ELECTROCHEMICAL INHIBITION BIOSENSOR ARRAY FOR RAPID DETECTION OF WATER POLLUTIONS BASED ON BACTERIA IMMOBILIZED ON SCREEN-PRINTED GOLD ELECTRODES

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This work reports on the development of a bacteria-based inhibition biosensor array for detection of different types of pollutions, i.e. heavy metal ions (Zn^{2+}), pesticides (DDVP) and petro-chemicals (pentane), in water. The biosensor chip for preliminary identification of the above water pollutants is based on three types of bacteria (*Escherichia coli*, *Shewanella oneidensis* and *Methylosinus trichosporium* OB3b) immobilized on screen-printed gold electrode surface via poly L-lysine which provides strong adhesion of bacterial monolayer to the electrode without losses of biological function. A series of optical measurements and DC electrochemical measurements were carried out on these three types of bacteria species immobilized on modified screen printed gold electrodes as well as on the bacteria in solution samples. The principle of electrochemical detection of pollutants is based on the facts that live bacteria adsorbed (or immobilized) on the electrode surface appeared to be insulating and thus reducing the electrochemical current, while the bacteria damaged by pollutants are less insulating. The results obtained demonstrated different effects of the three different types of analytes studied, e.g. Zn^{2+}, DDVP, and pentane, on the three bacteria used. The findings are encouraging for application of a pattern recognition approach for identification pollutants which may lead to development of a novel, simple, and cost-effective bio-sensing array for preliminary detection of environmental pollutants in water.

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Introduction

Extensive industrial and agricultural activities have contaminated the environment with large number of toxic chemicals, particularly, heavy metals, pesticides and petro-chemicals which are spread in the atmosphere and aquatic environment, and have negative impacts on all the living organisms.[1] Water Pollution has been described as any natural or human-made release of the chemical, biological, or radioactive elements to the aquatic environment which affect the health and wellbeing of the living species in water resources, and pose a serious threat to human, animals, plants, and microorganisms.[2] Such pollutants might cause either major destruction with direct visible effects on the environment, or minor destruction in the system of living organisms' life cycle due to disturbance of a delicate biological balance which becomes noticeable after a certain time.[3] The main sources of heavy metal contamination are associated with mining, manufacturing and chemical industries, and transport,[4] while pesticides have been released into the environment as a result of extensive agriculture,[5] and petro-chemical industry and transport heavily contribute to environmental pollution with a wide range of chemicals ranging from relatively harmless hydrocarbons, alcohols, and ketones to much more dangerous benzene derivatives (BTEX).[6] This study focuses on detection of three chemicals belonging to different classes of contaminants, i.e. Zn^{2+} ions, DDVP pesticide and pentane.

Great deal of effort has been made to develop technologies for monitoring pollutants in aqueous environment, because the evaluation of these contaminants is considered as one of the most serious current global problems.[7] A number of analytical methods, such as atomic absorption or atomic emission spectroscopies (AAS, AES), inductively coupled plasma mass spectroscopy (ICP-MS), cold vapour atomic fluorescence spectroscopy (CVAFS), and high pressure liquid chromatography (HPLC) are capable of detecting traces of toxic pollutants,[15,16] however these methods require sophisticated analytical equipment, specialised laboratories and highly qualified personnel, which make such analysis very expensive and time consuming. Therefore, the development of alternative detection technologies, for example, simple and inexpensive biosensor devices, capable of rapid detection of environmental pollutions, is urgently needed.[10]

A particular interest is in development of the inhibition type of bio-sensors which do not detect pollutants directly, but through the monitoring changes in functionality of bio-receptors caused by exposure to pollutants.[11] Such inhibition sensors (or rather senor arrays) are quite versatile and applicable for detection of various chemicals using a limited set of bio-receptors. The sensitivity and selectivity of these sensors may not be great but sufficient for quick detection and identification of suspected samples for further more detailed analysis; the cost and time of analysis can be substantially reduced as a result.

Enzymes are typically used as bio-receptors in inhibitions sensors.[12] The drawback of such enzyme-based inhibition sensors is in poor stability of enzyme.[13] Several inhibition

biosensors based on whole cells have been reported in the last two decades, which mostly use optical transduction techniques such as fluorescence.[14] However, these approaches rely heavily on genetically modified strains (e.g. strains with green fluorescent protein under the control of specific promoters), and hence have disadvantages of complexity and high cost.[15,16] There have also been reports on electrochemical sensors based on whole-cells.[17,18] Bacteria could be versatile bio-receptors for traces of toxic environmental pollutants because these molecules or ions interfere with biological processes (e.g. catabolism and photosynthesis) and modify bacterial activity that can be monitored in a number of ways.[19] It is relatively easy to immobilize whole bacterial cells on electrodes and electrochemically monitor redox species which participate in the cells' metabolic processes, e.g., oxygen, hydrogen peroxide or protons.[20]

