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A novel biosensor for *p*-nitrophenol based on an aerobic anode microbial fuel cell

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ABSTRACT

P-nitrophenol is one of the most common contaminants in chemical industrial wastewater, and *in situ* real-time monitoring of PNP cannot be achieved by conventional analytical techniques. Here, a two-chamber microbial fuel cell with an aerobic anode chamber was tested as a biosensor for *in situ* real-time monitoring of PNP. *Pseudomonas monteilii* LZU-3, which was used as the biological recognition element, can form a biofilm on the anode electrode using PNP as a sole substrate. The optimal operation parameters of the biosensor were as follows: external resistance 1000 Ω, pH 7.8, temperature 30 °C, and maximum PNP concentration 50 mg L^{-1}. Under these conditions, the maximum voltages showed a linear relationship with PNP concentrations ranging from 15 ± 5 to 44 ± 4.5 mg L^{-1}. Furthermore, we developed a novel portable device for *in situ* real-time monitoring of PNP. When the device was applied to measure PNP in wastewater containing various additional aromatic compounds and metal ions, the performance of the biosensor was not affected and the correlation between the maximum voltages and the PNP concentrations ranging from 9 ± 4 mg L^{-1} to 36 ± 5 mg L^{-1} was conserved. The results demonstrated that the MFC biosensor provides a rapid and cost-efficient analytical method for real-time monitoring of toxic and recalcitrant pollutants in environmental samples.

1. Introduction

P-nitrophenol (PNP), one of the most commonly used nitroaromatic compounds, is often found as a pollutant in chemical industrial wastewaters (Kitagawa et al., 2004; Shen et al., 2010; Takeo et al., 2008), potentially causing health and environmental damages due to its toxicity toward living organisms, its capacity to accumulate in food chains, and its persistence in different environments (Samuel et al., 2014). PNP has been listed as a priority pollutant by the U.S. Environmental Protection Agency and the European Union (Vincent, 1991). Several physical, chemical and biological techniques for treating PNP containing wastewaters have been reported (Kalbende et al., 2013; Peng et al., 2010; Samuel, et al., 2014). In order to apply these treatment processes adequately, analytical tools for real-time monitoring of PNP concentrations are required. Traditional PNP detection analytical methods usually focus on ultraviolet spectrometry, gas chromatography (GC), and high performance liquid chromatography (HPLC) (Chauhan et al., 2000; Yao et al., 1991). These methods are impractical for environmental samples because they involve complex pretreatment processes such as extraction, purification, and concentration. Furthermore, their use requires skilled technicians and results costly, time-consuming and unsuitable for *in situ* analysis (Raut et al., 2012). Therefore, developing user-friendly and cost- effective methods for *in situ* real-time monitoring of PNP is necessary.

Recently, biosensors have been widely used as promising tools for fast and selective detection of various analytes (Rodriguez-Mozaz et al., 2006). Traditional biosensors usually use fluorescence proteins or pigment molecules as indicators, which also introduces the need for costly transducers in order to generate measurable signals (Lei et al., 2006a). Other biosensors use purified enzymes (Lei et al., 2005, 2006b, 2007) and immobilized microorganisms (Lei et al., 2003; Mulchandani et al., 2002) as the biological sensing elements, and commercial electrodes as the transducers, which are relatively high cost and time-consuming (Xu et al., 2013). Therefore, an alternative biosensing technology based on microbial fuel cells (MFCs) was developed for *in situ* real-time environmental monitoring. This type of biosensor can directly generate a measurable electrical signal from substrates without the use of a signal transducer and external power source, resulting in cost-effective and time-saving detection processes (Sun et al., 2015).

MFC is an innovative technology for energy generation and pollution bioremediation using electrogenic bacteria as catalysts

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(Logan and Rabaey, 2012; Zhang et al., 2013). Since the electrical signal is proportional to the substrate concentration, MFCs can be applied as biosensors for environmental monitoring (Mathuriya and Yakhmi, 2014), such as biochemical oxygen demand (BOD) monitoring (Chang et al., 2005; Liu and Mattiasson, 2002), microbial activities assessment (Liu et al., 2011; Tront et al., 2008a), and chemical toxicants monitoring (Jiang et al., 2015; Stein et al., 2011). Furthermore, MFC-based biosensors have also been used to detect single substrates, such as glucose (Kumlanghan et al., 2007), acetate (Tront et al., 2008a), and lactate (Tront et al., 2008b). However, these biosensors have only been used to monitor easily degradable substrates under anaerobic conditions, which limits their applications for *in situ* monitoring, as their anode chambers need to be purged with nitrogen to maintain the anaerobic condition. To date, the use of MFCs as biosensors for recalcitrant pollutants monitoring under aerobic conditions is still scarce. PNP is a recalcitrant pollutant. In aerobic environments, it can be biodegraded completely to CO_2 by releasing nitrite through two oxidative pathways involving 4-nitrocatechol or hydroquinone (Zhang et al., 2009). Alternatively, PNP can easily be converted into more toxic aromatic amines through a six-electron transfer mechanism under anaerobic conditions (Razo-Flores et al., 1999). The toxic intermediates may affect the stability of the proton exchange membrane (Kumlanghan et al., 2007). It has been reported that PNP can be used as sole substrate in a two-chamber MFC for electricity generation by mixed cultures (Zhu and Ni, 2009). However, there is no study using pure cultures as anode catalysts and PNP as sole substrate for voltage generation in MFCs under aerobic conditions.

