Environmental Science
Processes & Impacts

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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POSSIBILITY OF USING A LITHOTROPHIC IRON-OXIDIZING MICROBIAL FUEL CELL AS A BIOSENSOR FOR DETECTING IRON AND MANGANESE IN WATER SAMPLES

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Running title: Using a lithotrophic iron-oxidizing MFC as an iron sensor

Keywords: microbial fuel cell, bioelectrochemical systems, iron biosensor, iron bacteria, iron oxidation, chemolithotrophs

Abstract

Iron-oxidizing bacterial consortia can be enriched in microbial fuel cells (MFCs) operated with ferrous iron as the sole electron donor. In this study, we investigated the possibility of using such lithotrophic iron-oxidizing MFC (LIO-MFC) systems as biosensors that monitor iron and manganese in water samples. When operated with anolytes containing only ferrous iron as the sole electron donor, the experimented LIO-MFCs generated electrical currents in response to the presence of Fe^{2+} in the anolytes. For the concentrations of Fe^{2+} in the range of 3-20 mM, a linear correlation between the current and the concentration of Fe^{2+} could be achieved ($r^2 = 0.98$). The LIO-MFCs also responded to the presence of Mn^{2+} in the anolytes but only when the Mn^{2+} concentration was less than 3 mM. The presence of other metal ions such as Ni^{2+} or Pb^{2+} in the anolyte reduced the Fe^{2+}-associated generation of electricity of the LIO-MFCs at various levels. Organic compounds, when present at a non-excessive level together with Fe^{2+} in the anolyte, did not affect the generation of electricity, although the compounds might serve as alternative electron donors for the anode bacteria. The performance of the LIO-MFCs was also affected at different degrees by operational parameters, including surrounding temperature, pH of the sample, buffer strength and external resistance. The results proved the potential of LIO-MFCs as biosensors sensing Fe^{2+} in water samples with a significant specificity. However, the operation of the system should be in compliance with an optimal procedure to ensure a reliable performance.

Introduction

In rural areas in developing countries (such as Vietnam), having no access to public water supply, people have to use water from underground sources without being aware of its quality. According to Winkel et al. (2011), of more than 16 millions people living on the Red River delta areas in northern Vietnam, 11 millions have no access to clean water [1]. There is a high chance that water from underground sources can be contaminated with metals such as iron and manganese. For example, also according to Winkel et al. (2011), 44 % of the water wells used by the mentioned people exceeds the WHO Fe and Mn guidelines (3 mg L^{-1} for Fe and 0.4 mg L^{-1} for Mn, as recommended by WHO). Exposure to elevated levels of these metals can cause several physiological malfuctions, particularly on nerve systems [2]. Currently, the detection of these toxic metals is based on chemical methods that can be done only in laboratories or by using kits and is thus time-consuming, not environment-friendly or not cost-effective. Thus an on-site biological device to detect metals such as iron and manganese in water sources would be contributive to a sustainable life of people in rural areas in developing countries.

A microbial fuel cell (MFC) based system can be a potential candidate for such a biological detector. A microbial fuel cell is a bioelectrochemical system where microorganisms catalyze electrochemical reactions to convert chemical energy comprised in electron donors to electrical energy [3, 4]. Due to this unique property, the electrical current produced by a MFC is relatively proportional to the concentration of substrates. By taking advantage of this phenomenon, Kim et al. (2003) have proposed MFC systems that can work as biosensors for monitoring biological oxygen demand (BOD) of wastewater [5, 6]. Similar systems to monitor the amount of organic

compounds in wastewater influents have been also reported recently [7, 8]. It should be noted that the bacterial community enriched in such a MFC is highly specific to the substrate supplied. For instance, in a MFC system where the substrates are rich in nutrients (high BOD values), the bacteria enriched are mostly copiotrophic [6]. MFCs enriched with these microorganisms can not measure low BOD values. In contrast, oligotrophic bacteria are specifically enriched in MFCs fed with low BOD artificial wastewater [9], enabling these systems to measure low BOD values. Molecular ecology analyses showed that the bacterial communities enriched in the two types of MFCs are distinctively different from each other [10]. In other studies where specific substrates, such as formate, acetate, or some other specific volatile fatty acids were used, the bacterial communities enriched are highly substrate-specific [8, 11, 12]. Thus, the possibility of enriching substrate-specific microbial communities in MFCs and using those MFCs as biosensors detecting special compounds appears convincing and promising. Moreover, such biosensors can have the advantages of MFC systems in general: (i) feasible on-site operation due to flexible sizes and operational procedures of MFCs; (ii) reusability, i.e. environment-friendliness, and thus (iii) cost-effectiveness. These advantages will certainly enable MFC-based biosensors to outcompete other sensing technologies based on chemical methods.

In a recent study, iron-oxidizing bacterial consortia were also specifically enriched in our MFC systems that can be operated with only Fe^{2+} as the sole electron donor [13]. These systems, designated as lithotrophic iron-oxidizing MFCs (LIO-MFCs), exhibited characteristics that can be exploited for detecting iron and manganese. Therefore, in this research, we attempt to investigate (i) whether the LIO-MFCs can be used as biosensors monitoring iron and manganese in water samples and (ii) factors that may affect their performance.

Materials and methods

The lithotrophic iron-oxidizing MFCs (LIO-MFCs) used in this study were developed by enriching neutrophilic iron-oxidizing bacterial consortia in modified NCBE-typed MFC reactors [13].

Fabrication of the MFCs [13]

Each reactor consisted of two large poly-acrylic frames (12 cm × 12 cm × 2 cm) and two small poly-acrylic rectangle-holed subframes of anode and cathode compartments (8 cm × 8 cm × 1.5 cm) (Fig. S1). The dimension of each rectangle hole on each subframe was 5 cm × 5 cm and thus each compartment had the dimension of 5 cm × 5 cm × 1.5 cm. Each compartment was filled in with graphite granules (3-5 mm in diameter), used as the electrode material, and packed enough so that the granules well contacted with each other and with a graphite rod (5 mm in diameter) to collect the electrical current. This rod penetrated the large frame of each compartment via a drilled hole (5mm in diameter) and stuck outside. The gaps between the rod and the frame were sealed up by epoxy glue to ensure that the compartment is closed. Also, for this purpose, rubber gaskets were placed between the poly-acrylic parts when the reactor was assembled. A 6 cm × 6 cm Nafion 117 membrane (Du Pont, USA) was used to separate the two compartments of each reactor. Each reactor was assembled using nuts and bolts penetrating holes at 4 corners of each large frame. Anode and cathode graphite rods were connected to crocodile clamps and through wires to a shared external resistor (of 10 ohm unless otherwise stated) and to a multimeter.

