measured according to the standard methods (APHA, 1998). Concentrations of Cl^-, NO_3^--N, NO_2^--N, SO_4^{2-}, and PO_4^{3-}-P were measured using an ion chromatograph (ICS-900, Dionex, USA). The concentrations of dissolved heavy metals such as Cr^{6+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, and As^{2+} were measured by using ICP-AES (OPTIMA 7300 DV, PerkinElmer, USA).

Results and discussion

Physicochemical parameters of Bukhan river water and swine wastewater are shown in Table 1. The pH and EC of Bukhan stream water were 7.7 ± 0.68, and, 98 ± 5 μS cm^{-1}, respectively. The NH_4^+-N, TN, NO_3^--N, and COD were 0.104 ± 0.047, 1.9 ± 0.102, 1.26 ± 0.25 and 2.7 ± 0.5 mg L^{-1}, respectively. However, heavy metals, such as Cr^{6+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, As^{2+}, and other organic pollutants such as phenol, chloroform, dichloromethane, benzene, ethyl benzene, toluene, dibromochloromethane, 1.4-dioxane, tetrachloroethylene, carbon tetrachloride, trichloroethylene, tetrachloroethylene, were not detected. Pollutants and contaminants, including heavy metals were below established water-quality standards or were not detected in the stream water. In contrast, swine wastewater had high COD and BOD as 12,000~20,000 and 4,500-7,500 mgL^{-1}, respectively. NH_4^+-N, T-P, Cl^-, TSS, and VSS were 2,500~4,000, 400, 2,000~2,500, 3,000~5,000, and 2,500~3,600 mgL^{-1}, respectively. Moreover, toxic heavy metals, such as Cr^{6+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, and As^{2+} were 0.08, 2.7, 1112.3, 0.005, 1.4, 0.31, and 0.01 mgL^{-1}, respectively.

Table 1

The toxicity monitoring system was operated for more than six months with stream water as influent. However, the SOB system could not detect any toxicity in the relatively-

unpolluted natural stream water. Pollutants and contaminants, including heavy metals were below established water-quality standards or were not detected in the stream water (Table 1).

Typical effluent EC values in the SOB reactors for either 12 hours or 1 month are shown in **Fig 2A and 2B**, respectively. For the R1 reactor operated under continuous mode, the effluent EC value remained constant at app. 0.9-1.10 mS/cm, while the influent EC values were 0.2~0.25 mS/cm. This means that there was oxidation of sulfur to sulfate by SOB. In the R2 and R3 reactors, during the period of rapid feeding (0.5 min) EC values sharply decreased to app. 0.56~0.6 mS/cm upon mixing with influent since influent EC is much lower than ECs in reactors 2 and 3. During the 30-min reaction, EC increased linearly to 1.37~1.47 mS/cm (R2) and to 1.55~1.67 mS/cm (R3).

Fig 2

After the SOB reactors had been operating for more than six months, diluted swine wastewater (50:1, stream water to wastewater) was pumped into the reactors. **Fig 3A** shows the effect of swine wastewater on SOB activity in the reactors under in the continuous and semi-continuous modes. The diluted swine wastewater was toxic to SOB since EC declined as soon as the wastewater was introduced into the reactor. The toxicity of diluted swine wastewater could be explained by the existence of toxic heavy metals and other pollutants such as COD, NH_4^+-N, Cl^-, and heavy metals (Table 1). In reactor R1, EC in effluent decreased to that of the influent within about one hour (influent EC of diluted swine wastewater was 1.4 mS/cm). This increase in influent EC could be explained by the presence of ions in diluted swine wastewater. In reactors R2 and R3, EC also declined after adding the diluted, swine wastewater. In all the reactors, the EC of the effluent reached the EC of the influent (diluted swine wastewater) within approximately one hour; thereafter, the EC value stabilized until stream water (without swine wastewater) was pumped into the reactors. The SOB reactors were

187 filled with diluted swine wastewater for nearly two hours and were then filled with unpolluted
188 stream water (i.e., without swine wastewater). In less than one hour after filling with unpolluted
189 stream water, the EC of the effluent increased in all reactors, while the EC of the influent
190 declined to match that of the stream water and then stabilized. These results indicate the
191 possibility of the recovery of the SOB biosensor after addition of stream water. Similar results
192 were observed with Gurung et al., (2011) who, revealed that the SOB biosensor could be
193 recovered from the toxicity of the textile industry effluent after dilution (100:1) as the
194 concentration of toxic chemicals is very low, thereby reducing the toxicity.

