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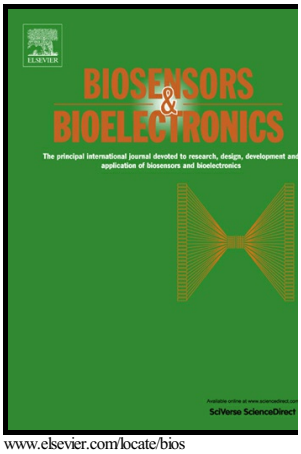

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PII: S0956-5663(15)30376-6
DOI: http://dx.doi.org/10.1016/j.bios.2015.08.055
Reference: BIOS7951

To appear in: *Biosensors and Bioelectronic*

Received date: 23 June 2015
Revised date: 21 August 2015
Accepted date: 22 August 2015

Cite this article as: Ilja Czolkos, Eva Dock, Erik Tønning, Jakob Christensen Margrethe Winther-Nielsen, Charlotte Carlsson, Renata Mojzíková, Petr Skládal Ulla Wollenberger, Lars Nørgaard, Tautgirdas Ruzgas and Jenny Emnéus, Prediction of Wastewater Quality Using Amperometric Bioelectronic Tongues, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2015.08.055

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Biosensors and Bioelectronics XX (2015) 1–4

Supplementary Material: Prediction of Wastewater Quality Using Amperometric Bioelectronic Tongues

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2. Materials and Methods

2.1. Wastewater Samples

The flux through the WWTP was approximately 100 mL/h and the temperature of the heat cover was set to 37°C. During one and a half months, 388 samples were collected continuously from the outgoing water at room temperature with an ISCO model 2700 autosampler. Each sample (200 to 400 mL) of outgoing water was collected for 2 to 4 hours. 24 samples were collected in each sequence before the autosampler was emptied. A fraction of 80-100 mL from each sample was stored at −18°C and the excess discarded.

The effluent quality during the treatment process was controlled to range from 'alarm' to 'normal' by adding nutrients N and P to the wastewater batch prior to the treatment. The batch wastewater was stored at 5°C. The process was monitored by measuring COD every second to third day. Starting with sample no. 352, the incoming batch was changed to wastewater with no nutrients added, and from sample no. 374 onwards, the thermostat (37°C) was turned off to perturb the process. These changes were brought about in order to regulate the quality from 'normal' towards 'alarm' again.

2.5. Chemicals

2.5.1. Array Type 1

Horseradish peroxidase (HRP, 263U/mg), soybean peroxidase (SBP, 108 U/mg), mushroom tyrosinase (TYR, 2590 U/mg), acetylcholinesterase from electric eel (AChE, 244U/mg), butyrylcholinesterase from horse serum (BChE, 345 U/mg), bovine serum albumin (BSA), glutaraldehyde, acetylthiocholine chloride (ATChCl), glucose, cellobiose, and catechol were obtained from Sigma (St. Louis (MO), USA). Glucose oxidase from *Aspergillus niger* (GOx, 270 U/mg) was purchased from Biozyme Laboratories (Gwent, UK). Cellobiose dehydrogenase from *Phanerochaete chrysosporium* (CDH, 2.45 g/L, $A_{420}/A_{280} = 0.64$) was purified according to the method of Henriksson et al. (1991). Chemicals for preparation of phosphate buffer saline (PBS, 50 mM phosphate buffer containing 0.1 M KCl at pH7) were from Merck (Darmstadt, Germany). All solutions were prepared using water purified in a Millipore Milli-Q system (Bedford (MA), USA).

2.5.2. Array Type 2

HRP (RZ II, 200 U/mg) and bovine hemoglobin (Hb) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Other enzymes were lactate oxidase from *Aerococcus*

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viridans (LOD, 21 U/mg) obtained from Genzyme (Neu-Isenburg, Germany), bovine liver catalase (CAT, 1990U/mg) purchased from Serva (Heidelberg, Germany), apo-glucose dehydrogenase (GDH, from *Acinetobacter calcoaceticus*, > 850 U/mg) and pyrroloquinoline quinone (PQQ) obtained from Roche (Indianapolis (IN), USA, and Mannheim, Germany). The HEPES-buffer solution of pH7.5 contained 25 mM HEPES (Roth, Karlsruhe, Germany), 100 mM KCl (Merck, Darmstadt, Germany), 1 mM $CaCl_2$ from Fluka (Taufkirchen, Germany), 10 mM D-glucose (Merck, Darmstadt, Germany), and 1 mM L-lactate (Li-salt) (Sigma-Aldrich, Taufkirchen, Germany). Purified water was taken from a Milli-Q system. Methanol from Roth was used to dissolve catechol (Sigma, Deisenhofen, Germany) for further dilution in aqueous buffer.