For the influent and effluent (of anolyte or catholyte), 2 holes (5 mm in diameter) were created on the large frame of each compartment and PVC pipes were sealed to them. The anode influent pipe was inserted with a three-way connector before

connected via a drip chamber to a bottle containing modified M9 medium (0.44 g KH_2PO_4 L^{-1}, 0.34 g K_2HPO_4 L^{-1}, 0.5 g NaCl L^{-1}, 0.2 g $MgSO_4.7H_2O$ L^{-1}, 0.0146 g $CaCl_2$ L^{-1}, pH 7) [14].

Operation of the MFCs [13]

The reactors were operated in batch mode at room temperature (25 ± 3 ^oC) (unless otherwise stated). Before a batch, the M9 medium bottle was sterilized, cooled and purged with nitrogen (Messer, Vietnam) for 30-60 min. to minimize the amount of oxygen, which potentially competes with the anode to accept electrons. To start a batch, a $FeCl_2$ solution (the source of ferrous ions) was syringed, together with a trace element solution (with the recipe following Clauwaert et al. (2007) [14]), into the anode compartment of each MFC through the three-way connector on the anode influent pipe (Fig. S1). The supplied volume and the concentration of the $FeCl_2$ solution were calculated so that the final concentration of Fe^{2+} in the anolyte will be as desired. The volume of the trace element solution was also calculated so that its final proportion in the anolyte was 0.1 % (v/v). Subsequently, sterilized and nitrogen-purged M9 medium was sucked from the containing bottle, with a syringe, and pumped into the anode compartment, also through the three-way connector. The volume of the pumped-in medium was calculated such that half of the anolyte was replaced (approx. 10 mL). Finally, a $NaHCO_3$ solution (the carbon source), was supplied into the anode compartment, in a similar manner, such that its final concentration in the anolyte was 2 g L^{-1} [14]. This sequence of supplying the components of the anolyte ensures that ferrous carbonate precipitate was not formed (experimentally checked, data not shown).

The cathode compartment of each MFC reactor contained only a buffer solution without any catalyst (0.44 g KH_2PO_4 L^{-1}, 0.34 g K_2HPO_4 L^{-1}, 0.5 g NaCl L^{-1}). At the

beginning of each batch, this catholyte was renewed completely. During a batch, the cathode compartment was aerated, through the cathode influent pipe, with an air pump (model SL-2800, Silver Lake, China) to supply oxygen, the final electron acceptor. The aeration rate was adjusted to be slightly above 50 mL min^{-1} to ensure that the catholyte was air-saturated [15] but did not evaporate fast.

A batch run was considered to start from the moment the anolyte was replaced in the device and lasted until when the current dropped down to the baseline (ca. 0.1 mA). The duration of such a batch was usually 2 hours. Each reactor was operated at least 3 batches per day (with 1 hour being the interval between 2 consecutive batches) and left standby during the night time. (This mode of operation did not affect the stability in the performance of the reactors).

Enrichment of iron-oxidizing bacteria in the MFCs [13]

Several MFC reactors were set up in this study. One MFC was not initially inoculated with any microbial source (designated as the biotic control, which is different from the abiotic control described below). Other MFCs, hereinafter designated as the lithotrophic iron-oxidizing MFCs (LIO-MFCs), were inoculated with a bacterial source (an inoculum) from a natural mud taken from a brownish water stream at the depth of 20 cm underneath the stream bottom, in Ung Hoa, Hanoi, Vietnam.

The inoculation was carried out in the first 3 days, during which the inoculum was daily supplemented into the anode compartment of each reactor (except the control) and the reactors were operated with 20 mM of Fe^{2+}. The inoculum was prepared by mixing 1 mL of sterile M9 medium with the pellet (after centrifuged at 4000 × g, for 5 min.) of 2 mL of the original bacterial source (the mud). After day 3, the reactors were operated without supplementation of inocula.

During the enrichment period (the first 4 weeks), all the MFC reactors were operated in the manner mentioned above with 20 mM of Fe^{2+} supplied into each anode compartment and the generation of electricity was monitored. After 4 weeks, neutrophilic iron-oxidizing bacterial consortia were successfully enriched in the MFC reactors [13] and the generation of electricity by the MFCs was stable. These functioning LIO-MFCs were subsequently used for experiments in this study.

In order to prove that the generation of electricity in the MFCs was not due to plain chemical reactions, an abiotic control was set up. The abiotic control was a reactor of the same MFC type, with the anode compartment (including the electrode but not the membrane) sterilized (at 121 ^{o}C, 1 atm, for 20 min.) before assembled with a brand-new membrane and the cathode compartment. After assembling, the anode compartment (then including the membrane) was washed 3 times with sterilized M9 medium and subsequently tested with different concentrations of Fe^{2+} during the first 3 hours after washing. That is, under such conditions, the anode compartment of this reactor is almost abiotic, having no or few microbes (already checked by plating, data not shown).

Measurement and calculation of electrical parameters

A digital multimeter (model DT9205A+, Honeytek, Korea) was used to measure the voltage between the anode and the cathode of each MFC. Electrical parameters (current I (A), voltage U (V), charge Q(C) and resistance R (Ω)) were measured and/or calculated according to Aelterman et al. (2006) and Logan et al. (2006) [4, 16]. Unless otherwise stated, all the values of average currents and charges reported in this study were the results of at least 3 repetitions.