195 **Fig 3**

196 As determined by the slope-intercept calculation (equation 2), we found diluted swine
197 wastewater was toxic to SOB. That is, we found that when the diluted, swine wastewater was
198 added to reactor R2, EC decreased from approximately 22 µS/cm/min to 12 µS/cm/min within
199 one hour (indicating a die-off of SOB). In reactor R3, EC decreased from 30 µS/cm/min to 10
200 µS/cm/min within an hour. In continuous operating mode, swine wastewater was also very
201 toxic to SOB, reaching almost 100% **(Fig. 3B),** while in the R2 and R3 reactors (semi-
202 continuous operation), the maximum rate of toxicity attained was approximately 80%. These
203 results reveal that SOB is very sensitive to contaminants in aquatic environments. After the
204 swine wastewater experiment, the reactors were filled with only stream water to clean the
205 reactors. Sulfur particles in the reactors were then replaced with sulfur from the SMCR to
206 prepare for the next experiment.

207 After resumption of pumping of stream water to the reactors (to obtain a baseline EC),
208 Cr^{6+} (toxicant) was introduced to the reactors. **Fig 4 (A** and **B)** shows the effect of Cr^{6+} on SOB
209 activity. As soon as 2 mg/L Cr^{6+} was added to the influent, the EC of the effluent gradually

decreased in all reactors (**Fig. 4A**). In the R1 reactor (continuously-fed), approximately 50% of the SOB activity was inhibited after four hours of operation. In the R2 and R3 reactors (semi-continuously fed), EC decreased sharply after introduction of Cr^{6+} to the system. The effluent's EC equaled the influent's EC in all the reactors after six hours of operation. Cr^{6+} was more toxic to SOB than swine wastewater because the recovery period was quite prolonged after spiking with Cr^{6+}. Percent toxicity also increased immediately after the reactors were fed with the toxicant. The toxicity value was higher in the R2 reactor than in the R1 and R3 reactors, but in all the reactors, toxicity persisted even after the system was fed with relatively unpolluted stream water.

Fig 4

After the reactors were again returned to baseline condition for the next experiment (flushing with stream water and adding sulfur particles from the SMCR), the system was spiked with 30 mg/L NO_2^--N. **Fig 5** shows the effect of NO_2^--N on SOB activity. As soon as the NO_2^--N was spiked to the reactors, SOB activity was inhibited (as evidenced by continuous decline in EC in the effluent EC) (**Fig. 5A**). In reactor R1, the addition of NO_2^--N decreased the EC of the effluent until it matched the EC of the influent EC after about 3-4 hours of operation. Similarly, in both reactors R2 and R3, EC rapidly declined until the EC of the effluent matched that of the influent (within approximately four hours). The pattern of toxicity (SOB) response) to NO_2^--N was very similar to that of the SOB response after to Cr^{6+} addition. NO_2^--N caused an average toxicity of approximately 95% to the SOB (**Fig. 5B**). The impact of NO_2^--N on toxicity persisted for 4-5 hours even after relatively unpolluted stream water had been flushed through the reactors.