2.6. Biosensor Preparation

2.6.1. Array Type 1

Biosensor preparations were performed as described in ref. (Solná et al., 2005a) except that the screen-printed carbon based electrodes were exchanged for electrodes sprayed with carbon paste. (Dock et al., 2005) Screen-printed arrays, consisting of eight platinum working electrodes ($\varnothing$ = 1 mm) arranged radially around a printed Ag/AgCl reference electrode, were from BVT Technologies a.s. (Brno, Czech Republic). Carbon based ink was sprayed on five of the platinum working electrodes. (Dock et al., 2005) The enzyme solutions were prepared in PBS. The following enzyme solutions were used (the concentration of the enzyme solution and the used electrode material are specified within the brackets): (1) CDH/HRP (2.45mg/mL and 5 mg/mL, respectively, sprayed carbon), (2) HRP/GOx (both 5 mg/mL, sprayed carbon), (3) SBP/GOx (5 and 10 mg/mL, respectively, sprayed carbon), (4) TYR (10 mg/mL, sprayed carbon), (5) AChE and (6) BChE (each enzyme solution was prepared as a mixture of 10 µL enzyme (20 nkat/µL) and 13 µL BSA (50 mg/mL) in 110 µL PBS, platinum). (Solná et al., 2005b) 1µL of each of the six enzyme solution was added on one electrode each in the array. One carbon and one platinum electrode were left unmodified. The prepared sensors were left in a Petri dish sealed with parafilm overnight at 4°C in vapours of glutaraldehyde (originating from a drop of a 3% glutaraldehyde solution). Before use, the array was rinsed with Milli-Q water.

2.6.2. Array Type 2

Screen-printed arrays consisting of eight platinum working electrodes were the same as above. All enzymes were immobilized in a polyurethane polymer (BST-Biosensor Technologies, Berlin, Germany) by adding the dry enzyme to the polymer solution. For the GDH-sensor 0.5 mg GDH and 6 mg PQQ were suspended in 100 µL polyurethane solution. (Rose et al., 2002) Typically, 0.5 µL of the respective enzyme-polymer mixtures were added onto the Pt-working electrodes and dried for 2 h at room temperature and then stored at 4°C. The array was coated with the following mixtures: (1) PQQ-GDH (0.25 U), (2) PQQ-GDH/CAT (0.5 U/35 U), (3) HRP/LOD (7 U/0.8 U), and (4) Hb/LOD (LOD: 0.8 U). Hb has pseudo-peroxidative activity. Replicates of the four different coatings were applied so that the identical coatings always were prepared on electrodes in the opposite position of the array to minimize for possible influences from neighbouring electrodes.

2.7. Measurements

2.7.1. Array Type 1

The biosensor array was inserted to a specially constructed amperometric cell, (Dock et al., 2005) which can be used either for steady-state or flow-injection measurements. Each of the eight working electrodes were independently controlled by an Ag/AgCl reference electrode printed on the same array using a multichannel potentiostat from Prof. J. Kulys, Laboratory of Enzyme Chemistry, Institute of Biochemistry, Vilnius, Lithuania. The working potentials for the sensors were as follows: +350mV for bare platinum and sensors based on cholinesterases, +400 mV for bare graphite and sensors based on CDH, −100mV for sensors based on peroxidase and tyrosinase. (Solná et al., 2005a)

Steady-state measurements were carried out in the cell using a rotating rod at a speed of 7 Hz, placed 1.8 mm over the array. (Dock et al., 2005) The array cell was filled with 4 mL PBS containing 0.5 mM glucose, 0.5 mM cellobiose and 0.5 mM ATChCl. These three chemicals act as substrates for enzyme activation and were also added to the catechol standard and samples prior to injection in order to keep their concentration constant throughout the experiment. Fig. 5 shows an array sensor response derived from one steady-state measurement according to the following: Baseline steady-state currents were recorded for 200 s. A