In recent years, a number of microbial biosensor systems based on different transducing methods have been developed for environmental, food, and biomedical applications including the detection of various inorganic and organic pollutants in water, such as heavy metal ions, pesticides, hydrocarbons, etc.[21,22] Similar to this study, electrochemical detection principles have been exploited in the study of inhibition effects of environmental pollutants on *E. coli* bacteria.[23,24]

In our previous study of optical and electrical properties of solutions of two types of bacteria (*E. coli* and *Deinococcusr adiodurans*), a correlation between the optical density and electrical conductivity and the bacteria concentration in liquid samples was established, and then utilized for identification of two types of pollutants, e.g. heavy metals and radionuclides, by their inhibition effects on the above bacteria.[25] This approach was further developed recently.[26] This work has focused mostly on electrochemical detection of heavy metal ions (Hg^{2+}) using two types of bacteria (*E. coli* and *Shewanella oneidensis*) which were either free in solution or immobilized on the electrode surface. In the current work, we went further by expanding the bacterial sensor array (three types of bacteria were used *E. coli*, *S. oneidensis*, and *Methylosinus trichosporium* OB3b) as well as the range of pollutants tested (Zn^{2+} ions, DDVP, and pentane which belong to different classes of chemicals). The main aim of this work was to develop the inhibition bacterial sensor array for detection of different types of water pollutants using pattern recognition principles. The results of this work may lead to development of a novel, simple and cost-effective bio-sensing technology for preliminary detection (screening) of water pollutants.

Experimental methodologies

Preparation of bacteria samples.

Three diverse bacterial strains were selected for this work: (i) the model Gram-negative *E. coli* K12, known to be sensitive to various types of pollutants including heavy metals, pesticides, and hydrocarbons,[27] (ii) *S. oneidensis* MR-1, a Gram-negative bacterium known to tolerate and interact with heavy metals[28] and (iii) the methanotrophic bacterium *M. trichosporium* (OB3b) strain (a Gram-negative bacterium that grows on methane and is also able to co-oxidise a range of other hydrocarbons and hydrophobic organic molecules.[29,30] *E. coli* cultures were grown at 37 °C for 16 h in LB broth and LB agar.[31] *S. oniedensis* cultures were grown at 30 °C for 24 h in the same medium,[32] while *M. trichosporium* OB3b was grown at 30 °C for 2 weeks in flask cultures in nitrate mineral salts (NMS) medium and on NMS agar plates, using methane as the carbon source, as described previously.[33]

The surface of screen-printed gold electrodes was modified with a (0.1%) solution of poly L-lysine (PLl; Sigma-Aldrich; 0.1 mg/ml in deionised water) for 1 h at 37 °C. The bacteria were immobilized by pippeting a liquid culture at a stationary phase of the appropriate strain onto the electrode and keeping it in contact with the surface for 1 h, then washing off non-bound bacteria with phosphate buffered saline (Sigma-Aldrich 14200-067).

Preparation of analytes

The samples of $ZnCl_2$, DDVP and pentane (all from Sigma-Aldrich) were prepared at concentrations of 0.1, 1, 10, 100 mM by consecutive dilution of 1 M stock solutions in deionised water. The stock solution of pentane was prepared in a 40% (v:v) ethanol:water mixture. Liquid bacteria culture samples were mixed with these solutions in 1:1 ratio and incubated for 2 h at 22-23 °C. The samples of immobilized bacteria were treated similarly by immersing the electrode functionalized with bacteria into the required solution of pollutants for 2h.[26]

Optical and SEM characterization of bacteria

The effect of the above pollutants on the bacterial cultures was examined using three different optical experimental techniques: fluorescence microscopy, UV–visible spectrophotometry, and flow cytometry. A Becton-Dickinson FACS Calibur flow cytometer was used to count live and dead bacteria after staining them with the BacLight live/dead bacterial viability kit (Molecular Probes). Fluorescence microscopy of liquid cultures and bacteria immobilized on the screen printed gold electrodes was performed using an Olympus-BX60 instrument using liquid bacterial samples also stained with the BacLight kit.[34,35] Optical density of bacterial cultures with measured at 600 nm with a 6715 UV/Vis spectrophotometer (Jenway). For scanning electron microscopy (SEM) immobilized bacteria were fixed on double-sticking carbon tape mounted on a sample holder and coated with a few-nanometer-thick layer of carbon using a carbon evaporator (Edwards E306A; Edwards, United Kingdom). The scanning electron microscopy using in all experiments is FEI-Nova SEM. The system was operated at 1 to 15 kV for high-resolution secondary electron imaging and elemental analysis.