Some known heavy metals such as Cr^{6+} and Cu^{2+} cause cellular toxicity related to protein damage, oxidative damage, DNA damage, and membrane leakage (Abskharon et al., 2010;

Van Ginkel et al., 2011). Toxic effects of NO_2^--N or Cr^{6+} could be explained by the free, protonated, undissociated form (HNO_2, $H_2Cr_2O_7$). The pKa value of HNO_2 is 3.1, while, $H_2Cr_2O_7$ has two pKa's: -0.2 and 6.51. When pH < pKa, these acids are predominantly protonated and capable of entering the bacterial cell and deprotonate at the higher pH inside the cell. This raises intracellular $H^+$and; acts as an uncoupler, thereby weakening the proton motive force for energy conservation (Ma et al., 2010; Van Ginkel et al., 2011). The high sensitivity of the SOB biosensor at low concentrations could be related to its low operating pH. In addition, under acidic conditions, NO^+ or NO radicals form spontaneously from NO_2^-. The susceptibility of bacteria to the toxicity of NO_2^- results fom the formation of metal nitrosyl complexes that occurs when NO^+ or NO radicals interact with bacterial enzymes (Zumft, 1993). The nitrosyl complexes are more toxic than that of NO_2^--N itself (Van Ginkel et al., 2011).

Fig 5

The sulfur particles in the reactors must be replaced with the sulfur particles in the SMCR when toxic chemicals are introduced to the reactors and SOB activity is inhibited. **Fig 6** shows the typical change in EC after the sulfur particles were replenished. Right after the replenishment, the pattern of EC became more or less irregular, but EC maintained a stable, baseline pattern showing no lag phase.

Fig 6

In recent years, biomonitoring systems have been developed to monitor water quality in real-time in aquatic environments (Kholodkevich et al., 2008; Storey et al., 2011). Despite recent advances in both biomonitoring capabilities and communication technologies, there is as yet no universally-accepted monitoring procedure for assessing water quality and detecting toxic substances in aquatic environments (Storey et al., 2011; Bae and Park, 2014). Although

257 many biomonitoring methods incorporate microsensors (Waich et al., 2007) and use
258 nanotechnology (Riggs et al., 2011) to make real-time measurements of toxicity, these
259 approaches are not at a developmental stage where they can be readily installed in on-line
260 monitoring stations (Storey et al., 2011). Existing toxicity technologies are either expensive or
261 complicated and await improvements before being universally adopted (Kholodkevich et al.,
262 2008; Storey et al., 2011). In contrast, our SOB system is simpler to initiate, more sensitive to
263 pollutants, easier and more economical to operate, and relatively more inexpensive than
264 currently available commercial, toxicity-monitoring systems. We believe that the
265 biomonitoring system we created and tested can be integrated into water safety and security
266 networks as a component of an early-warning, online system that could rapidly alert water
267 managers of the presence of toxic chemicals in water.

268 **Conclusions**

269 In this study, an online toxicity monitoring system based on SOB was developed and operated
270 for a real-time assessment of water quality of the natural stream water. The important
271 conclusions are highlighted below:

272 1. An online toxicity monitoring system based on SOB was operated successfully for
273 more than six months during which the system could not detect any toxicity in the
274 stream water due to very low concentrations of toxic chemicals in the water.

275 2. When the reactors were fed with diluted swine wastewater, SOB activity was inhibited
276 by approximately 90% within one hour under both a continuous and semi-continuous
277 mode of operation, which could be explained by the presence of different toxicants in
278 the swine wastewater.

3. The addition of 2 mg/L Cr^{6+} or 30 mg/L NO_2^--N to the influent resulted in the complete inhibition of SOB activity under both a continuous and semi-continuous mode of operation, leading to average toxicity of more than 80%.

4. The recovery period for SOB with the non-polluted stream water is longer in response to Cr^{6+} pollution than the response to diluted swine wastewater and NO_2^--N.

5. Immediately after replacement of the sulfur particles in the reactors with sulfur from the SMCR, EC maintained a stable, baseline pattern showing no lag phase.