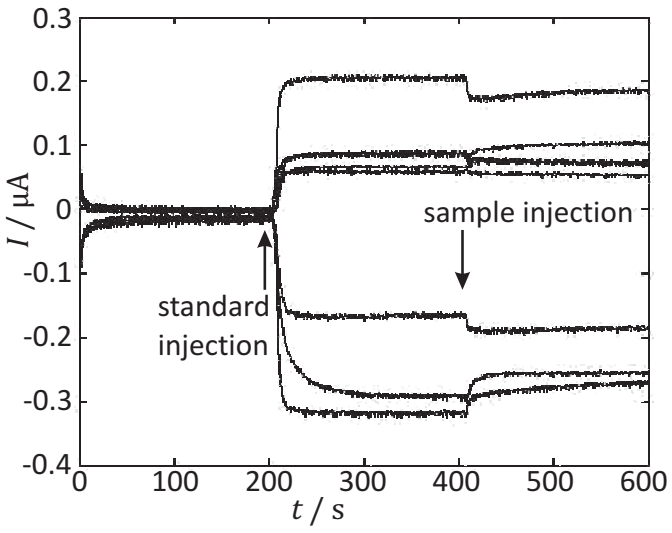

Figure 5. Measurements using an eight-electrode array of type 1 analyzed with the steady-state technique. The first signal succeding baseline stabilization originates from injection of the standard (15µM catechol) while the second signal corresponds to an injected sample.

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standard of 15 µM catechol in 0.5 mL PBS spiked with the above mentioned enzyme substrates was then added and recorded for 200 s. Finally a sample (wastewater or catechol) spiked with the enzyme substrates was added and recorded for 200 s. The cell was rinsed with Milli-Q water and dried before the next experiment was initiated.

For flow-injection measurements, an inlet flow of 1.87 mL/min was delivered using a peristaltic pump (Gilson minipuls 2) through the rotator into the cell. The same pump was also used to transfer solution away from the cell via a needle glued to the inner wall. The rotator was spinning with a speed of 12 Hz during the measurement at a distance of 0.16 mm over the array surface. The analyte was injected through a 200 µL injection loop connected to a Rheodyne (Berkeley (CA), USA) six-port injection valve. Each experiment lasted 600 s. Fig. 6a shows a typical flow-injection measurement. After 100 s a standard containing 10 µM catechol and 0.5 mM ATChCl mixed with the enzyme substrates (0.5 mM glucose and 0.5 mM cellobiose) in PBS was injected. The second injection was performed at 350 s with the sample (wastewater or catechol) diluted 1:1 in PBS, spiked with 0.5 mM ATChCl, 0.5 mM glucose, and 0.5 mM cellobiose. The recording was continued for another 250 s.

2.7.2. Array Type 2

The multielectrode array was placed in a home-made flow cell (Analytical Biochemistry, University of Potsdam), designed for flow measurements with low buffer consumption (cell volume < 50 µL). An injector of an Eva-Flow Injection Analysis system from Eppendorf (Hamburg, Germany) was used for time-controlled sample injection and to ensure a constant sample injection volume of 100 µL. An Abimed perstaltic pump from Gilson (Villiers-le-bel, France) was also used in the flow system. As for array 1, each of the eight working electrodes was controlled independently against a Ag/AgCl reference electrode using a multichannel potentiostat from Prof. J. Kulys. The applied potentials were +500 mV for the GDH-electrodes and −50 mV for the HRP- or Hb-based electrodes. The samples and calibration solutions were diluted 1/4 with concentrated buffer solution to finally contain 25 mM HEPES-buffer at pH7.5, 100 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose, and 1 mM L-lactate. Each measurement was dedicated to a single sample and consisted of three time-controlled injections. After a constant baseline was obtained, the above-mentioned HEPES buffer with 5 µM catechol was injected into the buffer stream. The signal was used for calibration of the electrodes. The second injection was a diluted sample (1/4) spiked with 5 µM catechol followed by the third injection of only the respective sample, also in a final dilution of 1/4. The system was thoroughly rinsed with buffer between each injection and between each measurement. The flow rate in the system was 0.9 mL/min, corresponding to a velocity of the liquid stream above the working electrodes of 2.4 mm/s.

2.8. Data Pre-processing

The signal pre-processing procedures, similar to the methods used in reference (Tønning et al., 2005) were performed in MatLab version 6.5. The essence of these procedures is described below. All samples were measured in random order.