Experiments with different concentrations of ferrous iron

To investigate the Fe^{2+}-sensing capability and the detection limits of the LIO-MFCs, three of them were operated as described above but in their anolytes, different concentrations of Fe^{2+} were tested, including 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 and 50 mM. In parallel, for comparison, the biotic control and the abiotic control were also tested with 5, 10, 15 or 20 mM of Fe^{2+} in their anolytes.

Starvation experiment

In order to test the endurance of the LIO-MFCs to starvation, those at their steady state were not fed, i.e. their anolytes were not renewed, for a period of time. After that period, they were fed and operated again as usual, i.e. with 20 mM of Fe^{2+}. The tested periods of starvation included 7 days, 14 days and more than 14 days (15-21days).

Tests with Manganese

A LIO-MFC was operated as described but with its anolyte containing only Mn^{2+} as the sole electron donor, at different concentrations varying from 0.1, 0.3, 0.6 and 1 mM to 2, 3, 4 and 5 mM. (5 mM of Mn^{2+} is stoichiometrically equivalent to 10 mM of Fe^{2+} because Mn^{2+} can be oxidized to Mn^{4+}). After these tests, the MFC was operated again with only Fe^{2+} (20 mM) as the electron donor.

Specificity experiments

For tests with Ni^{2+} and Pb^{2+} (two potential alternative metallic electron-donors), a LIO-MFC was operated as described above, but with an anolyte containing 20 mM of Fe^{2+} and either Ni^{2+} or Pb^{2+} (by adding the corresponding chloride salt into the anolyte). The concentration of the other metal ion varied from its prevalent concentration in groundwater to higher levels (in the range equivalent to 20 mM of Fe^{2+}). According to that, the tested concentrations of Ni^{2+} were 0.1, 0.2, 0.5, 0.7, 1, 2 and 5 mM, while those of Pb^{2+} were 0.0006, 0.006, 0.06, 0.6, 6, 10 and 50 mM. After

tested with the highest concentration of the other metal, the LIO-MFC was operated again with only 20 mM of Fe^{2+}.

For tests with organic compounds as potential alternative electron donors, a LIO-MFC was operated as described above, but with an anolyte containing 20 mM of Fe^{2+} and an organic substance (acetate or lactate) (by adding the corresponding sodium salt into the anolyte) as potential electron donors. It was also operated with an anolyte containing only the organic matter as a potential electron donor. Two concentrations of the organic matter were tested, including: the prevalent concentration in groundwater (corresponding to 50 ppm COD (chemical oxygen demand)) and the concentration stoichiometrically equivalent to 20 mM Fe^{2+}. Thus, our calculation showed the tested concentrations of acetate were 0.8 mM and 2.5 mM and those of lactate were 0.52 mM and 1.7 mM.

A LIO-MFC was even operated with an anolyte containing 20 mM of Fe^{2+} and a mixture of glucose/glutamate with a BOD (biological oxygen demand) concentration of 50 ppm, 200 ppm or 500 ppm, or with an anolyte containing only that mixture. 50 ppm BOD is the common BOD content that groundwater may be contaminated with. 200 ppm and 500 ppm were two representative BOD values of heavily-contaminated water to be tested.

Experiments testing the effects of operational parameters

To test the effect of pH of the sample, a LIO-MFC was operated with half of the anolyte being the M9 medium and the other half being a "sample solution". This is also our intended mode of operation if the MFC is to be used for practical measurement. The sample solution contained 40 mM of Fe^{2+} so that the final Fe^{2+} concentration in the anode chamber was 20 mM as usually tested. The MFC was

tested with different sample solutions of various pH values, including 2, 5, 7, 9, 11 and 13. The pH of the sample solution was adjusted by using NaOH 1N or HCl 1N.

To test the effect of buffer strength, a LIO-MFC was tested with various buffer strengths of both the anolyte and the catholyte. Accordingly, the tested buffer concentrations included 50 (the usual concentration), 5 and 0.5 mM.

To test the effect of surrounding temperature, a LIO-MFC was operated at different temperatures by being placed in temperature-controlled chambers. The tested temperatures included 13, 20 and 23 ^{o}C as low temperatures; 35 and 38 ^{o}C as moderate temperatures; and 40, 42 and 47 ^{o}C as high temperatures.

To investigate the effect of external resistance, a LIO-MFC was operated with different resistors at its external circuit. Resistors of various magnitudes, including 5, 10, 50, 100, 500 and 1000 ohm were tested.

Data analysis

All the experiments, unless otherwise stated, were repeated three times. Data were analyzed using basic statistical methods: differences in data were evaluated by *t*-Test analysis; errors among replicates were expressed in the form of standard deviations.

Results

Correlation between the generation of electricity and the concentration of ferrous iron in a lithotrophic iron-oxidizing MFC

The lithotrophic iron-oxidizing MFCs (LIO-MFCs) used in this study were developed by enriching iron-oxidizing bacterial consortia in modified NCBE-typed MFC reactors from a natural microbial source and with a modified M9 medium containing only Fe^{2+} (20 mM) as the sole electron donor [13]. These LIO-MFCs could generate stable electrical currents in the range of 0.4-0.6 mA (depending on each MFC) after

two weeks of operation, and harbor neutrophilic iron bacteria in their anode chambers [13]. In order to evaluate the performance of these LIO-MFCs as potential sensors detecting iron, they were operated with different concentrations of Fe^{2+}. It is noticeable that the change of the current generated by a LIO-MFC was corresponding to the change of the concentration of Fe^{2+} supplied (Fig. 1), and so was that of the per-batch amount of charge (Fig. S2). Particularly, the current and the charge generated by a LIO-MFC were well proportional to the concentration of Fe^{2+} from 5 mM to 20 mM, no matter whether the concentration of Fe^{2+} was tested in an ascending direction or a descending direction. The response time of the MFC (i.e. time for the current to reach a steady state in any test) was about 60 sec. when the concentration of Fe^{2+} was step increased. When the concentration of Fe^{2+} was step decreased, the response was usually only clear after a period of one batch run (Fig. S3). If no Fe^{2+} was present in the anode buffer, the MFCs generated almost no current (data not shown). The above-described phenomena were not observed for MFC 1, a biotic control uninoculated but probably containing bacteria contaminating from surroundings, as well as for the abiotic control with a sterilized anode chamber containing no bacteria (Fig. 1).