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Figures

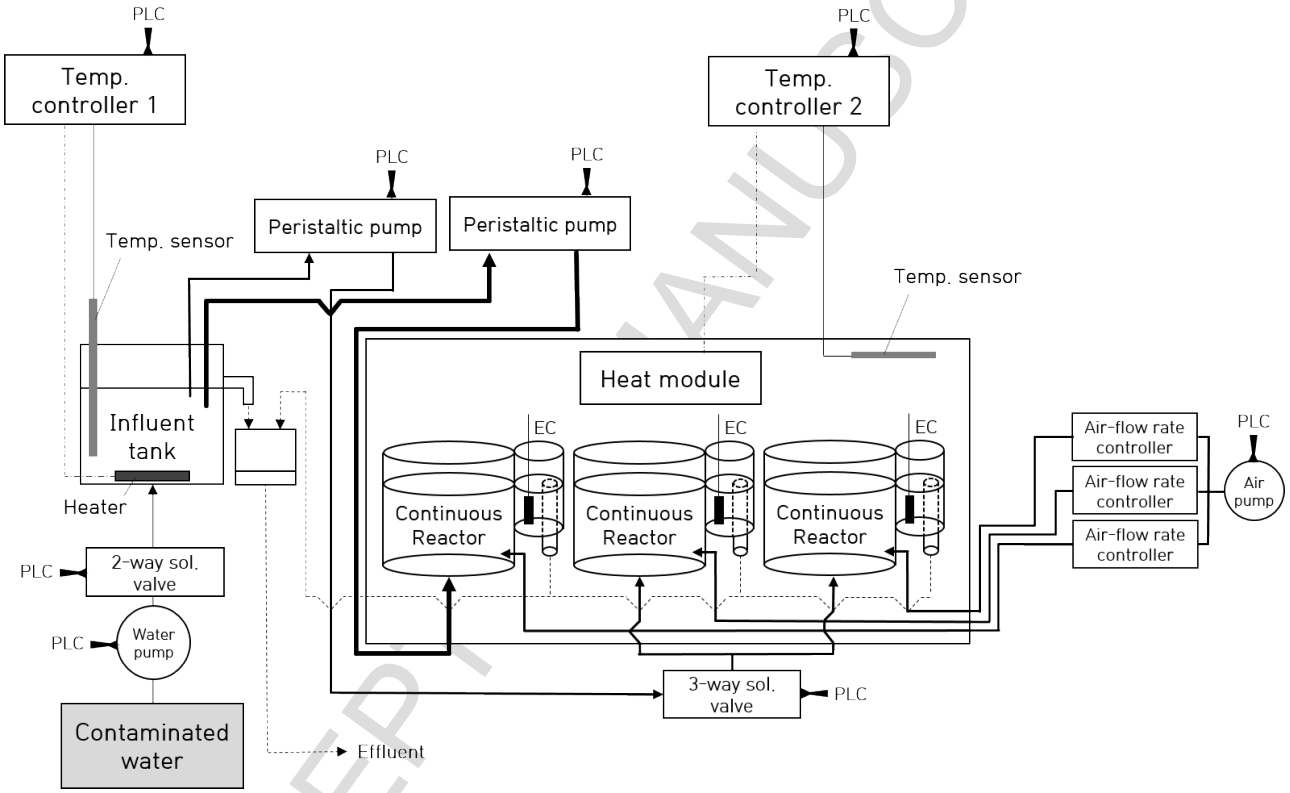
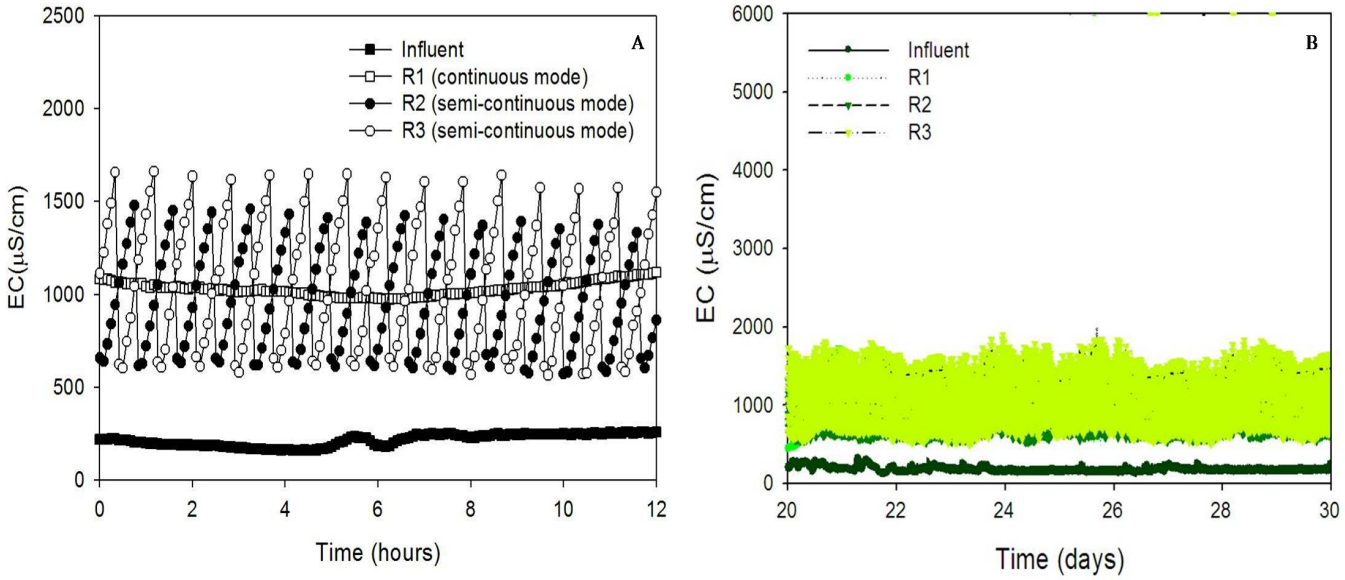