Electrochemical measurements

Cyclic voltammograms (CVs) were recorded in a voltage range from -0.5 V +0.5 V using DropSens gold screen-printed three-electrode assemblies (which include Ag/AgCl reference electrode) and a DropSens microSTAT4000P potentiostat. CVs of liquid bacterial cultures were recorded

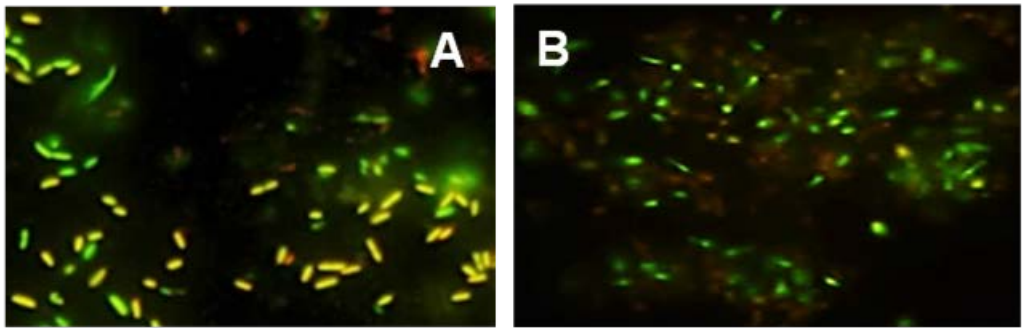

Figure 1. Fluorescence microscopy images of immobilized *Shewanella oneidensis* before (A) and after (B) treatment with $ZnCl_2$ salt (1 mol).

on screen-printed electrodes immersed into the bacterial suspension. Measurements were taken on liquid samples of all three bacteria before and after treatment with each pollutant at each concentration. The CV measurements of the electrodes with freshly immobilized bacteria were carried out in LB broth before and after treatment with each pollutant at each concentration.

Results and Discussion

Optical characterization

Fluorescence microscopy images in Figure 1 show the effect 1M solution of $ZnCl_2$ on *Shewanella oneidensis* bacteria immobilized on screen printed gold electrodes and treated with the BacLight live/dead stain.[36] The exposure to Zn^{2+} ions reduced the number of live bacteria (green or yellow) and increases the dead ones (red or orange). Similar experiments were carried out for all three types of bacteria and for all analytes used, and the resulted counts of live (green or yellow) and dead (red or orang) bacteria on recorded images of identical dimensions are summarized in Table 1.

Table 1. The numbers of live and dead bacteria immobilized on modified screen printed gold electrodes for all three bacteria before and after treatment with 1M solutions of the three pollutants for 2 h.

Bacteria	Pollu-tants	Before exposure		After exposure	
		Live	Dead	Live	Dead
E. coli	$ZnCl_2$	75	19	21	65
S. oneidensis	$ZnCl_2$	57	11	52	28
M. trichosporium	$ZnCl_2$	69	21	34	79
E. coli	DDVP	62	17	31	73
S. oneidensis	DDVP	79	18	25	41
M. trichosporium	DDVP	81	25	23	65
E. coli	Pentane	43	13	19	51
S. oneidensis	Pentane	49	22	38	17
M. trichosporium	Pentane	93	20	62	14

These data revealed that *E. coli* and *M. trichosporium* (OB3b) more severely affected by large concentrations of Zn^{2+} ions than *S. oneidensis*. The negative effect of DDVP is dramatic and more or less similar for all three bacteria. Pentane, however, did not affect *M. trichosporium* (OB3b) strain, though it reduced the viability of both *E. coli* and *S. oneidensis*. The pattern of responses of immobilized bacteria to the above pollutants is similar to what was previously observed for the same bacteria strains in solution.[26] The results of optical density (OD_{600}) study of liquid bacteria samples in Figure 2 shows the effect of exposure to a high concentration (1 M) of $ZnCl_2$. The results were similar to those obtained via fluorescent microscopy i.e., all bacteria appeared to be affected by $ZnCl_2$ though this effect was less pronounced for *S. oneidensis*. It is important to state that the optical density measurements, which are based on light scattering, could be affected by different motility of the bacteria studied, whereas this would not affect the bacteria held in place on the gold electrode surface. Similarly, death of bacteria without lysis or morphological change would not contribute to a change in OD.