According to previous investigations, several aerobic PNP-degrading bacteria could be immobilized onto electrodes in microbial biosensors for PNP monitoring (Lei, et al., 2003; Mulchandani, et al., 2002). However, there is no specific reports on the use of MFC-based biosensors for PNP determination. In this study, we screened a strain named *Pseudomonas monteilii* LZU-3, which is able to degrade PNP aerobically and generate voltage in a two-chamber MFC with an aerobic anode chamber. The MFC was tested as a biosensor for PNP monitoring. The effects of different operation parameters on the performance of the biosensor were investigated including external resistance, anode biofilm, pH, temperature and PNP concentration. Furthermore, the characteristics of the biosensor were also studied in terms of stability and accuracy. In addition, we developed a portable PNP monitoring system based on the correlation between the maximum voltages and the PNP concentrations, which can achieve *in situ* real-time monitoring of PNP in wastewater. To our knowledge, this is the first report that uses an aerobic strain in a two-chamber MFC with an aerobic anode chamber for *in situ* real-time monitoring of recalcitrant pollutants.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Successive enrichment method was employed to screen the PNP-degrading strains (Chen et al., 2014). The modified mineral salt medium (MS, 0.85 g L^{-1} $K_2HPO_4 0 \cdot 3H_2O$, 0.02 g L^{-1} KH_2PO_4, 0.09 g L^{-1} $MgSO_4 0 \cdot 7H_2O$ and 0.0239 g L^{-1} $FeSO_4 0 \cdot 7H_2O$) supplemented with 100 mg L^{-1} PNP was used as the enrichment medium (Samuel, et al., 2014). PNP was provided as a 10 g L^{-1} stock solution and filter-sterilized before use. Thirteen bacteria strains able to degrade PNP aerobically were isolated from the soil samples collected from the Lanzhou Reaches of the Yellow River. We also tested their abilities to generate voltage in a two-chambered MFC without adding any exogenous electron mediator. The strain LZU-3 was found to have the ability to generate voltage using PNP as a sole substrate. Furthermore, we identified the strain based on its 16S rRNA sequence (GenBank accession number: KP056323), which showed 99.8% similarity with *Pseudomonas monteilii*. The strain was then named as *Pseudomonas monteilii* LZU-3 (CCTCC M 2015454) and selected for further studies.

P. monteilii LZU-3 was first cultured in Luria–Bertani (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} sodium chloride) medium for 24 h at 30 °C and 180 rpm, and the cultures were then centrifugalized at 8000 g, for 5 min. The cell pellets were washed three times with MS medium and re-suspended in the same medium. The suspensions were maintained at a 1.35 optical density at 600 nm, and inoculated at a 10% (v/v) proportion into the anode chamber containing 100 mg L^{-1} PNP, maintaining the initial optical density at $\approx$0.2 at 600 nm. Depending on the time at which the anolyte color changed from yellow to colorless, appropriate sampling intervals were chosen and 3 ml of samples were removed to determine the cell growth and PNP concentration.

2.2. Biosensor design and operation

A two-chamber MFC was constructed as the biosensor. The MFC was made of two borosilicate glass bottles separated from each other by a proton exchange membrane (PEM, Nafion117, DuPont, USA). Each chamber was equipped with an injection cap, in which three distinct holes, 1 mm, 2 mm and 5mm diameters, were drilled. The 1 mm hole allowed a titanium wire through, the 2 mm hole allowed the reference electrode through, and the 5 mm hole allowed a temperature sensor through. The PEM was pretreated as described by Biffinger et al. (2008). The working volume of each chamber was 240 ml. The anode and cathode chambers were both designed with two inlets and one outlet. To maintain the constant oxygen supply in the anode chamber, the two inlets were covered with 8 layers of gauze and tightened with hollow caps. The two inlets of the cathode chamber were tightened with solid caps. For convenient sampling, the outlets of the MFCs were sealed with clamps. Carbon felt electrodes with a geometric surface area of 16 cm^2 were soaked in 1 M HCl and 1 M NaOH to remove possible metals and impurities before use (Bond and Lovley, 2003). The anode and cathode electrodes were connected with a resistor box to set a desired external resistance. Ag/AgCl reference electrodes (3 M KCl) were inserted into the anode chamber to monitor the anode potential. Before assembly, the MFCs were sterilized by autoclaving at 121 °C for 15 min. The titanium wires and pretreated carbon felt electrodes were also autoclaved, but the reference electrodes were sterilized using 75% ethanol before use. The anolyte was MS medium supplemented with a desired concentration of PNP as the sole substrate. The catholyte was a 100 mM potassium ferricyanide solution in 50 mM phosphate buffer (pH 7.0). To increase the conductivity of the anolyte, 300 µl of trace minerals (Balch et al., 1979) were added to it. The anode chamber was inoculated with 10% (v/v) of the prepared inoculums. Dissolved oxygen of the anolyte was determined using a portable dissolve oxygen meter (JPB-607A, Shanghai, China). The MFCs were placed on a magnetic stirrer and the temperature sensors were inserted into each chamber to maintain the temperature constant. To reduce the mass transfer resistance, the speed of the stir bars was controlled and maintained at 180 rpm. The voltage across the resistance was recorded at 1 min intervals by an 8-channel data acquisition apparatus connected to a computer.

A portable biosensor device was developed for *in situ* real-time monitoring of PNP, and an application was also created to control and monitor the biosensor automatically, which contained an Analogue-to-Digital conversion chip (AD) and a Micro-programmed Control Unit (MCU) (Fig. 1a). The AD converts the voltages to digital signals which can be read and analyzed by the