Fig.1. A schematic representation of SOB biosensor.



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413 **Figure 2.** Typical results showing the stabilized EC values of SOB reactors: (A) 12 hours and
414 (B) 1 month.

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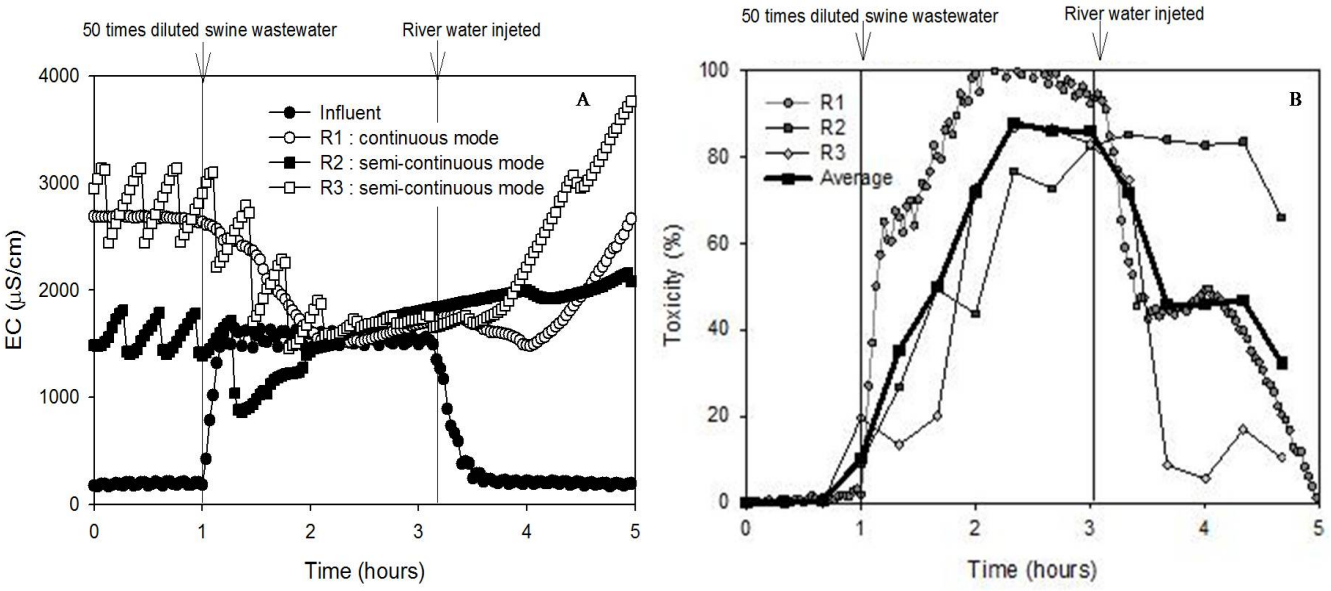
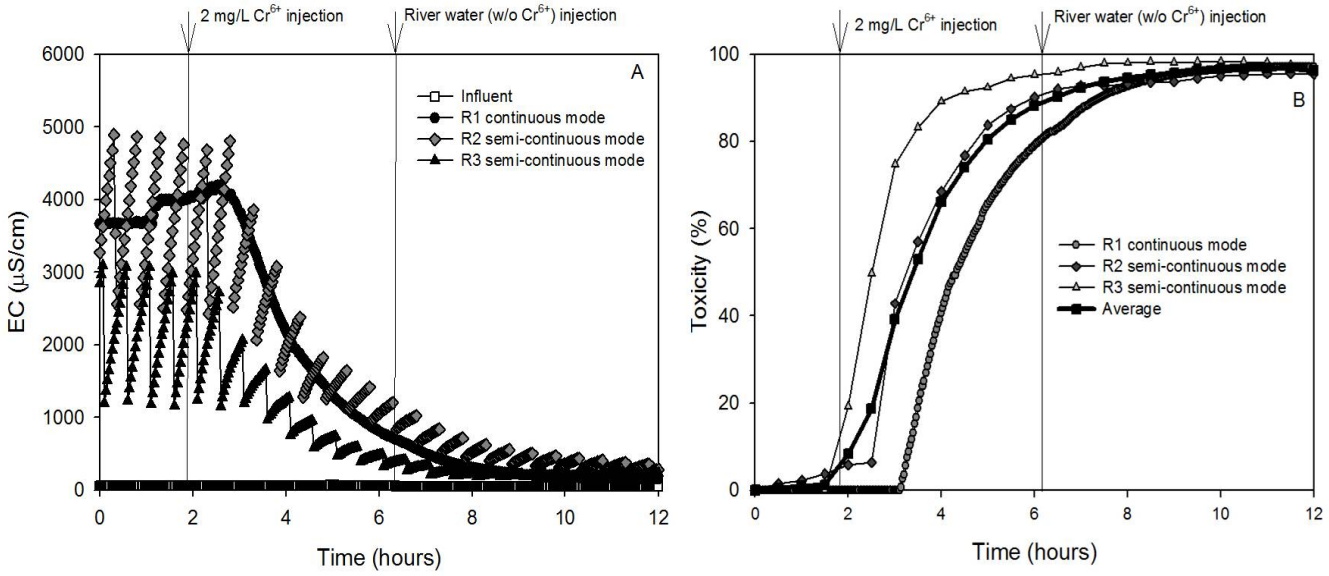