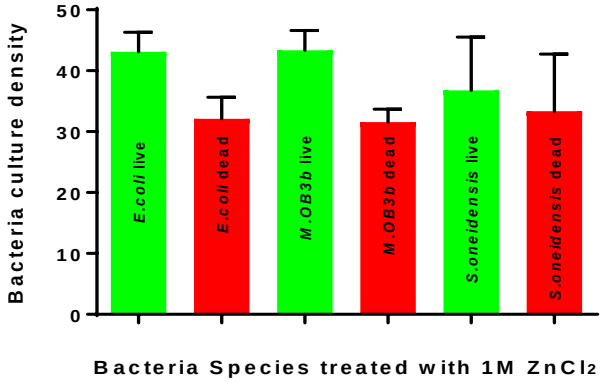

Figure 2. Bacteria culture concentration before and after treatment with large concentrations (1 M) of $ZnCl_2$.

Flow cytometry measurements combine the advantages of fluorescence microscopy and optical counting of individual cells. Typical results of flow cytometry for cultures of all three bacteria before and after treatment with a 1M solution of $ZnCl_2$ for 2 h and subsequent staining with the BacLight are presented in figure 3. The increase in the counts of dead bacteria after exposure to $ZnCl_2$ is apparent for all three types of bacteria studied. In addition to that, after $ZnCl_2$ treatment, dead *E. coli* and *M. trichosporium* (OB3b) bacteria appear mostly in bottom-left quadrant of the graph in Figure 3C and 3B indicating the increase in the bacteria size most-likely due to the enlargement or rapture of cell walls. *S. oneidensis* was less affected by $ZnCl_2$ as one can see in Figure 3A. Flow cytometry tests were carried out for the other two pollutants, e.g. DDVP and pentane, and the results were summarized in table 2 as the percentage of live and dead bacteria.

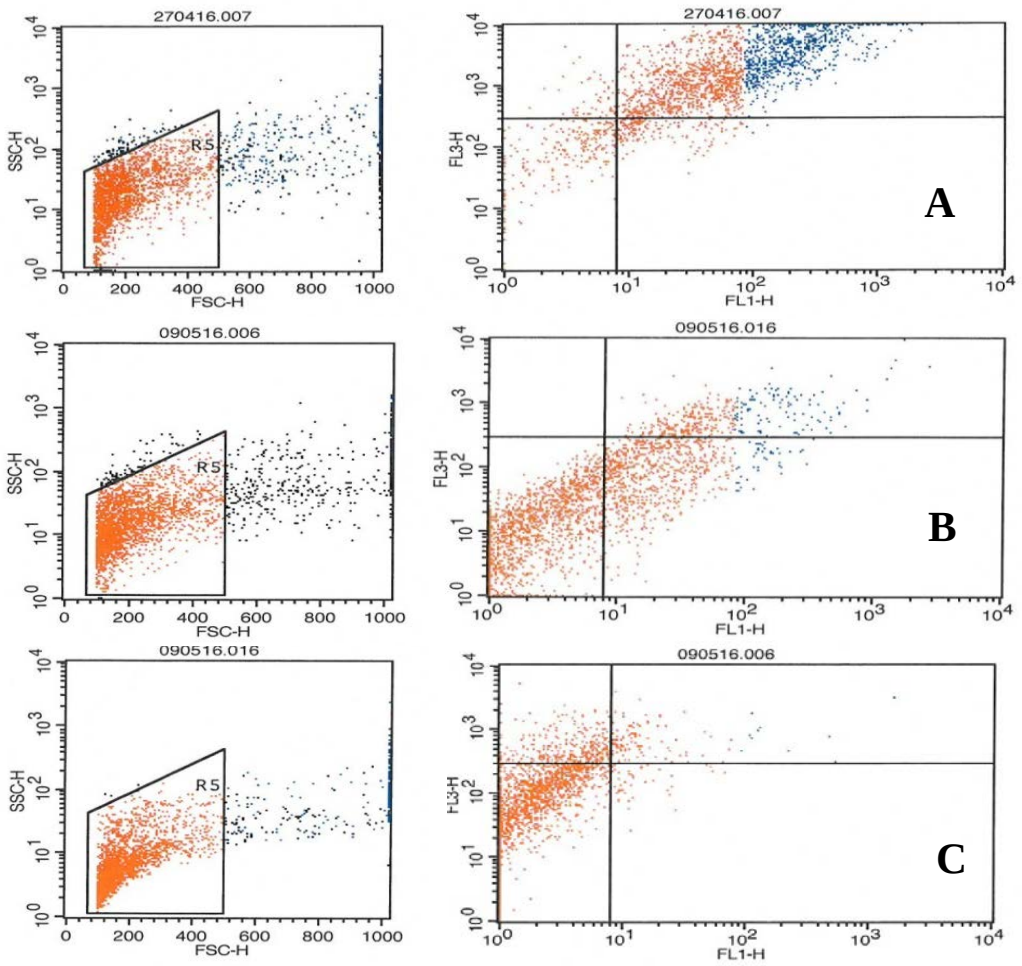

Figure 3. Flow cytometry results for *E. coli* after treatment with Pentane (1 M) (A) ,*S. oneidensis* after treatment with $ZnCl_2$ (1 M) (B) and *M. trichosporium* (OB3b) after treatment with DDVP (1 M) (C) , respectively.