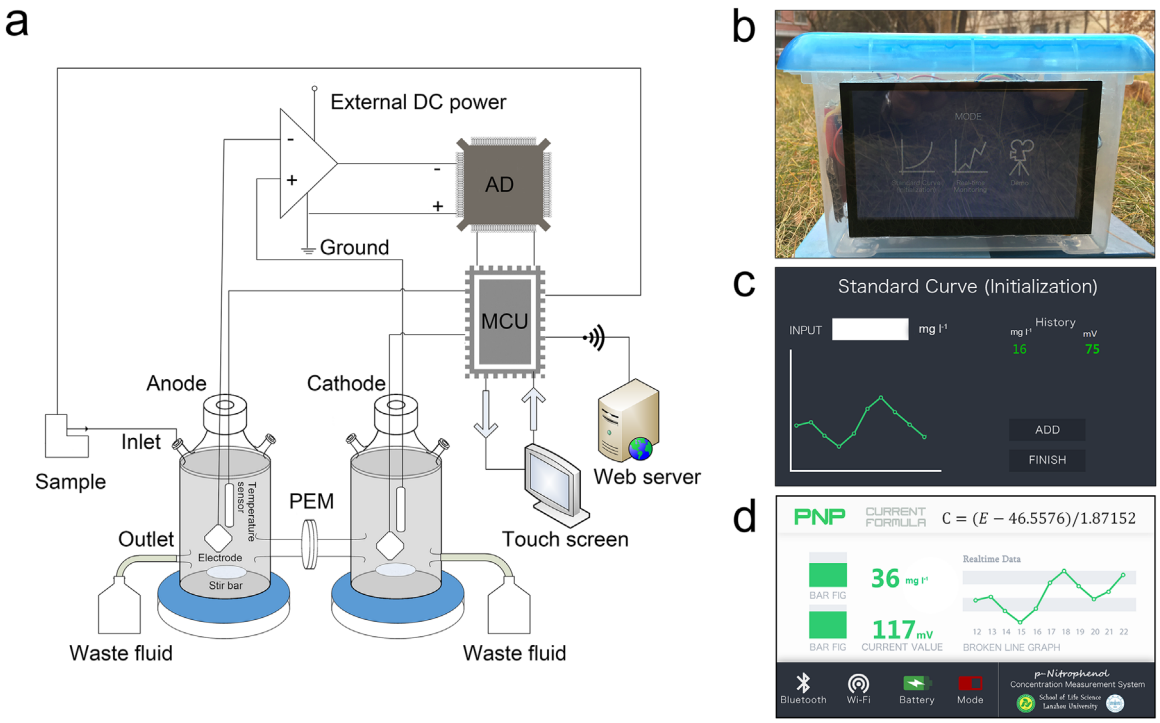

Fig. 1. Portable biosensor design and operation flowchart. (a) Schematic diagram of the MFC-based biosensor. AD: Analogue-to-Digital conversion chip; MCU: Micro-programmed Control Unit. (b) The operating desk of the biosensor. (c) Standard curve plotting desktop. (d) Real-time monitoring desktop.

MCU. The MCU analyses and records the maximum voltage, and when the voltage decreases to the baseline value, controls the wastewater discharging device, as well as the sample collecting device, automatically. Fig. 1b shows the operating desk of the biosensor. Under standard curve mode, when the different concentrations of PNP standard solutions were added to the biosensor, the MCU chooses the maximum voltages and fits a standard curve (Fig. 1c). The MCU can calculate the PNP concentration from the standard curve (Fig. 1d). Thereafter, when a sample is added to the biosensor, the MCU repeats the above procedures. Under WiFi or Bluetooth conditions, the data can be sent to the web server in real-time. Thereby, the biosensor can achieve *in situ* real-time monitoring of PNP.

2.3. Analytical methods

PNP concentrations were determined by UV spectrophotometry. In brief, a full-wavelength scan was performed between 200 and 800 nm. The resulting maximum absorption wavelength (400 nm), under alkaline conditions, was chosen. In our experiments, 50 µl samples were added to 3 ml NaOH (0.1 M), and the absorbance was then measured at 400 nm to determine the PNP concentration, using a standard curve.

According to Ohm's law, $I=V/R$, where I is the current, V is the determined voltage, and R is the external resistance. $P=VI$, where P is the power. The current density (mA m^{-2}) and power density (mW m^{-2}) were normalized to the anode geometric surface area (16 cm^2). Open-circuit voltage (*OCV*) is the maximum voltage obtained when the external resistance is infinite. The polarization curve was obtained by decreasing the external resistance from 100,000 to 100 Ω (Logan et al., 2006). Cyclic voltammetry (CV) analysis was performed to investigate the electron transfer mechanism. To carry out the CV measurements on the anode solutions, a conventional three-electrode system, with the MFC anode used as the working electrode, Ag/AgCl as the reference electrode, and a platinum wire as the counter electrode, was used. A potential range of −1.8–1.8 V at a scan rate of 5 mV s^{-1} was applied. For *in situ* CV tests on anode biofilms, the MFC anode, cathode, and an Ag/AgCl electrode were used as working electrode, counter electrode, and reference electrode, respectively. A potential range of −0.5–0.9 V at a scan rate of 5 mV s^{-1} was used. All CV analyses were performed using a potentiostat (CHI604E, Shanghai, China) and were each repeated three times.

Scanning electron microscopy (SEM) was employed to analyze the anode biofilm formation. A 0.5 cm × 0.5 cm section was cut from the carbon felt electrodes and placed in a 1.5 ml tube, and then fixed overnight in 2.5% glutaraldehyde in buffer at 4 °C. Subsequently, the samples were fixed with 1% osmium tetroxide in buffer for 2 h, and then dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 100%, and 100%). Finally, the samples were freeze-dried and coated with sputtering gold, and then viewed using SEM (S-3400, HITACHI, Japan) (Bond and Lovley, 2003).

2.4. Biosensor test

To evaluate the performance of the MFC, in addition to the experimental MFC, we also designed a non-inoculated control to eliminate the effect of the anolyte on voltage generation. In order to optimize the operation parameters of the MFC, the following experiments were carried out. Experiment 1: The effects of different concentrations of PNP on voltage outputs (50, 100, 200, 300, and 400 mg L^{-1}). Experiment 2: The effects of different pH (5.7, 6.4, 7.1, 7.8, 8.9, and 11.2) and temperatures (27, 30, 37, and 45 °C) on voltage outputs. The pH was adjusted using 1 M HCl or 1 M NaOH after the MFCs were assembled, because the metal ions of the anolyte could form precipitations with hydroxide ions at high temperatures, potentially decreasing its pH. All experiments were repeated three times and the mean values were calculated, while the error bars were also shown in the figures. Once the optimal operation parameters were determined, the linearity, stability, accuracy and application studies of the biosensor were performed under these conditions.

3. Results and discussion

3.1. Voltage generation and PNP degradation by P. monteilii LZU-3

P. monteilii LZU-3 was tested for its voltage generating and PNP degrading abilities using PNP as sole carbon and energy source in the MFC. The voltage gradually increased to a maximum value of 139 ± 9 mV (12.9 ± 0.05 mW m^{-2}) and the PNP concentration decreased from 100 ± 4.85 to 6.4 ± 3.5 mg L^{-1} in 12 h (7.8 ± 0.29 mg L^{-1} h^{-1}). In the non-inoculated control, both the voltage and PNP concentration remained unchanged over the whole cycle (Fig. 2a). The results demonstrated that the voltage generation was due to the PNP degradation and the growth (data not shown) of *P. monteilii* LZU-3. When the PNP concentration was below 6.4 mg L^{-1}, the voltage began to decrease gradually (Fig. 2a). According to the polarization curve obtained (Fig. 2(b)), the maximum power density, 13.06 ± 0.05 mW m^{-2}, was produced with an external resistance of 1000 Ω. Therefore, a resistance of 1000 Ω was selected for subsequent experiments. To determine the effects of biofilm formation on the voltage generation and PNP degradation in the MFC, the anolyte and catholyte solutions were replaced with fresh ones simultaneously. The results showed that the regeneration of voltage (Fig. 2c) and continuous degradation of PNP (Fig. 2d) could be maintained over five cycles. Scanning electron microscopy (SEM) images demonstrated that the microbes formed biofilms onto the exposed electrodes