These results confirm that the generation of electricity by the LIO-MFCs is indeed due to the iron-oxidizing activity of the bacteria enriched in their anodes and suggest that the LIO-MFCs could be potentially used as sensors to detect iron (via detecting Fe^{2+}), and even to measure the amount of ferrous iron (within a range) in a water sample.

Detection limits of a lithotrophic iron-oxidizing MFC for Fe^{2+}

It can be seen from the results (Fig. 1) that when the concentration of Fe^{2+} was over 20 mM, the linear correlation between the generated electricity and the concentration of Fe^{2+} was no longer applicable and the current tended to be stable or reduced. Thus

20 mM can be considered as the upper limit concentration of Fe^{2+} that the LIO-MFCs can measure.

By testing concentrations of Fe^{2+} from 1 to 5 mM, we observed that the LIO-MFCs did not respond to the change of the concentration of Fe^{2+} when the latter was below 3 mM (Fig. 1, inlet). A correlation between the current generated and the concentration of Fe^{2+} appeared only when the latter was 3 mM or above. Therefore, 3 mM was determined as the lower detection limit of the devices for Fe^{2+}.

Starvation and recovery

Three LIO-MFCs were subjected to starvation (being fed without Fe^{2+} in the anolyte) for 7 days or 14 days or more. The MFCs appeared to generate electricity again and still responded well to the concentration of Fe^{2+} after starvation no matter whether the starvation period was 7 days or 14 days (Fig. 2). However, if the starvation lasted for more than 14 days, the generation of electricity could not be restored (data not shown).

Figure 2

These results suggest that the LIO-MFCs can endure a starvation and can recover (restore their capability of generating electricity) after the starvation, which should not last for more than 14 days.

The responses of a lithotrophic iron-oxidizing MFC to manganese

Based on the theory that iron-oxidizing bacteria can also oxidize manganese, our hypothesis was that a LIO-MFC could also detect and sense Mn^{2+} and thus one LIO-MFC was tested with different concentrations of Mn^{2+} (as the sole e^- donor) in the anolytes. A proportional relationship between the generated current and the concentration of Mn^{2+} was observed only when the concentration of Mn^{2+} was not more than 3 mM (Fig. 3). Indeed, above that concentration, the current decreased as the concentration of Mn^{2+} increased (Fig. 3). When the MFC was operated again with

Figure 3

Fe^{2+}, the generation of electricity could be restored. Even when the same concentrations of Mn^{2+} were tested in the anolyte containing also Fe^{2+} (20 mM), similar results were observed (data not shown).

These results suggest that bacteria in the LIO-MFCs can possibly use Mn^{2+} as an electron donor (or as a "fuel") as expected but the upper detection limit for Mn^{2+} is pretty low (3 mM).

Specificity of a lithotrophic iron-oxidizing MFC in respect of sensing Fe^{2+}

A LIO-MFC was tested with an anolyte containing Fe^{2+} and another metal ion, either Ni^{2+} or Pb^{2+}, which are usually present in groundwater and could possibly act as alternative electron donors to Fe^{2+}. As can be seen in Fig. 4, the more Ni^{2+} was present in the anolyte, the lower the current generated by a LIO-MFC was. When the MFC was fed again with only Fe^{2+} and without Ni^{2+}, the current could not be restored to the previous levels. In the case of Pb^{2+}, at low concentrations (less than 10 mM), this ion did not cause reductions of electricity generation, but had an effect similar to that of Ni^{2+} at concentrations of over 50 mM and the effect was not reversible, either (Fig. 4). These results suggest that the two metal ions did not act as competing electron donors but possibly as inhibitors on the anodic microbes.

Acetate, lactate or a mixture of glucose and glutamate were tested in the anolyte of the LIO-MFCs in order to investigate whether organic compounds can act as potential alternative electron donors for the anode bacteria. The presence of acetate (at the concentration of 0.8 mM, corresponding to 50 ppm COD) in the anolyte already containing 20 mM of Fe^{2+} did not lead to any increase of the electricity generation of a LIO-MFC (Fig. 5). When only acetate was present in the anode influent, the current decreased (Fig. 5). The decrease was even more in the case the concentration of acetate was higher (at 2.5 mM, stoichiometrically equivalent to 20 mM of Fe^{2+}).

These results suggest that acetate can be a substrate but not a favorable one for the anode bacteria. This is more supported by the restoration of the current levels when the MFC was fed again with only Fe^{2+}. The tests with lactate, another organic acid, produced almost similar results (Fig. 5). The only difference is that the currents generated when the anolyte contained only lactate as the electron donor at 1.7 mM (stoichiometrically equivalent to 20 mM of Fe^{2+}) were equivalent to those in the case the anolyte contained only 20 mM of Fe^{2+} (Fig. 5). The generated current was still at the same level even in the case a LIO-MFC was tested with an anolyte containing Fe^{2+} (20 mM) and a glucose/glutamate mixture with its BOD value of 50 ppm or 200 ppm (Fig. 5). However, the presence of this mixture alone could result in improved currents, by ca. 33% when the anolyte BOD was 50 ppm and 42% when it was 200 ppm (Fig. 5). When the anolyte BOD was 500 ppm, the current increased 50-60%, no matter whether Fe^{2+} was present or not (Fig. 5). All the results above suggest that the microbial consortium in the anode of a LIO-MFC can use organic compounds as electron donors/substrates but it still seems to specifically favor Fe^{2+} if the concentration of organic compounds is not high. Thus the presence of organic compounds, if not at excessive levels, in the anode did not interfere with the generation of electricity from the oxidation of Fe^{2+}.

Effects of operational parameters on the performance of a lithotrophic iron-oxidizing MFC

Our intended method of operating the MFC as a sensor is to combine one volume of the sample with one volume of the M9 medium (without electron donors) in an anolyte. In such a manner, the anolyte is still buffered. However, it is still intriguing to study how changes of the pH of the sample may affect the performance of the LIO-MFCs. As can be seen in Fig. 6 (A), the pH of the sample did not significantly affect

Figure 6

the generation of electricity by a LIO-MFC. However, it was clear that samples with pH values falling in the range of 7-9 could lead to about 20% higher levels of currents in comparison with those with other pH values ($p < 0.05$) (Fig. 6 (A)).

