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► **To cite this version:**

Aliki Tsopela, Ahmet Lale, Emilie Vanhove, Olivier Reynes, Isabelle Séguy, et al.. Integrated electrochemical biosensor based on algal metabolism for water toxicity analysis. *Biosensors and Bioelectronics*, 2014, 61, pp.290 - 297. 10.1016/j.bios.2014.05.004 . hal-01504978

HAL Id: hal-01504978

<https://hal.science/hal-01504978v1>

Submitted on 10 Apr 2017

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Integrated electrochemical biosensor based on algal metabolism for water toxicity analysis

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Abstract

An autonomous electrochemical biosensor with three electrodes integrated on the same silicon chip dedicated to the detection of herbicides in water was fabricated by means of silicon-based microfabrication technology. Platinum (Pt), platinum black (Pt Bl), tungsten/tungsten oxide (W/WO₃) and iridium oxide (Pt/IrO₂) working ultramicroelectrodes were developed. Ag/AgCl and Pt electrodes were used as reference and counter integrated electrodes respectively. Physical vapor deposition (PVD) and electrodeposition were used for thin film deposition. The ultramicroelectrodes were employed for the detection of O₂, H₂O₂ and pH related ions H₃O⁺/OH⁻, species taking part in photosynthetic and metabolic activities of algae. By measuring the variations in consumption-production rates of these electroactive species by algae, the quantity of herbicides present at trace level in the solution can be estimated. Fabricated ultramicroelectrodes were electrochemically characterized and calibrated. Pt Black

ultramicroelectrodes exhibited the greatest sensitivity regarding O₂ and H₂O₂ detection while Pt/IrO₂ ultramicroelectrodes were more sensitive for pH measurement compared to W/WO₃ ultramicroelectrodes for pH measurement. Bioassays were then conducted to detect traces of Diuron herbicide in water samples by evaluating disturbances in photosynthetic and metabolic activities of algae caused by this herbicide.

Keywords: ultramicroelectrodes; oxygen; hydrogen peroxide; pH measurement; algal metabolism; pesticides detection

1. Introduction

Water quality assessment has been an issue of particular concern over the past few years and is actually generating major interest. Contamination of water resources brought about by agricultural and industrial practices can pose possible threats and have a long-term impact on the environment and all living organisms. Pesticides are frequently employed in agriculture to protect crops and control non-crops and can easily leach from the soil in underground water supply and be often detected in rivers, lakes and later in consumption water (Barbash et al., 2001). It is therefore a major challenge to rapidly detect contamination by monitoring the quantities of different toxicants present in water.

Conventional analysis methods like gas (Schellin et al., 2004) and liquid (Jáuregui et al., 1997) chromatography performed in highly equipped laboratories do still provide the most accurate and sensitive means of detecting water contamination but they encounter considerable limitations as they are expensive and time consuming (Eltzov and Marks, 2011). Electrochemical detection systems are a potential alternative to these methods as they are easily miniaturized and portable enabling on-site analysis and providing rapid, real-time feedback information on the presence of pollutants (Lagarde and Jaffrezic-Renault, 2011; Grieshaber et al., 2008; Li et al., 2013). More specifically, lab on chip based, electrochemical biosensors meet the requirement for a rapid, cost-effective, accurate and sensitive analytical system and give information on water quality by detecting wide range of chemical species (Jang et al., 2011). They convert a biochemical phenomenon into a detectable and measurable electrical signal that is proportional to the analyte concentration and provide an early indication by sorting the samples needed to be further analyzed by conventional techniques.

Electrochemical biosensors can be amperometric, potentiometric and conductometric.

The operating principle of amperometric biosensors lies in applying a potential and

recording the current corresponding to oxidation and reduction of electroactive species on electrode surface. Current variations result from variations in consumption-production rates of electroactive species and reflect the biochemical phenomenon taking place (Ben-Yoav et al., 2009). Amperometric biosensors have been widely used for the oxygen monitoring in wastewaters (Chan et al., 2000), detection of organophosphates (Mulchandani et al., 2001), phenols (Mulchandani et al., 2005), cyanide (Ikebukuro et al., 1996) and heavy metal ions (Lehmann et al., 2000).

Potentiometric biosensors are designed to measure the difference in potential between working electrode and a stable reference electrode with no current flowing through the system (Lagarde and Jaffrezic-Renault, 2011). Potential values are then correlated to the concentration of the analyte (Lei et al., 2006). Although pH electrodes have been widely used for detection of pesticides in agriculture (Mulchandani et al., 2001; Gaberlein et al., 2000), other ion selective electrodes, such as chloride ion selective electrode, have also generated major interest in wastewater treatment domain (Han, 2002).