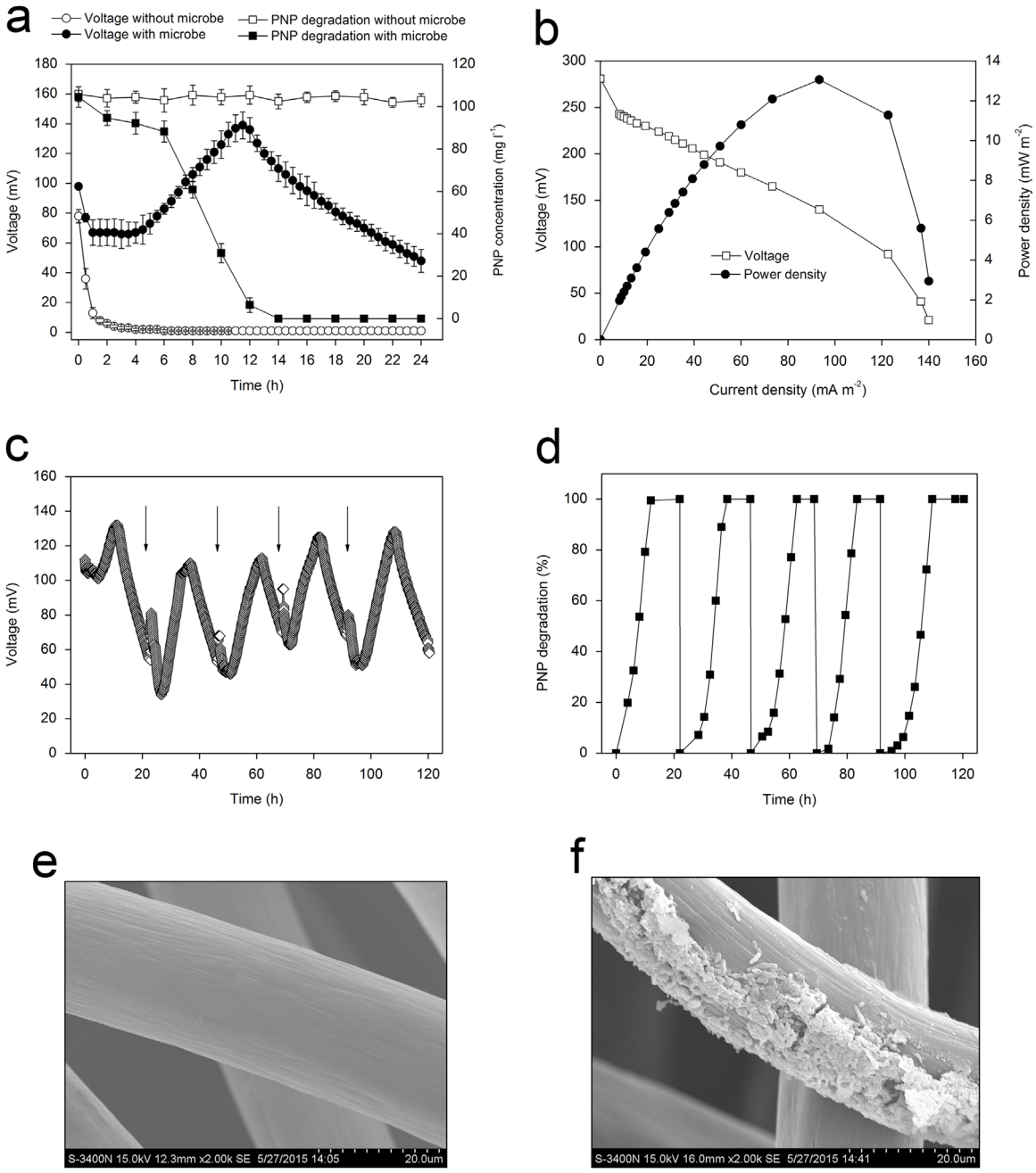

Fig. 2. Voltage generation and PNP degradation by *P. monteilii* LZU-3. (a) Voltage generation and PNP degradation in the MFC with an external resistance of 1000 Ω, under aerobic conditions. (b) Polarization and power density curves. The current and power densities were normalized to the anode geometric surface area (16 cm^2). (c) Voltage regeneration and (d) PNP continuous degradation in the MFC over a period of five cycles, with an external resistance of 1000 Ω, arrows indicate simultaneous replacement of both catholyte and anolyte solutions, without inoculation. (e) Scanning electron microscope images of carbon felt electrodes without microbes and (f) with microbes at a 20 μm scale. Error bars represent the standard deviations of triplicated tests.

(Fig. 2f), while the control electrode showed natural characteristics (Fig. 2e). The results of the CV analysis showed that the biofilm formed by *P. monteilii* LZU-3 exhibited electrochemical activities and that a pair of oxidation/reduction peaks appeared at 85 mV and 314 mV, respectively, Furthermore, no peaks were observed in the anode without microbes (Fig. S1a). Additionally, the CV curve obtained for the anode solution showed a pair of oxidation/reduction peaks at −371 mV and 676 mV, respectively, while no peaks were observed for the MS medium (Fig. S1b). These results indicate that *P. monteilii* LZU-3 can generate electron mediators within the anode chamber, potentially involved in extracellular electron transfer.

A previous study reported that PNP could be used as a sole substrate for current generation in a MFC inoculated with mixed cultures (Liu, et al., 2013). However, the maximum power density obtained was only 1.778 mW m^{-2}, which was much lower than the 12.9 ± 0.05 mW m^{-2} yielded in the present MFC with an aerobic anode chamber. Furthermore, PNP can be mineralized completely to CO_2, in conjunction with a nitrite release, in aerobic environments (Kulkarni and Chaudhari, 2007), and can easily get converted into more toxic aromatic amines through a six-electron transfer mechanism, operated by microorganisms, under anaerobic conditions (Razo-Flores et al., 1999). Moreover, the biofilm can further transfer the anode electrons, generated by the oxidation of substrates, from the cell to the surface of the electrode (Lovley, 2012). As a result, our MFC design is more suitable for voltage generation and PNP degradation. It has been reported that oxygen exposure could promote fuel diversity (Biffinger, et al., 2008), also supporting the application of aerobic strains in MFCs. In addition, the PNP-degrading organisms *Moraxella* sp. and *Arthrobacter* sp. have previously been immobilized on a dissolved oxygen electrode for PNP monitoring (Lei, et al., 2003; Mulchandani, et al., 2002). Therefore, *P. monteilii* LZU-3 might be applied as a biological recognition element in MFCs for PNP determination. Unlike immobilized cells (Lei, et al., 2003, 2005; Mulchandani, et al., 2002), *P. monteilii* LZU-3 can directly form biofilms on the surface of the anode electrodes, making it a cost-effective and time-saving method.