Table 2. Flow cytometry data showing the percentage of live and dead bacteria before and after treatment with different pollutants.

Type of bacteria	Type of pollutant	Before		After	
		Live	Dead	Live	Dead
E. coli	$ZnCl_2$	73.88%	26.12%	26.11%	73.89%
S. oneidensis	$ZnCl_2$	52.32%	37.68%	67.68%	32.32%
M. trichosporium (OB3b)	$ZnCl_2$	71.49%	28.51%	24.49%	75.51%
E. coli	DDVP	85.13%	14.87%	29.43%	70.57%
S. oneidensis	DDVP	71.32%	28.68%	66.71%	33.29%
M. trichosporium (OB3b)	DDVP	85.33%	14.67%	32.33%	67.67%
E. coli	Pentane	86.54%	13.46%	31.54%	68.46%
S. oneidensis	Pentane	77.71%	22.29%	33.68%	66.32%
M. trichosporium (OB3b)	Pentane	79.47%	20.53%	62.58%	37.42%

Direct evidence of cell enlargement was obtained from SEM study. SEM images (Figure 4) show the rupture of *E. coli* and *M. trichosporium* (OB3b) bacteria (Fig. 4A and 4C) and enlargement of *S. oneidensis* cells (Fig. 4B) caused by exposure to high concentration (1 mol) of $ZnCl_2$. These observations are similar to previously reported SEM studies of bacteria.[37,38] In contrast, *S. oneidensis* bacteria were affected much less by $ZnCl_2$ than the other bacterial strains and appeared slightly elongated (Figure 4B). Similar elongation has been observed in *S. aureus* due to exposure to high salt concentration as a specific response to other stress conditions.[39] Also, a significant increase in bacteria length was found in *S. oneidensis* exposed to UV radiation.[40] The data above are consistent with the conclusion that *E. coli* cells are strongly inhibited by all three pollutants, while *S. oneidensis* are less affected by Zn^{2+} ions as compared to the strong inhibition effect of DDVP and pentane. *M. trichosporium* (OB3b) cells are severely affected by Zn^{2+} ions and DDVP, while pentane/ethanol mixture may even stimulate their growth.

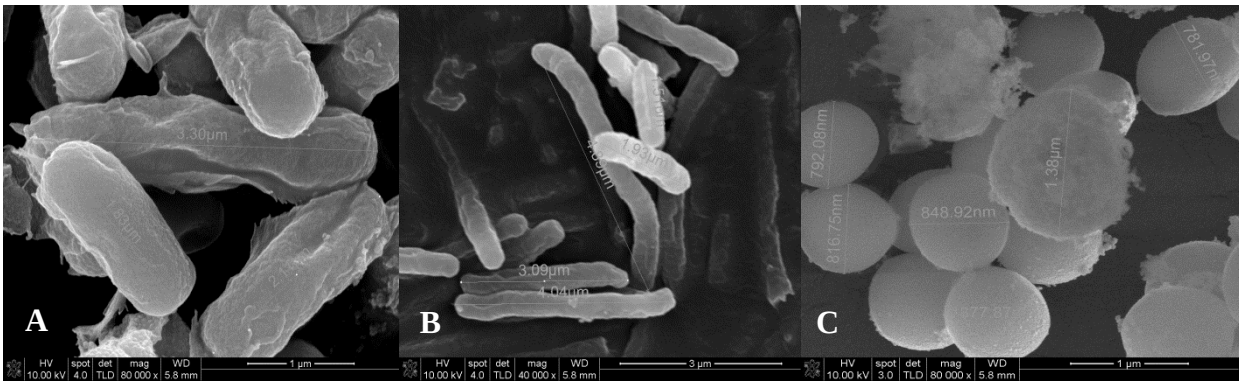

Figure 4. SEM images of (A) *E. coli* after treatment with pentane (1 M), (B) *S. oneidensis* after treatment with $ZnCl_2$ (1 M) and (C) *M. trichosporium* (OB3b) after treatment with DDVP (1 M).

Among the three optical methods used to determine the proportion of live and dead bacteria, flow cytometry appeared to be the most reliable.[41] Flow cytometry is also expected not to be affected by different motility of *E. coli*, *S. oneidensis*,[26] and *M. trichosporium* (OB3b). Immotile dead bacteria may sediment more readily, which may affect the results of static fluorescent microscopy and optical density measurements.