Conductometric biosensors record variations in conductivity of the electrolyte containing charged species in contact with the electrode (Lagarde and Jaffrezic-Renault, 2011). These variations result from the production or consumption of charged species during different reactions and can therefore indicate a biological process taking place (Lawrence and Moores, 1972; Jaffrezic-Renault and Dzyadevych, 2008). They have found various applications in environmental monitoring for detection of heavy metal ions and pesticides (Chouteau et al., 2004; Chouteau et al., 2005).

As far as biological recognition elements are concerned, algae are often used in biochemistry for trace detection of environmental toxic compounds as they are sensitive to changes in their environment induced by the presence of toxicants and can therefore provide information on toxic effect of pollutants on living organisms (Giardi and Pace, 2005; Brayner et al., 2011; Moreno-Garrido, 2008; Carrilho et al., 2003). Due to their high sensitivity to toxic substances, even

lower detection limits (LOD) can be reached (Brayner et al., 2011). Microalgae and photosynthetic cyanobacteria are used in biosensors industry as they are capable of providing information on pollution levels based on modifications in photosynthetic or metabolic activity (Kasai and Hatakeyama, 1993; Lagarde and Jaffrezic-Renault, 2011). Monitoring of species such as oxygen, hydrogen peroxide and pH related ions that participate in algae's metabolism and photosynthesis can indicate the presence of herbicides. For instance, amperometric microalgae sensors are designed to monitor photosynthetic oxygen production and can therefore indicate pollutant related photosynthetic activity inhibition (Pandard and Rawson, 1993). Furthermore, hydrogen peroxide, the concentration of which is related to potential stress exerted on algae, could be useful indicator of the presence of herbicides. As reported by Mittler (Mittler, 2002), reactive oxygen species (ROS) can be stress indicators and signals for defense pathways activation. Different types of stress (pathogens, extreme temperatures, light, heavy metals, salt, pollutants) can cause metabolic disruptions resulting in an increase in the production of ROS (Mittler et al., 2004). Along with O_2 and H_2O_2 , pH monitoring can also provide information on metabolic activity modifications induced by herbicides and other inhibitors based on changes in medium basification and acidification rates (Schubnell et al., 1999).

As a matter of fact, different sensors have been developed for the detection of the species mentioned above, viewed as indicators of water pollution (O_2 , H_2O_2 , H_3O^+/OH^- ions). Two basic detection methods, amperometry and potentiometry have been discussed and various electrode materials have been mentioned.

As far as oxygen monitoring is concerned, noble metals (Pt or Au) are primarily used in literature as working electrode materials so that the electrode surface does not participate in the chemical reaction (Lee et al., 2007). Platinum is the most suitable candidate for oxygen detection as it is physically stable (Zhang and Wilson, 1993), mechanically robust (Ben-Amor et al., 2013) and compared to gold and other metals is the best electrocatalyst for the oxygen reduction reaction (Maja N. Desic, 2005). Platinum microdiscs (Sosna et al., 2007) and array of platinum

microelectrodes (Wittkamp et al., 1997) have been employed as dissolved oxygen sensors in environmental monitoring. Different techniques have been followed to enhance sensitivity by increasing electro-active surface area and porosity of platinum electrodes and therefore enhancing mass transport of species towards electrode surface (Qiang et al., 2010; Lee and Park, 2011). Although platinum is the most commonly applied material in oxygen sensors, gold electrodes have been designed to measure dissolved oxygen in environmental samples (Liu et al., 2007), bacterial films (Lee et al., 2007) or during cell culture (Pereira Rodrigues et al., 2008). Carbon nanotubes integrated on a screen-printed algal biosensor have also been used to detect oxygen produced by algae (Shitanda et al., 2009).