3.2. Optimization of operating parameters

In order to optimize the operating conditions, the effects of different PNP concentrations, pH, and temperatures on the performance of the MFC were carried out in batch mode. The results showed that the maximum voltages produced by the MFC increased from 115 ± 5 mV (8.8 ± 0.02 mW m^{-2}) to 150 ± 8 mV (15 ± 0.04 mW m^{-2}) when increasing the PNP concentrations from 50 to 200 mg L^{-1} (Fig. 3a), revealing a linear relationship ($R^2 = 0.99763$) between these two parameters (Fig. 3b). When the PNP concentration was increased up to 300 mg L^{-1}, the maximum voltage decreased down to 137 ± 13 mV (12.5 ± 0.11 mW m^{-2}). When further increasing the PNP concentration up to 400 mg L^{-1},

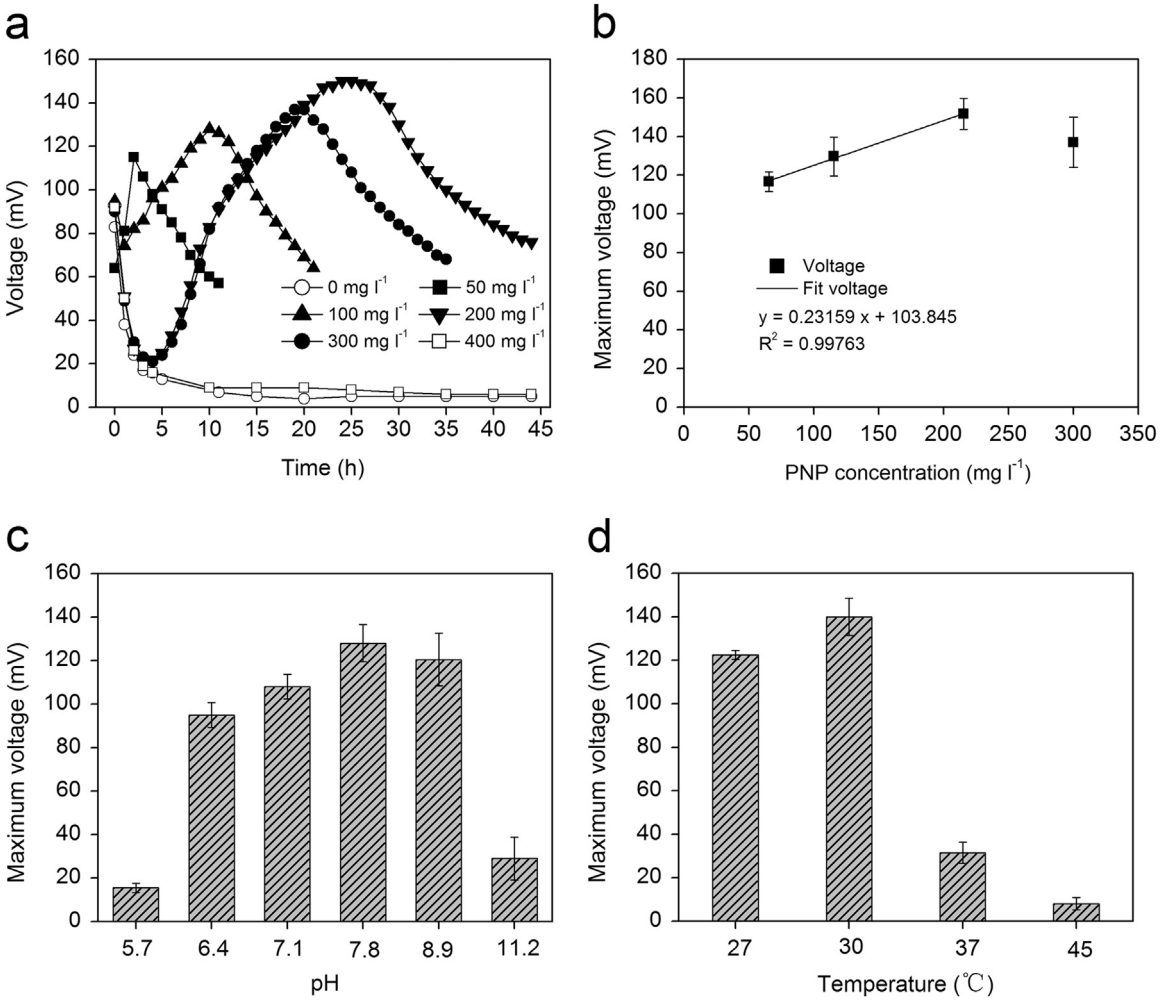

Fig. 3. Optimization of operating parameters. (a) Effects of PNP concentration on voltage generation. (b) The correlations between the PNP concentrations and the maximum voltages. (c) Effects of different pH and (d) temperatures on voltage generation. Error bars represent the standard deviation of triplicated tests.

the voltage generation was inhibited completely. These results demonstrated that the voltage generation by the MFC was directly related to the PNP concentration. This correlation can potentially be used toward the development of a biosensor for real-time monitoring of PNP. However, when the PNP concentration was higher than 50 mg L^{-1}, the maximum voltage output needed to go through a longer lag period, making the sensor unsuitable for fast detection of PNP. Therefore, we selected 50 mg L^{-1} as the PNP concentration for the subsequent experiments.

To further optimize the performance of the MFC, we tested the voltage generation at different pH and temperatures using 50 mg L^{-1} PNP as sole substrate. For the pH experiments, the temperature was fixed at 30 °C. The results showed that the MFCs were able to generate voltage with pH ranging from 6.4 to 8.9. The highest voltage obtained was 128 ± 8.5 mV (10.9 ± 4.8 mW m^{-2}) for pH 7.8, which was 18.5% and 6.2% higher than those obtained at pH 7.1 and 8.9, respectively (Fig. 3c). The results revealed that the alkaline pH was more suitable for the voltage generation by *P. monteilii* LZU-3, which was accordance with the results previously obtained with *Shewanella oneidensis* MR-1 (Yong et al., 2013). For the temperature experiments, the pH was adjusted to 7.8. The results showed that the optimal temperature for the voltage generation was 30 °C, with the highest voltage reaching 140 ± 8.4 mV (13.1 ± 4.7 mW m^{-2}). A further increase in temperature (up to 45 °C), inhibited almost completely the voltage generation (Fig. 3d). Therefore, 30 °C and 7.8 were selected as the optimum temperature and pH, respectively, for the subsequent biosensor experiments.