Electrochemical study of bacteria in suspensions and immobilized bacteria

The effect of Zn^{2+} ions, DDVP, and pentane in of all three bacterial strains, both in suspension culture and immobilized on the screen printed gold electrodes, was studied with cyclic voltammetry. A typical series of CVs recorded on *E. coli*, *S. oneidensis*, and *M. trichosporium* (OB3b) samples are shown in Figure 5. The CV curves in Figure 4 are almost featureless in the selected voltage range from -0.5 V to +0.5 V, which was chosen in order to avoid electrochemical reactions on the electrodes, with the scan range limited to where both cathodic and anodic currents just began to rise. The values of both cathodic and anodic current at -0.5 V and +0.5V, respectively, depend on the bacteria concentration in solution,[25,26] however the effect on anodic current is more pronounced and it was therefore used for analysis in this work.

CV cycles shift progressively upwards upon increasing the pollutants concentration from zero to 1 mol (Figure 5). The characteristic parameter in this study, e.g. the value of anodic current at +0.5 V, increases with the increase in pollutant concentration for all three bacteria in both liquid and immobilized forms. This means that the electrical conductivity is controlled by bacteria adsorbed on the surface of gold electrodes and acting as insulating layer reducing the current. The correlation between bacteria cell density and the electric current (or conductivity) values is very important for further study of the effect of pollutants, and such measurements were always carried out first.[25,26] The presence of pollutants (Zn^{2+} ions, DDVP, and pentane in our case) causes the damage of bacterial cells, and therefore bacteria became less insulating, in-turn leading to the increase in the anodic current, which is observed in Figure 5.

To analyse the effect of pollutants on electrical properties of immobilized bacteria, the values of anodic current (I_A) at +0.5V from CV measurements were normalised by the currents values of uncoated electrodes in PBS with the addition of a particular pollution of particular concentrations (I_{A0}) to construct the values of relative changes of anodic current. For example, for *S. oneidensis* bacteria treated with 1 mM solution of pentane (Figure 5A), the reference was recorded on uncoated electrodes in PBS containing 1mM of pentane.

The relative changes in anodic current are presented in Figure 6 for all three bacteria studied as concentration dependences of the three pollutants. As one can see the effects of $ZnCl_2$, DDVP, and pentane on *S. oneidensis,M. trichosporium* (OB3b) strain and *E. coli* are distinctly different. *E. coli* appeared to be affected by $ZnCl_2$, DDVP, and pentane even at low concentrations since the $\Delta I_A/I_{A0}$ values increase monotonically in Figure 6A, 6B, and 6C, respectively. This means that *E. coli* is equally inhibited by all three pollutants and becoming less electrically resisting. In contrast, *S. oneidensis* is almost unaffected by $ZnCl_2$ at low concentrations of all pollutants up to 10 mM, and then $\Delta I_A/I_{A0}$ started to increase at high concentrations of 100mM and 1M. Such behaviour of immobilized *E. coli* and *S. oneidensis* bacteria is similar to those in liquid as reported in.[26.]

M. trichosporium (OB3b) responded to $ZnCl_2$ (Figure 6A) and DDVP (Figure 6B) similarly to the other two bacteria studied,though the changes in $\Delta I_A/I_{A0}$ are more pronounced at high pollutant concentrations, particularly for pentane. However, *M. trichosporium* (OB3b) is not affected by pentane (Figure 6C) even at high concentration; moreover an overall trend of small decrease in $\Delta I_A/I_{A0}$ is observed. Such behaviour was expected since methanotrophic bacteria can oxidise many hydrocarbons.[29]

The results presented in Figure 6 show a possibility of pattern recognition the three pollutants studied. The relative responses of the three bacteria (*E.coli*, *M. trichosporium*, and *Shewanella oneidensis*) to the three pollutants ($ZnCl_2$, DDVP, and pentane) presented in a pseudo-3D plot in Figure 7, clearly demonstrated this. The experimental points for $ZnCl_2$, DDVP, and pentane in concentrations up to 1M shown in different colours are well-separated in the 3D-graph in Figure 7. This is a clear indication that pattern recognition principles can be applied for identification of pollutants using different types of bacteria.