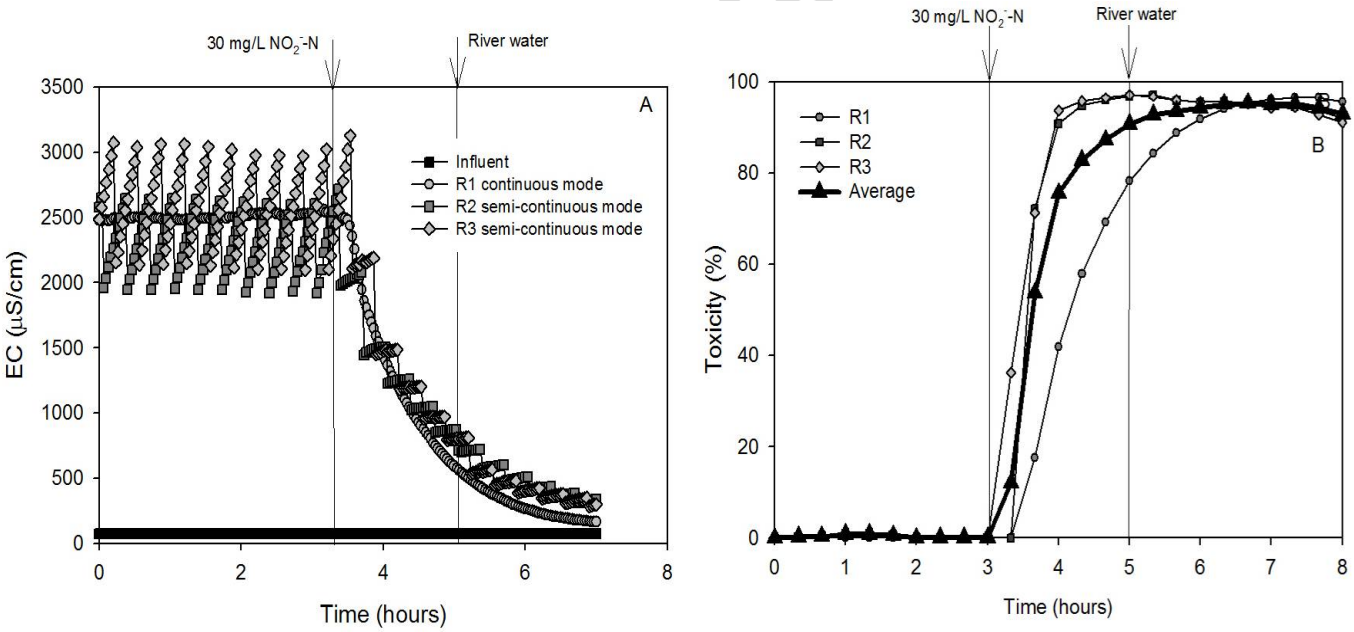
416

417 **Figure 3**. Responses of SOB to diluted swine wastewater: (A) effect on EC and (B) toxicity
418 due to swine wastewater.



419

420 **Figure 4.** (A) Effect of Cr^{6+} on EC and (B) toxicity of Cr^{6+} to SOB.



421

422 **Figure 5**. (A) Effect of NO_2-N on EC and (B) toxicity of NO_2-N on the SOB activity.