2.8.1. Array Type 1

Each steady-state response sequence constists of 1230 time channels (2.05 s^{-1}, Fig. 5). Of these, the last 411 time channels were chosen for further analysis since they contain the second steady-state plateau after sample addition. These responses were corrected for shift in baseline by subtracting the mean value of the time channels 800 through 810, *i.e.*, the baseline level just before introducing the sample into the cell. The 411 time channels containing the sample response were further corrected for reduced sensitivity by dividing the response with the mean height of the first plateau originating from the catechol standard, *i.e.* mean of time channels 507 through 773 minus mean of time channels 400 through 410 just before injection of the standard. After correction, one sample measurement resulted in a 411×8 matrix where each column represents the sample response from one sensor electrode in the array. To perform a 2-way multivariate analysis, this matrix was rearranged so that one sample was described by a column of $411 \times 8 = 3288$ time channels, sequentially listing all eight different sensor responses.

One flow-injection measurement sequence consists of 4917 time channels (8.2 s^{-1}, Fig. 5b). The data were smoothed using a 15-point moving average. For convenience, the data were padded with more channels towards the end of the recording to obtain a value of 5000 time channels. The number of time channels was reduced to 500 by keeping only each tenth point for further analysis. Of these 500 data points, 100 points describing the sample peak were corrected for shift in baseline and reduced sensitivity by dividing the sample response with the adjacent catechol standard peak height. The data matrix for the sample responses after correction then had the size of 100×8. Before multivariate analysis, the matrix was rearranged into a column of 800 time channels based on eight different flow-injection peaks from eight different sensors.

2.8.2. Array Type 2

The current of each of the eight electrodes was recorded at a data rate of 4.1 points per second. Each of the three peaks from each of the eight current *vs.* time curves in the measurement run was treated separately. The first peaks, data from 390–439 s, were taken and baseline-corrected by subtracting the mean of the current from 350–389 s. The second peaks were extracted from 662–711 s, and the third peaks from 936–985 s, and corrected with their respective baseline means calculated from time 622–661 s and 896–935 s, respectively. The area under each peak was calculated. Finally, the area under the second and third

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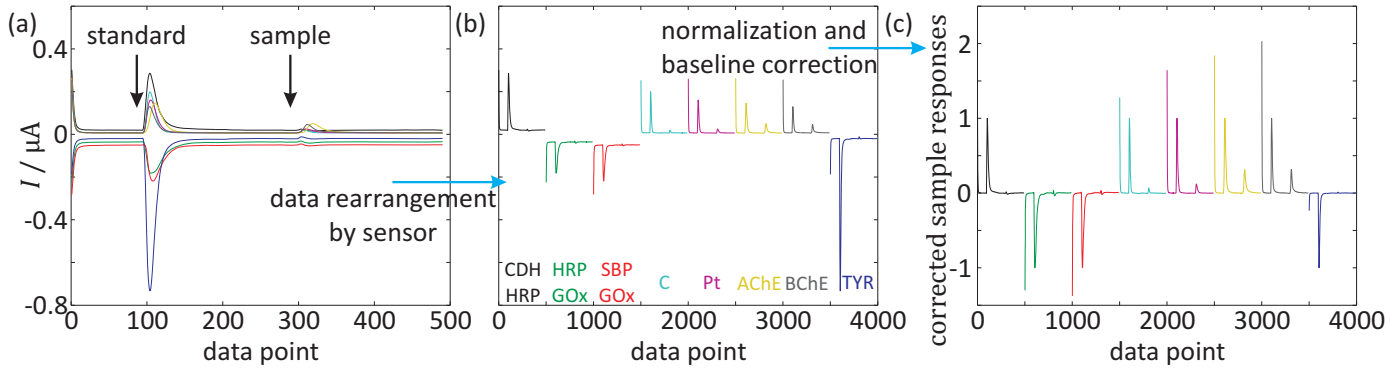

Figure 6. Illustration of data treatment of flow-injection responses from one of the tested arrays of type 1 (not necessarily proceeded in this order). (a) A typical sensor response to two injections of a standard (10 µM catechol, 0.5 mM ATChCl added to the buffer) and a sample diluted in buffer, spiked with 0.5 mM ATChCl. (b) The individual traces from each sensor were placed in consecutive order to obtain a one-dimensional trace for all sensors, and (c) the baseline-corrected and normalized traces. The data points that were selected as standard and sample injection peaks are shown in fig. 1 (main article).

peak were normalized to the first peak, *i.e.* the calibration peak. Each sample therefore provided two datasets with eight values for peak areas. Since no effect of catechol on the sample signal was observed, only the dataset with the unspiked sample was used for further analysis.

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