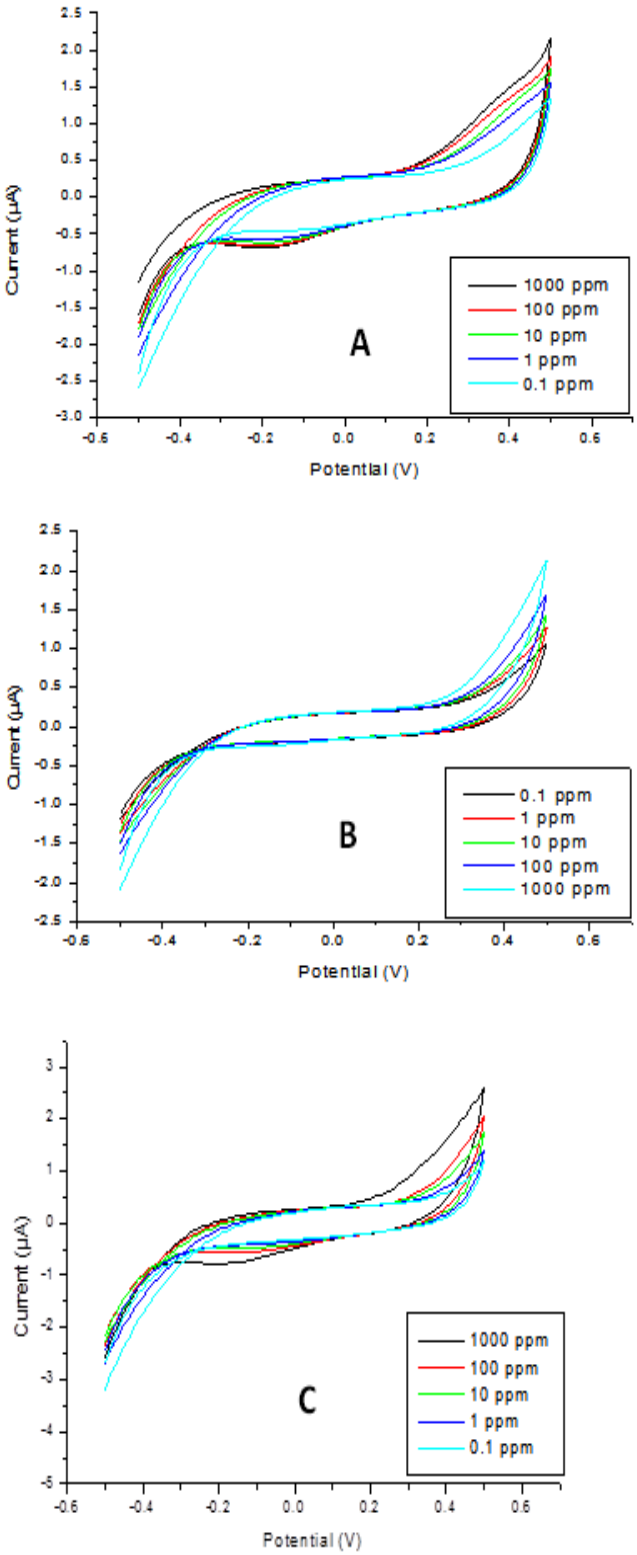

Figure 5. Cyclic voltammogram recorded (A) on immobilized *S. oneidensis* treated with different concentration of pentane, (B) *E. coli* treated with different concentration of DDVP and (C) *M. trichosporium* (OB3b) treated with different concentration of $ZnCl_2$; CV curves for clear LB broth are shown on all graphs.