As already stated, hydrogen peroxide is an essential species to monitor as it is taking part in various biological processes (Li et al., 2013). Gold (Brett et al., 1997), platinum (Zhang and Wilson, 1993; Yokoyama et al., 1997), carbon based (Cho et al., 2010; Nowall and Kuhr, 1997), enzyme modified (Ruzgas et al., 1996) and Prussian Blue modified (Karyakin et al., 2009) electrodes are situated in the main stream of research interest towards suitable working electrode materials for the detection of H_2O_2 . Platinum is still the preferred option as it provides a high catalytic surface for H_2O_2 oxidation (Arbault et al., 1995) and it is even possible to increase active surface area of the electrodes by platinization. Indeed, sensors with platinized working electrodes yield greater sensitivity due to their nanoscale structure (Vaddiraju et al., 2010) and the large number of electroactive sites that facilitates electron transfer (Li et al., 2013). Furthermore, on platinum black (Pt Bl) microelectrodes, H_2O_2 oxidation is taking place at a lower potential compared to bare Pt electrodes, minimizing interferences induced by other electroactive species (Arbault et al., 1995). Platinum black microelectrodes have been widely used to study ROS species released during cellular oxidative bursts (Li et al., 2013) and monitor the production of H_2O_2 by mitochondria during aerobic metabolism (Ben-Amor et al., 2013). They have also found various applications as amperometric glucose sensors (De Corcuera et al., 2005).

Potentiometry is widely applied for pH monitoring and glass membrane electrodes are commonly used. However, glass fragility and size limitation impede in situ measurement in complex configurations and mediums (Ruffien-Ciszak et al., 2008; Huang et al., 2011) giving way to metal/metal oxide based pH sensors that are easily miniaturized and provide a well-defined metal/metal oxide interface (Daomin Zhou, 2008). More specifically, iridium oxide films can be formed by various physical and chemical methods (Ges et al., 2005) and exhibit many advantages among which high pH sensitivity, biocompatibility, chemical stability and fast response time (Daomin Zhou, 2008; Marzouk et al., 1998). IrO₂ films have therefore found applications in cell metabolism monitoring in cell culture volumes (Ges et al., 2005) and microenvironmental studies (Bezbaruah and Zhang, 2002). Tungsten/tungsten oxide sensors represent another attractive alternative for pH monitoring as tungsten is cheaper compared to other metals such as Pt or Au. Additionally, stable tungsten oxides can be easily formed by electrochemical oxidation of the metal at low potential (Ruffien-Ciszak et al., 2008). W/WO₃ sensors have been developed for skin pH monitoring (Ruffien-Ciszak et al., 2008), determination of extracellular pH values for cultured cells (Yamamoto et al., 2003) and pH measurement of environmental water samples (Dimitrakopoulos et al., 1998).

Thus, elaboration of detection materials and analytical methods led to the development of different systems applied for detection of water pollution. In this study, an autonomous fully integrated three-electrode electrochemical microsystem is developed by means of microfabrication technology on a silicon chip. The performance of the microsystem is evaluated through electrochemical characterization and calibration is performed by amperometric and potentiometric methods for the three species of interest: dissolved oxygen (O₂), hydrogen peroxide (H₂O₂) and pH related ions (H₃O⁺/OH⁻). The electrochemical microsystem is further used in bioassays, serving as screening prior to lab-analysis, to detect traces of toxicants in water samples by evaluating their effect on a biological sensing element, such as algae. Pollution level was estimated by measuring disturbances in photosynthetic and metabolic activities of algae

caused by herbicides. These disturbances are represented by variations in consumption-production rates of the three electro-active species (dissolved oxygen, hydrogen peroxide and pH related ions) by algae and could therefore be detected by the electrochemical microsensor. A variation of oxygen production during algal photosynthesis is evidenced in the presence of Diuron herbicide.

2. Experimental

2.1 Electrode materials and configuration

Electrochemical microcells designed for this work consist of three integrated electrodes (working, reference, and counter electrodes) on the same silicon chip (Christophe et al., 2013). Working electrode materials were determined according to previously mentioned electro-active species needed to be detected. Platinum and functionalized Platinum Black (Pt BI) working electrodes were fabricated for H₂O₂ and dissolved O₂ measurements whereas tungsten/tungsten oxide (W/WO₃) and iridium oxide (Pt/IrO₂) electrodes were used for pH monitoring. The autonomous, miniaturized electrochemical cell was completed by integrating a platinum counter electrode and a silver based electrode in the structure. The latter was further electrochemically oxidized to form the Ag/AgCl reference electrode. In order to determine the arrangement and form of the three integrated electrodes, different electrode configurations were patterned; parallel and concentric arrangements were tested. For concentric arrangement, working electrode is encircled by the reference and counter electrodes that have a ring shape while for parallel arrangement, all electrodes are placed in parallel with reference electrode placed in the middle of the structure to reduce ohmic drop. Both single ultramicroelectrodes and ultramicroelectrode arrays were evaluated in terms of detection properties. In this way, favorable properties of ultramicroelectrodes and planar array configurations were used to enhance the efficiency of the sensors. Dimensions and distance between electrodes in the array were determined so that diffusion profiles of adjacent electrodes will not overlap.