3.3. Characterization of the biosensor

Based on these results, the optimal conditions for operating the biosensor were obtained, and used to evaluate the performance of the biosensor.

3.3.1. Starting-up the biosensor

To start-up the biosensor, three parallel MFCs were operated at the optimal conditions. When the maximum voltage decreased down to 30 mV, the anolyte and catholyte solutions were replaced with fresh ones. Once the maximum voltage decreased to 30 mV again, we could confirm that the anode biofilm had been established and that the biosensor had been started-up successfully. The whole initiation phase of the biosensor took about 10 h. Compared with BOD biosensors inoculated with activated sludges (Peixoto et al., 2011; Di Lorenzo et al., 2009), which need 3–8 weeks to form anode biofilms, the pure cultures needed shorter times to form biofilms on the surface of anode electrodes. Previous studies also reported the use of *Shewanella oneidensis* MR1 (Tront et al., 2008b) and *Geobacter sulfurreducens* (Tront et al., 2008a) as sensing elements toward the monitoring of lactate and acetate in biosensors, showing biofilm formation times varying from 24 h up to 10 days. Compared with these easily degradable substrates, refractory compounds are more beneficial to the biofilm formation in biosensors due to the inducing effect of toxic compounds, a bacterial resistance mechanism against environmental stress (Dunne, 2002).

3.3.2. Calibration of the biosensor

The calibration of the biosensor was performed with PNP standard solutions in continuous mode. After each cycle, the anode and cathode solutions were replaced with fresh ones without additional inoculation. The results showed that the voltage increased stepwise as the PNP concentrations increased from 16 ± 5 mg L^{-1} to 44 ± 4.5 mg L^{-1} (Fig. 4a). A good linear relationship between the PNP concentrations and the maximum voltages of the biosensor were obtained within this range ($R^2 = 0.98457$) (Fig. 4b). Further increasing the PNP concentration to 55 ± 6 mg L^{-1} led to a decrease in voltage output. The results showed that the performance of the continuous mode MFC was not as good as in batch mode. When the PNP concentration was lower than 10 mg L^{-1}, the voltage was maintained at the baseline value of 30 mV (data not shown). Therefore, the maximum detection limit of the biosensor was determined as 44 ± 4.5 mg L^{-1}. With previous biosensors for PNP determination, the maximum detection limit was as low as 6.96 mg L^{-1} (Lei, et al., 2003; Mulchandani, et al., 2002). In comparison with these biosensors, our biosensor is more suitable for environmental monitoring due to its relatively wide PNP detection range. The response time of the biosensor was almost unchanged when increasing the PNP concentration, remaining at 27 ± 4.8 min (Fig. 4c). However, a previous report, describing a MFC-based biosensor, showed an increased response time when increasing BOD concentrations (Zhang and Angelidaki, 2011). Compared with this result, our biosensor showed an advantage in terms of reactivity when responding to different concentrations of substrates. The recovery time of the biosensor increased with increasing PNP concentrations, showing a linear relationship ($R^2 = 0.95483$) within the detection range (Fig. 4d). This behavior is consistent with observations made in a previous study, showing that the higher the concentration of volatile fatty acids in the samples, the longer the time needed for the MFC-type biosensor to consume the substrate (Kaur et al., 2013). Previous studies also reported immunoassay-based techniques for PNP monitoring, however, these required hours to enable a response and involved the use of expensive antibodies (Oubiña et al., 1999; Nistor et al., 2001). Compared with previously developed biosensors, our system shows some competitive advantages such as short response time and low cost.

3.3.3. Stability and accuracy of the biosensor

To evaluate the stability of the biosensor, a 25 mg L^{-1} PNP standard solution was tested with the biosensor over a period of 10 cycles. Each analysis was performed in triplicate. The results showed that the biosensor exhibited a fairly stable voltage output over the whole testing period (Fig. 5a). This outcome is similar to that of a previous study involving a MFC-based biosensor was designed for the fast analysis of glucose, in which the voltage output remained stable over 11 days (Kumlanghan, et al., 2007). Furthermore, the average maximum voltage of the present biosensor was 84.05 ± 3.75 mV (Fig. 5b), which is much higher than that obtained by Kumlanghan, et al. (1.07 ± 0.06 mV) (2007). The average response time of the biosensor was 30.25 ± 1.63 min and the average recovery time was 5.52 ± 0.48 h over the 10 cycles of successive testing (Fig. 5c). The results demonstrated that the biosensor possess an excellent long-term stability for PNP monitoring.

To further investigate the accuracy of the biosensor, a 50 mg L^{-1} PNP standard solution was diluted with distilled water to prepare working solutions at concentrations ranging within the detection limit, and measurements were made. The results showed good correlations with the PNP concentrations measured using the spectrophotometer ($R^2 = 0.99179$) (Fig. 5d), demonstrating that the biosensor provides high accuracy for PNP monitoring. The results are consistent with previous data obtained for another PNP microbial biosensor determination (Mulchandani, et al., 2002). The present results also provide evidence to support the feasibility of real-time monitoring of refractory pollutants using a single strain in a MFC-based biosensor under aerobic conditions.

3.3.4. Application study

To allow the application of the biosensor in the field, a novel portable biosensor system was developed (Fig. 1), which can