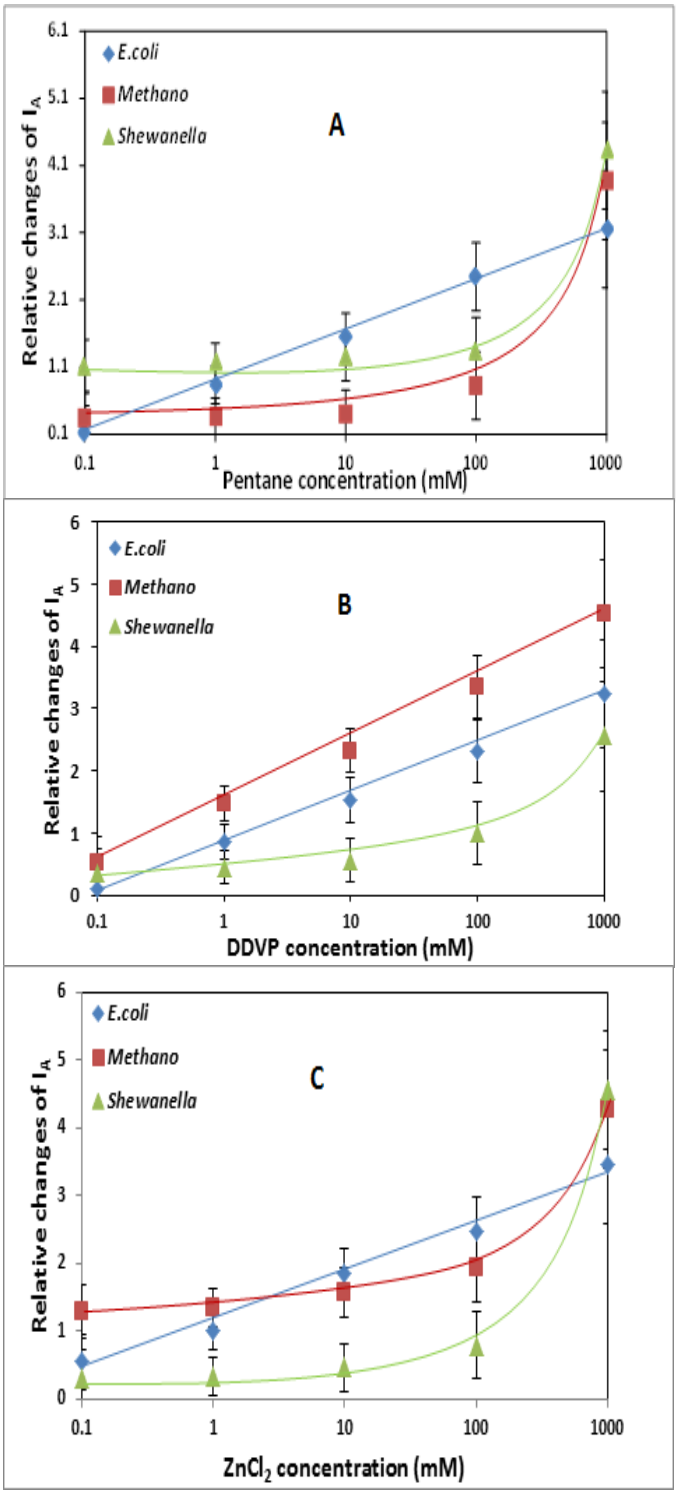

Figure 6. Comparison of relative changes of anodic current (IA) at +0.5V of all three types immobilized bacteria samples on modified electrodes exposure to: (A) pentane, (B) DDVP and (C) $ZnCl_2$.

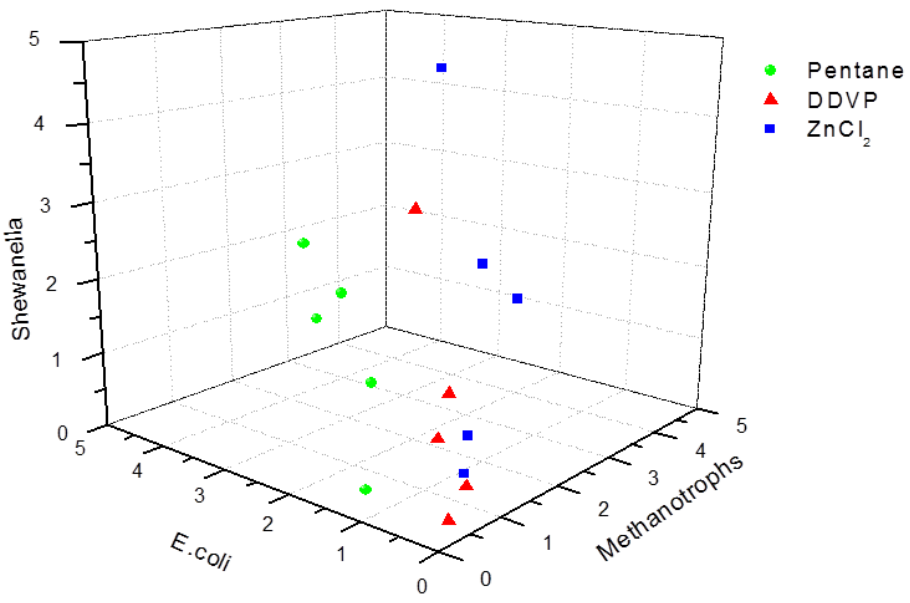

Figure 7. 3D plot of relative changes in anodic current for *E. coli*, *M. trichosporium* (OB3b) and *S. oneidensis* caused by different pollutants. Points show the direction of the pollutants' concentration increase from 0.1mM to 1000mM.

The concentration of pollutants could be evaluated to using the appropriate calibration and data extrapolation.

Conclusions and future work

This study gives proof-of-principle for the use of a panel of diverse wild-type bacteria to detect and discriminate different pollutant molecules. First of all, the values of anode (or cathode) current were found to correlate with bacteria concentration and thus with the concentration of different pollutants acting as inhibitors for bacteria. It shows simple electrochemical tests, i.e. cyclic voltammograms, either on gold electrodes immersed into liquid bacteria samples or (even better) on screen printed gold electrodes with immobilized bacteria have distinctive characteristic responses to different pollutants, and the pattern recognition principles can be applied for identification of pollutants.

This work paves the way for the development of novel, simple, and cost effective electrochemical bacteria-based

sensor array for preliminary assessment of the presence of pollutants in water. Future work which is currently underway will focus on extending the range of pollutants (different heavy metals, pesticides, and petrochemicals) and using advanced data processing tools such as (ANN) Artificial Neuron Network for analysis of real water samples.

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