In order to save the material cost, the buffer strength might be reduced and thus it is at first necessary to investigate how more diluted buffers affect the performance of a LIO-MFC. As can be seen in Fig. 6 (B), a 10-fold diluted buffer only reduced the generation of electricity by about 15%. Thus, the effect of the buffer strength did not appear to be critical.

For practical applications, it is important to investigate how the surrounding temperature affects the performance of a LIO-MFC. As can be seen in Fig. 6 (C), surrounding temperatures lower than 30^{o}C or higher than 40^{o}C significantly reduced the current generated by a LIO-MFC ($p < 0.05$). The optimal surrounding temperature for the MFC appeared to be around 35^{o}C (Fig. 6 (C)). The level of the currents generated at this optimal temperature was 3 times higher than that under temperatures lower than 30^{o}C and 2 times higher than that under temperatures higher than 40^{o}C.

In most MFC studies, it is also essential to investigate what external resistance is appropriate to enable an optimal performance of a LIO-MFC as a Fe^{2+} sensor. It was evident that the higher the external resistance was, the lower the current could be generated, but the relationship between these two parameters was not merely inversely linear. With the resistances higher than 50 ohm, the level of the current was significantly low (lower than 0.15 mA) ($p < 0.05$) and less reduced as the resistance increased.

The results reported above suggest that surrounding temperature and external resistance seriously affect the generation of electricity of a LIO-MFC while pH of the sample and buffer strength only had mild effects.

The stability in performance of the LIO-MFC

After 12 months of operation, a reduction of about 25% of the current generated by a LIO-MFC could be observed (Fig. S4). However, the responses of the system to changes of Fe^{2+} or other factors in the anolyte still followed the same tendencies as described above (data not shown).

Discussion

The potential use of lithotrophic iron-oxidizing MFCs as biosensors to detect Fe and Mn

In the term of iron sensing, it is clear from the results that our LIO-MFCs could produce electrical currents only when ferrous iron was present and that a linear correlation between the current and the concentration of Fe^{2+} could be applied within the concentration range of 3-20 mM (r^2 = 0.98). Such a linear correlation was also observed in BOD sensor type MFCs for the BOD concentration range from 0-200 ppm [5, 6]. Thus, it is solid that the LIO-MFCs can be used to detect iron in water samples (based on the appearance of electrical current). The presence of ferrous iron will reflect the presence of iron the samples. The presence of iron in a water sample usually indicates the presence of other metals [1]. Thus the detection of iron by the LIO-MFCs can be also regarded as a warning about the presence of other metals in a water sample. The good linear correlation mentioned above suggests that the MFCs also have a potential to be used as biosensors to monitor iron, although several limitations need to be overcome, as discussed below.

Although the linear current-[Fe^{2+}] correlation could be achieved, it can be seen that the levels of the currents generated by different MFCs are not always the same. In addition, as mentioned earlier, the current of a LIO-MFC may decrease after a significant time of operation (e.g. 12 months), although the tendency of response is

unchanged. Thus, it is obvious that for any LIO-MFC to be applied for detecting iron, a calibration before measurement is compulsory. This is also because under a certain circumstance, operational parameters (temperature, pH,…) can also affect the generation of electricity of the MFCs, as shown by the results. Another precaution is that measurement should always be repeated (at least 3 times as practiced in this study) to ensure a reliable accuracy, since the response time of the system was longer when the concentration of Fe^{2+} was decreased. Indeed, similar response time observations were reported elsewhere for other MFC systems [17, 18].

As reported previously, the iron concentration in groundwater, for example in Vietnam, can reach 140-160 mg L^{-1}, equivalent to 2-3 mM [1]. The Fe^{2+} detection range of the LIO-MFCs in this study (3-20mM) might thus not be ideal for monitoring the iron content in groundwater, in general. However, the MFCs can be used particularly to detect waters over-polluted with Fe. Further improvements are needed in order to lower the lower detection limit of the LIO-MFCs.

Regarding the capability of the LIO-MFCs to detect Mn, although the results suggest that Mn^{2+} can be used as an electron donor by the bacteria in the systems, the narrow detection range for Mn is unexpected. There has been evidence that Mn^{2+} can exert inhibitory effects on bacteria, including iron bacteria [19, 20]. This could be an explanation for the poor responses of the LIO-MFCs to Mn^{2+} and even to Fe^{2+} when Mn^{2+} was also present, which may imply that the application of the MFCs for monitoring Mn is limited. Perhaps the neutrophilic iron-oxidizing bacteria enriched in the MFCs [13] are even more sensitive to Mn^{2+}. Nevertheless, it should be noted from the results that the effect of Mn^{2+} could be reversible.

It should be noted that the current generated by a LIO-MFC was significantly high (0.34 ± 0.035 mA) when the concentration of Mn^{2+} was 3 mM. Such a level of the

current is equivalent to those when higher concentrations of Fe^{2+} were tested. This phenomenon is possibly due to the higher affinity of the anode bacteria in the LIO-MFC to Mn^{2+} or the higher Mn^{2+}-oxidizing rate of these bacteria, although they might be more sensitive to Mn^{2+}. The fact that Mn^{2+} can be further oxidized up to Mn^{5+} or Mn^{7+}, while Fe^{2+} only to Fe^{3+}, might be also an explanation.

Considering factors affecting the specificity of the LIO-MFCs, our first suspicion was that other metal ions such as Ni^{2+} or Pb^{2+} might act as electron donors for bacteria in the LIO-MFCs, thus competing with Fe^{2+} and causing false positive electrical signals. However, this is not the case, as supported by the results. On the other hand, these metal ions appeared to have some inhibitory effects on the anode microbial consortia. The effects seemed irreversible; unlike in the case Mn^{2+} was tested. Toxic effects of heavy metals, including Ni and Pb, on bacteria have been reported [21, 22]. According to these reports, metabolic processes of bacterial cells and particularly their substrate utilization are significantly affected (reduced) under metal stresses. The effect of Ni also appeared to be more serious than that of Pb [21], similar to observations in this study (Fig. 4). These metal effects imply that the field measurement of Fe^{2+} by the LIO-MFCs can be seriously influenced by the presence of metals toxic to bacteria.