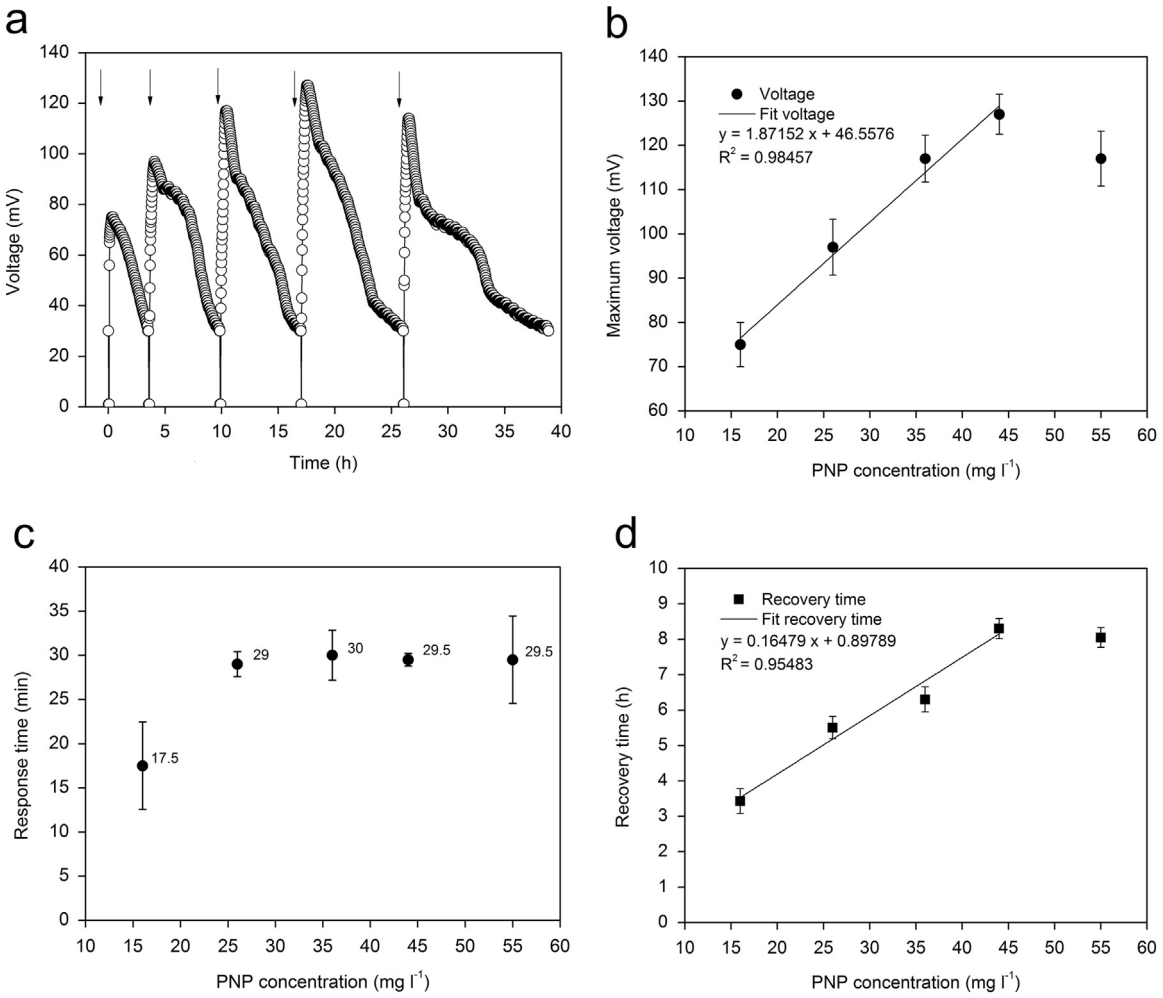

Fig. 4. Calibration of the biosensor under pH 7.8 and temperature of 30 °C with an external resistance of 1000 Ω. (a) Continuous voltage generation under different PNP concentrations, arrows indicate simultaneous replacement of both catholyte and anolyte solutions. (b) Correlations between the maximum voltages and PNP concentrations. (c) Response time and (d) recovery time of the biosensor. Error bars represent the standard deviations of triplicated tests.

achieve *in situ* monitoring of PNP in wastewater, and send the data in real-time on the web server for further analysis. A water sample (without PNP) was collected from a chemical industrial wastewater discharging site in the Lanzhou reaches of the Yellow River. The sample was filtered through a 0.45 μm filter and diluted three times with phosphate buffer (10 mM, pH 7.8), before supplementing it with different concentrations of PNP. In order to test the selectivity of the biosensor and its ability to resist against interferences, we also added the following most common toxic substances into the wastewater: 2-nitrophenol, 5 mg L^{-1}; 3-nitrophenol, 5 mg L^{-1}; nitrobenzene, 5 mg L^{-1}; toluene, 5 mg L^{-1}; phenol, 5 mg L^{-1}; NaCl, 50 mg L^{-1}; $FeSO_4 0 \cdot 7H_2O$, 20 mg L^{-1}; and $ZnCl_2$, 7 mg L^{-1}. The results demonstrated that structurally similar aromatic compounds and metal ions did not affect the performance of the biosensor and that the maximum voltages still showed a linear relationship (R^2=0.98933) with PNP concentrations ranging from 9 ± 4 mg L^{-1} to 36 ± 5 mg L^{-1}, confirming that the biosensor offers high selectivity (Fig. 6). The results were consistent with previous studies that had shown that phenol derivatives, nitrophenols and chlorophenols did not affect the performance of a microbial biosensor for PNP determination (Lei, et al., 2003; Mulchandani, et al., 2002). Although the regression equations and the maximum voltages of the biosensor were different when testing either standard solution or wastewater samples, the detection limit of the biosensor was almost unchanged. Additionally, the PNP was found in relatively high amount in various industrial wastewaters, ranging from 2.8 to 1220 mg L^{-1} (Zhang et al., 2011). At the later phase of PNP treatment, our biosensor can monitor PNP concentrations within the detection range. The results revealed that the biosensor could be used for *in situ* real-time monitoring of PNP contaminated wastewater.

To date, numerous biosensors were developed for PNP monitoring such as immunosensors (Nistor et al., 2001; Oubiña et al., 1999), microbial biosensors (Lei et al., 2003; Mulchandani et al., 2002), and amperometric microbial biosensors (Lei et al., 2005; Mulchandani et al., 2005). However, immunosensors require expensive antibodies, complicated analysis protocols, and skilled operators (Oubiña et al., 1999); microbial biosensors usually involve the use of immobilized cells as sensing elements and commercial dissolved oxygen electrodes as the transducers (Lei et al., 2003); amperometric microbial biosensors also require costly voltammetric analyzers to maintain the desired potential (Mulchandani et al., 2005). Therefore, these techniques are high-cost and time-consuming. Compared with these biosensors, our biosensor uses a single strain as the sensing element, able to quickly form biofilms on the surface of the anode electrodes and directly generate a measurable electrical signal, without any external signal transducer, and is, therefore, cost-efficient and time-saving. The present study provides an alternative method for *in situ* real-time monitoring of PNP in wastewaters.