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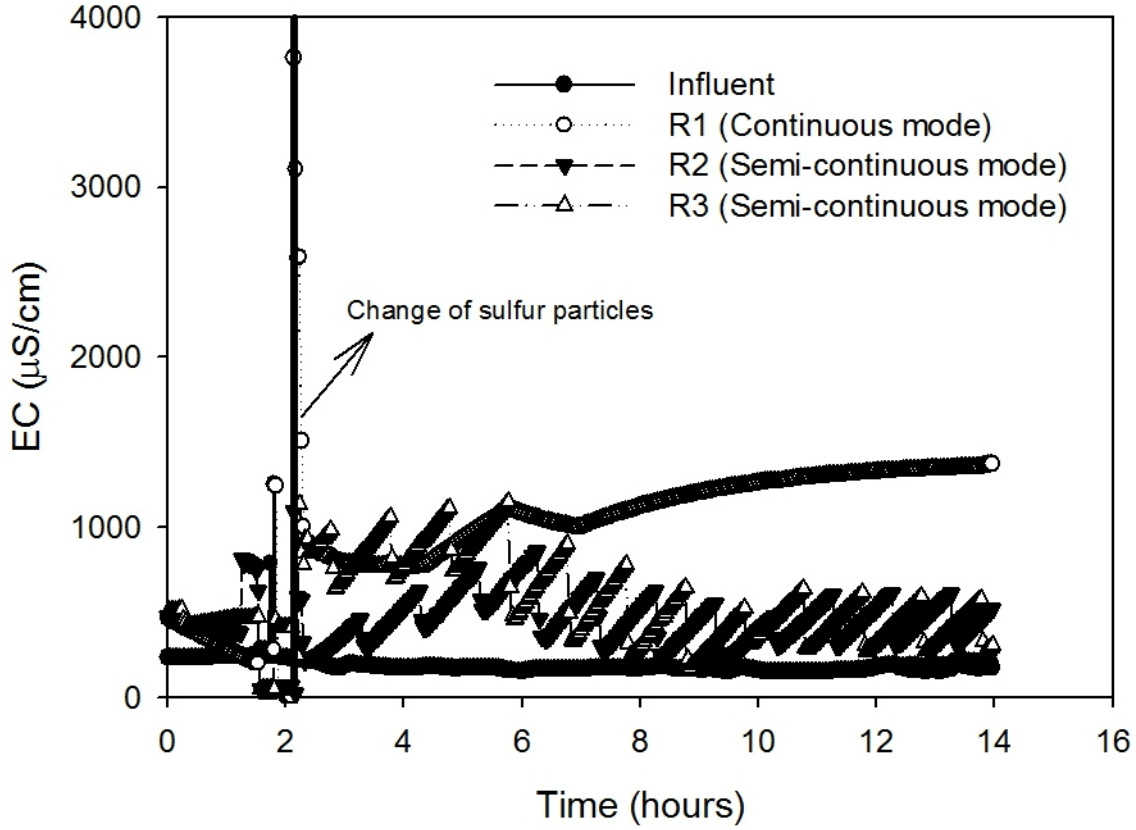

Figure 6. Typical results showing the effects of changing the sulfur particles in the reactors.

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Table 1. Physicochemical parameters of Bukhan river and swine waste water.		
Parameters	Bukhan river	Swine wastewater
pH	7.7 ± 0.68	8.3±0.53
EC (μS cm^{-1})	98 ± 5	-
TSS (mg L^{-1})	-	3000-5000
VSS (mg L^{-1})	-	2500-3600
NH_4^+-N (mg L^{-1})	0.104 ± 0.047	2000-4000
COD (mg L^{-1})	2.7 ± 0.5	12000-20000
Cl^- (mg L^{-1})	29 ± 2.6	2000-2500
T-N (mg L^{-1})	1.9 ± 0.102	3000-4500
NO_3^--N (mg L^{-1})	1.26 ± 0.25	-
T-P (mg L^{-1})	0.013 ± 0.008	400
BOD (mg L^{-1})	1.4 ± 0.1	4500-7500
TOC (mg L^{-1})	1.9 ± 0.5	-
Cr^{6+} (mg L^{-1})	ND	0.08 ± 0.001
Zn^{2+} (mg L^{-1})	ND	2.7 ± 0.03
Cu^{2+} (mg L^{-1})	ND	112.3 ± 1.3
Cd^{2+} (mg L^{-1})	ND	0.005 ± 0.0001
Ni^{2+} (mg L^{-1})	ND	1.4 ± 0.01
Pb^{2+} (mg L^{-1})	ND	0.31 ± 0.03
As^{2+} (mg L^{-1})	ND	0.01 ± 0.0001
Mg^{2+} (mg L^{-1})	-	39.5 ± 0.4
Ca^{2+} (mg L^{-1})	-	27.2 ± 0.3
ND= not detected., - = not available		

- Biomonitoring of toxic chemicals is the most effective method in aquatic ecosystems.
- Real time monitoring system based on sulfur oxidizing bacteria has been developed.
- The SOB biosensor can detect toxic chemicals in continuous & semicontinuous modes.
- The addition of 2 mg/L Cr^{6+} to SOB biosensor results in complete inhibition of SOB.