Another specificity-related issue might be that organic compounds present in water samples could interfere with the responses of the LIO-MFCs to Fe^{2+}, because in any bacterial consortium, it is highly possible to find some individual species with flexible metabolism that can utilize other electron donors. Thus, the fact that Fe^{2+} was the favored substrate or electron donor over organics such as acetate, lactate or BOD materials (when present at inevitably non-excessive levels) is astounding. This is because considering the redox aspect, ferrous oxidation was much less favored in comparison with the oxidation of organic substances [23]. Our hypothesis is that the

anode bacterial consortia in the LIO-MFCs were so specialized to adapt to lithotrophic electrochemical conditions that their switch to utilize energy-rich organic compounds is slow.

With respect to the effect of operational parameters, as shown by the results, pH of the sample, buffer strength, surrounding temperature and external resistance may affect the generation of electricity of the LIO-MFCs upon the feeding of Fe^{2+} at various degrees. Therefore, it is highly recommended that based on real conditions, adjustments (calibrations) should be done when using the levels of the currents to quantify the amount of Fe^{2+}. Similar effects of operational parameters on the performance of BOD sensor type MFCs have been discussed [18, 24, 25]. Particularly, Gil et al. (2003) reported similar effects of pH, buffer strength and external resistance [24]. Stein et al. (2012) also reported similar effects of external resistance and furthermore showed that its magnitude could also affect the response time and the recovery time of their MFC when challenged with toxic substances [25]. In our study, no matter what magnitude of the resistance was tested, the LIO-MFC always responded immediately (e.g. in less than 60 sec.) to any change in the concentration of Fe^{2+} in the anolyte. Thus, for the LIO-MFC, it is only necessary to select an external resistance that enables the generation of the highest current so that changes of the current are the most conceivable.

Our results, altogether suggest that a LIO-MFC may reach an optimal performance when operated at temperatures from 30-35^{o}C, with a phosphate buffer strength of 5 mM (to save chemicals), with a sample of pH 9 and with an external resistance of 10 ohm. Besides, as mentioned, in order to milden the effect of pH, we always supply buffer in the anolyte (at the ratio of 1:1 to the sample). Those optimal conditions may

not be fully practical but they can be used as references when applying the MFCs in practice.

Recently, novel systems that monitor the organic content or detect toxic substances of the anode influents have been also reported [7, 18, 26, 27]. However, there has been no research on a system for specifically detecting iron by using a specific iron-oxidizing bacterial consortium enriched from a natural source. Our study is therefore the first to report such a system. One of the toxicity detecting sensors mentioned above can respond to Cr^{6+} or Fe^{3+}, but the response is based on the inhibition of these metal ions to non-specific bacteria in the anode [26] and will therefore not be specific. Webster et al. (2014) reported a system, in which an engineered *Shewanella oneidensis* strain was used, for detecting specifically arsenic [28] but the use of such an axenic culture requires strict handling. Our LIO-MFC system, with a specific iron-oxidizing bacterial consortium enriched from a natural source, can have a specific response to Fe^{2+} and can be operated as an open system without special care.

Propositions to improve the performance of lithotrophic iron-oxidizing MFCs as iron biosensor

The first proposition is to replace the anode material. Due to our laboratory conditions, we could not test graphite felt as the anode material. Our current systems with graphite granules in the anode chambers appear to favor suspending bacteria that electrochemically function by self-produced mediators [13]. This may not ensure a steady operation of the system because when the anolyte is washed out, the number of acting bacterial cells decreases and so does the performance of the system. The MFC systems operated with graphite felts as anode materials usually harbor biofilms formed on their anode surfaces [29-31]. Such a biofilm would ensure a stable microbial community that can last long and have a steady function [32].

The second proposition can be to reduce the volume of the anode chamber. It has been reported that by reducing the volume of the anode chamber, the sensitivity and detection limit of a BOD sensor could be significantly improved [18]. The high lower detection limit of our LIO-MFCs for Fe^{2+} might be due to the fact that the volume of the anode chamber is still not small enough. Thus further experiments trying smaller volumes of the anode chambers are expected to expand the detection range of the MFCs.

Lastly, operating the LIO-MFCs in a continuous mode operation might be also a worth-trying proposition. Combining with the use of graphite felt as the anode material, the operation of the LIO-MFCs in the continuous mode should significantly improve its iron sensing capability. Operating MFCs in the batch mode always produce batch-type current patterns that may not be always consistent due to many affecting factors [24]. A continuous mode might ensure the generation of a continuous current that is stable (much less affected by environmental factors) and reflects the change of substrate concentration in the anolyte in a real-time manner [6].

In summary, in this study, we have demonstrated that with a proper procedure, including calibrations, a lithotrophic iron-oxizing MFC could be used as a biosensor sensing Fe^{2+} in water samples. The same application for manganese might be limited due to the significant inhibitory effect of manganese on the bacteria in the system. The iron sensing capability of the MFC has a significant specificity although the presence of other metals does affect the current. The system should be operated after optimizing operational parameters to ensure a good performance. Furthermore, further studies on the anode material, the volume of the anode chamber and the operational mode are required to warrant the application of the MFC as an efficient iron biosensor.

Acknowledgement: This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.03-2012.06. It also received some equipment support from Korea Institute of Science and Technology (KIST) IRDA Alumni Program and International Foundation for Science (IFS – Sweden) (grant number W-5186). The authors assure that there is no conflict of interest from any other party regarding the content of this paper.

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Figure legends:

Figure 1. The correlation between the electrical current generated and the concentration of Fe^{2+} fed to the anode of a LIO-MFC. Notes: 3 LIO-MFCs were tested. The biotic control was not inoculated with any microbial source at the beginning. The abiotic control had its anode chamber sterilized right before the experiments, in which different concentrations of Fe^{2+} were tested in only some hours after sterilization. Each MFC was operated with a 10 ohm external resistor, at 25 ^{o}C.