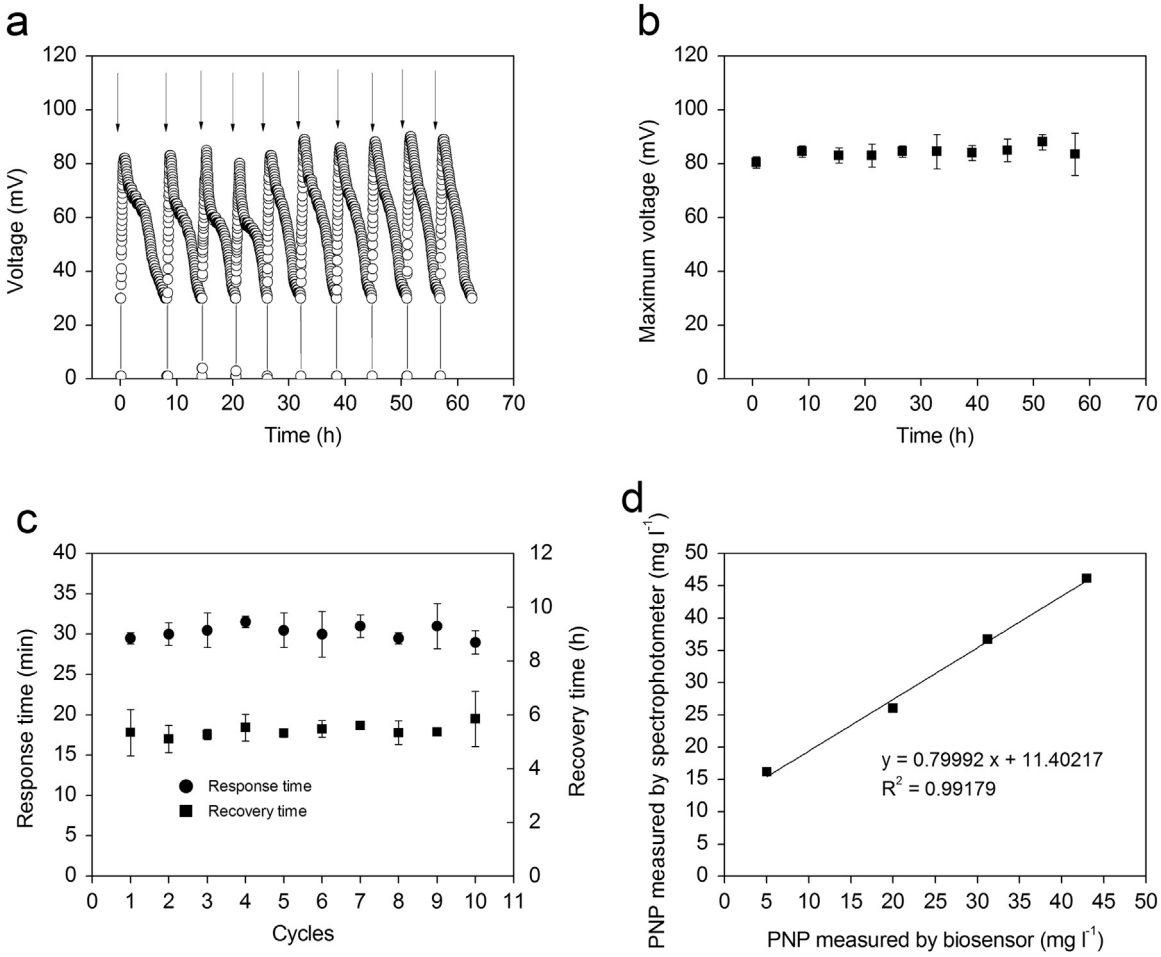

Fig. 5. Stability and accuracy of the biosensor. (a) Voltage generation in the biosensor with a PNP concentration of 25 mg L^{-1}, under optimal conditions over 10 cycles. Arrows indicate simultaneous replacement of both catholyte and anolyte solutions. (b) Maximum voltage of the biosensor. (c) Response time and recovery time of the biosensor. (d) Accuracy of the biosensor, error bars represent the standard deviations of triplicated tests.

4. Conclusions

This study describes a two-chamber MFC, with an aerobic anode chamber, tested as a biosensor for PNP monitoring. The biosensor was operated using pure cultures of *P. monteilii* LZU-3 as the sensing element and PNP as sole substrate, yielding an excellent stability and accuracy for PNP detection. Furthermore, we developed a novel portable biosensor system for *in situ* real-time monitoring of PNP in wastewater. Finally, the results demonstrate the potential of the biosensor to be applied for *in situ* real-time monitoring of refractory contaminants.

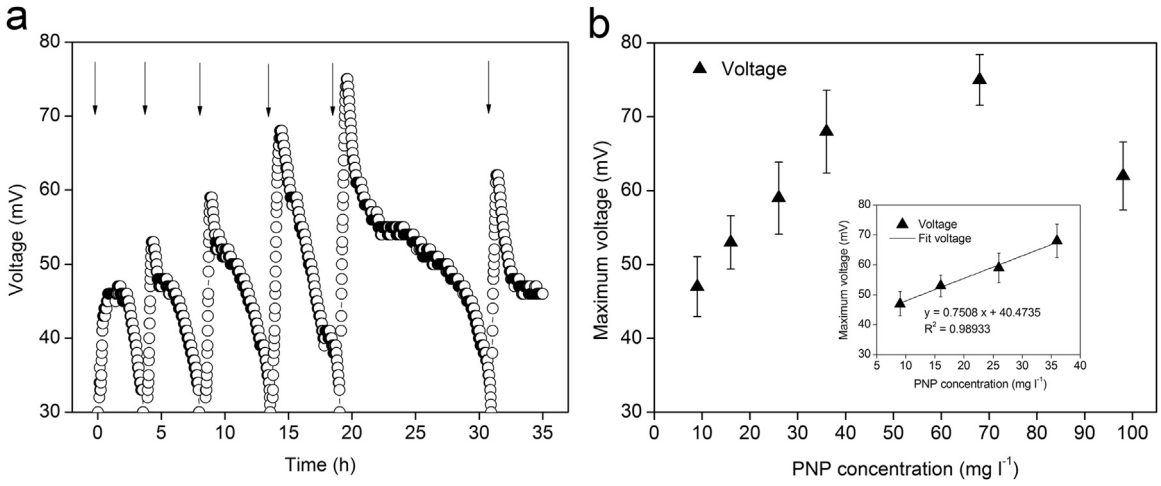

Fig. 6. Application of the biosensor for the monitoring of PNP-containing wastewater. (a) Voltage generation of the biosensor, arrows indicate simultaneous replacement of both catholyte and anolyte solutions. (b) Correlations between the maximum voltages and PNP concentrations. Error bars represent the standard deviations of triplicated tests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.06.007.

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