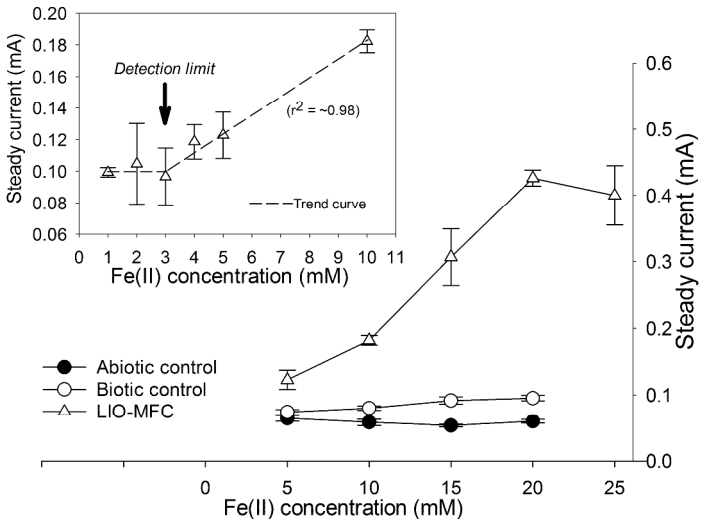
Figure 2. Effect of a 14-day starvation on the generation of electricity by a LIO-MFC and its recoverability after the starvation. Notes: During the starvation, the MFC was not fed. The MFC was operated with a 10 ohm external resistor, at 25 ^{o}C.

Figure 3. The electrical responses of a LIO-MFC to various concentrations of Mn^{2+} in the anolyte. Notes: Before and after testing, the MFC was operated with Fe^{2+} (20mM) and without Mn^{2+} in the anolyte. The MFC was operated with a 10 ohm external resistor, at 25 ^{o}C.

Figure 4. The effect of some metal ions (Ni^{2+} and Pb^{2+}) co-present in the anolyte. (Numbers in brackets indicate concentrations in mM). Notes: The MFC was operated with an anolyte containing Fe^{2+} (20mM) and another metal with the concentration indicated in each test. After the tests, the MFC was operated again with only Fe^{2+} (20mM). The MFC was operated with a 10 ohm external resistor, at 25 ^{o}C.

Figure 5. The effect of organic compounds present in the anolyte (with or without Fe^{2+}) on the generation of electricity of the LIO-MFCs. Notes: For a better comparison, the value of the current in each test was normalized to the percentage of the current before the test, i.e. when the tested MFC was operated with only Fe^{2+} (20mM) in the anolyte (default operation). (Numbers in brackets indicate concentrations in mM, except for that of BOD, which is in ppm).

Figure 6. Effects of different operational parameters on the performance of the LIO-MFCs. Notes: The MFCs were operated with 20 mM of Fe^{2+} in the anolytes. Unless changed for experimenting, the surrounding temperature was 25 ^{o}C and the external resistance was 10 ohm.



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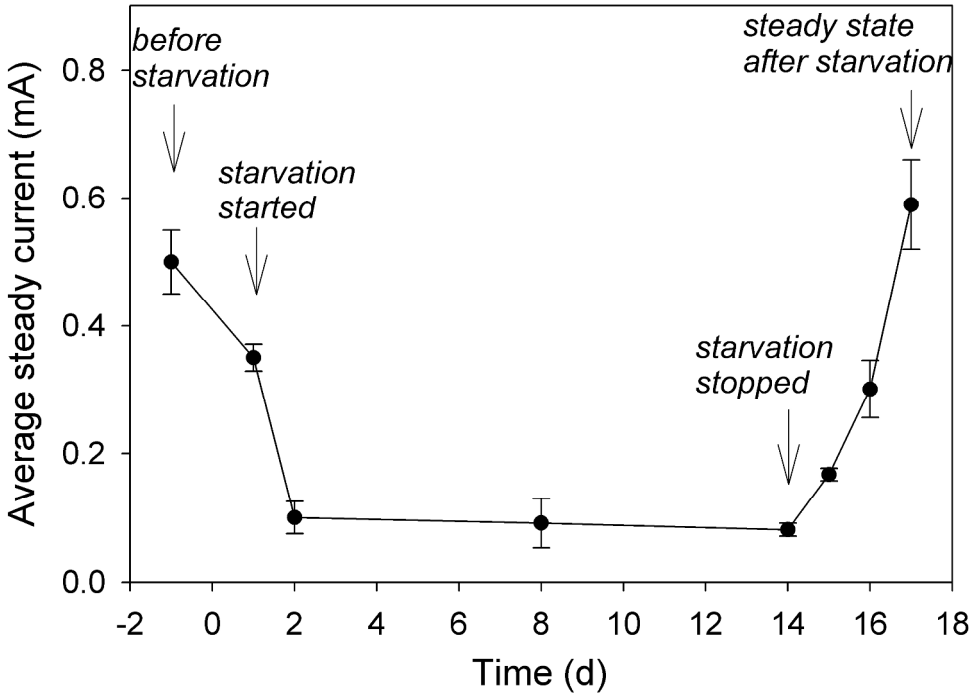

Effect of a 14-day starvation on the generation of electricity by a LIO-MFC and its recoverability after the starvation. Notes: During the starvation, the MFC was not fed. The MFC was operated with a 10 ohm external resistor, at 25 ^{o}C.
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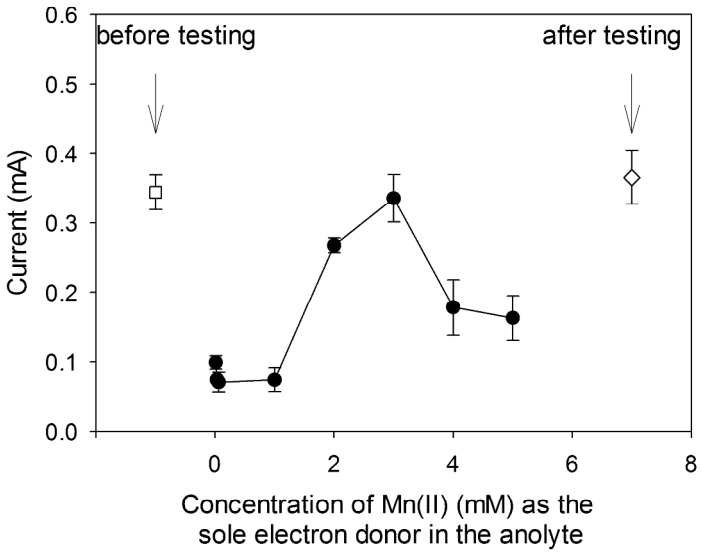

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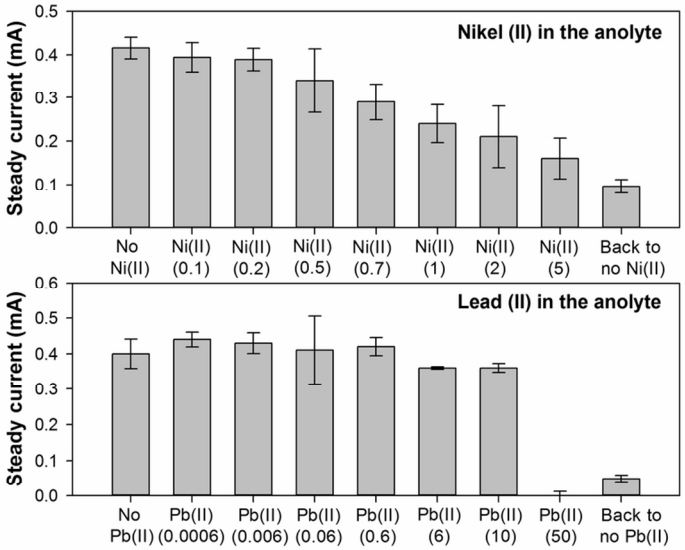
248x170mm (300 x 300 DPI)



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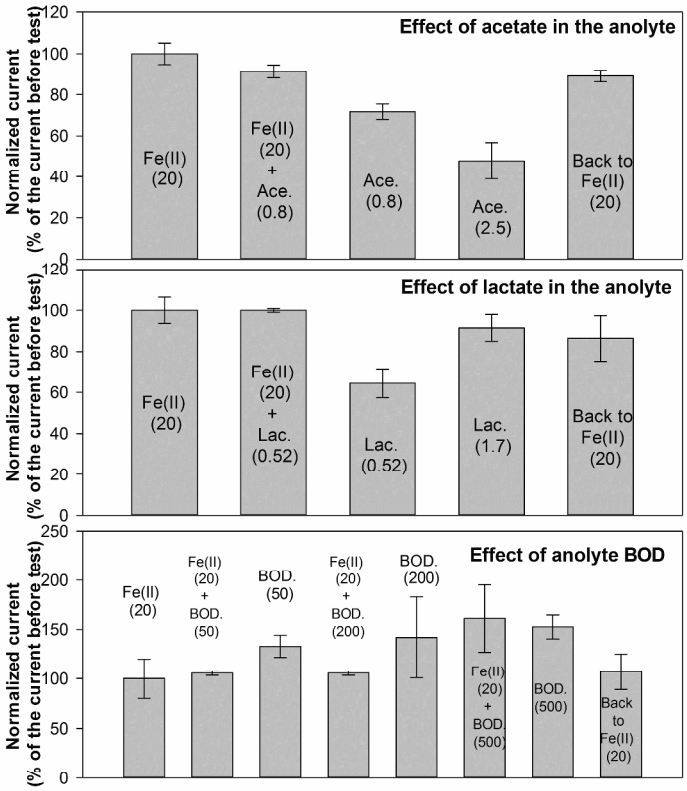

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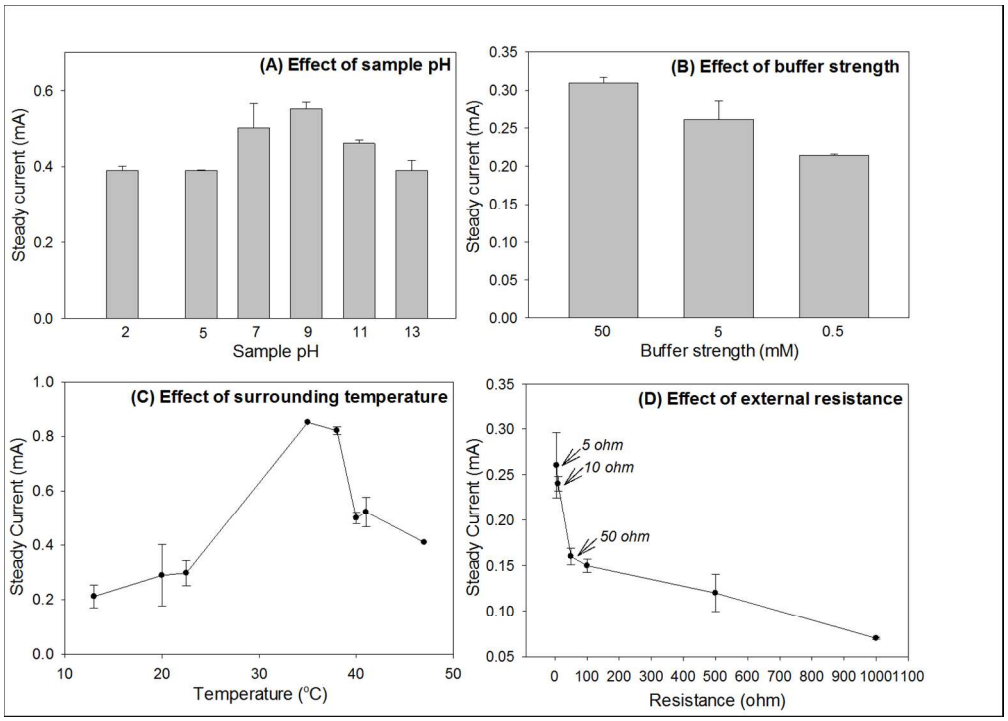

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267x190mm (150 x 150 DPI)