2.2 Fabrication of the electrochemical microcells

Electrochemical microcells were fabricated on a thermally oxidized silicon wafer (SiO_2) (oxide layer thickness: $\sim 1 \mu\text{m}$). All thin metal layers were evaporated on the oxidized silicon substrate (Figure 2a) and conventional photolithography followed by lift-off technique was conducted in order to obtain metal patterns. Platinum (Pt) (150 nm) and tungsten (W) (500 nm) layers correspond to working electrodes while silver (Ag) layer (400 nm) represents the future reference electrode. Platinum (Pt) (150 nm) also serves as the counter electrode. The underlying titanium (Ti) layer (20 nm) assures adhesion of platinum on SiO_2 substrate. Silver was deposited on a platinum layer, which in this case serves as a barrier layer, in order to avoid diffusion of titanium atoms in the silver layer. All layer thicknesses were selected so that no mechanical stress is exerted on the silicon wafer. Following the development of all metal layers, electrode's active surface area needed to be precisely defined as it will be later used to correlate current intensity recorded to the quantity of species to be detected. Low temperature Inductively Coupled Plasma Chemical Vapour Deposition (ICP-CVD) was conducted for the deposition of high quality silicon nitride (Si_3N_4) insulation layer (100 nm) (Figure 1a). ICP-CVD process yields conformal deposition that enables perfect covering of the whole structure -even the lateral parts of metal layers. However, in order to enable patterning of a conformal coating, a specific double layer lift-off process has been developed and the thickness of the Si_3N_4 film was optimized accordingly (Vanhove et al., 2013). This insulation layer also contributes to long-term stability of electrochemical microcells in contrast to organic insulation layers that do not provide sufficient stability through time in complex, corrosive mediums (Vanhove et al., 2013). After fabrication, devices were diced and attached on a printed circuit board (PCB). Wedge bonding was subsequently carried out to complete packaging procedure and wire bonds were covered in a silicon glob-top to assure mechanical protection and avoid electrical short circuit (Figure 1b).

2.3 Surface functionalization of ultramicroelectrodes

In order to attain high sensitivity of platinum working electrodes, porosity and surface area were enhanced by forming “black platinum” electrodes. Platinization procedure was carried out by reducing a solution of hexachloroplatinic acid (32 mM in phosphate buffer solution) which contains lead acetate (1.23 mM) at -60 mV versus Ag/AgCl/KCl_{sat} (Ben-Amor et al., 2013). The amount of Pt deposited was controlled by the coulometric charge versus time plots recorded during platinization so that a nanostructured surface will be obtained. Platinization charge deposited on single Pt ultramicroelectrodes ($d = 50 \mu\text{m}$) was $0.5 \mu\text{C}.\mu\text{m}^{-2}$ compared to $0.25 \mu\text{C}.\mu\text{m}^{-2}$ on the arrays of Pt ultramicroelectrodes (25 disks of $10 \mu\text{m}$ diameter).

Regarding pH electrodes, tungsten W surface was oxidized to form a WO₃ film by cyclic voltammetry in a 2 M H₂SO₄ solution, from +1.0 to +2.0 V vs Ag/AgCl/KCl_{sat}, at the scan rate of $20 \text{ mV}.\text{s}^{-1}$ for 20 cycles (Yamamoto et al., 2003). For the deposition of IrO₂ on Pt electrodes, an iridium tetrachloride based solution was prepared as described elsewhere (Marzouk, 2003). A constant potential (0.6 V/ECS) was applied and the duration of the deposition was indicated by the total charge transferred ($0.5 \text{ C}.\text{cm}^{-2}$) (Ges et al., 2005).

In order to form the Ag/AgCl reference electrode and obtain a fully integrated electrochemical cell, Ag surface was electrochemically oxidized. Silver surface was first examined by conducting cyclic voltammetry in acidified potassium nitrate (KNO₃) solution of 0.1 M. The electrode was then oxidized in 10^{-2} M potassium chloride solution (KCl) by linear sweep voltammetry within the potential range of 0.1-0.25 V/ECS and a low scan rate of $1 \text{ mV}.\text{s}^{-1}$ which yields stable and homogeneous Ag/AgCl structure (Christophe et al., 2013). Indeed, higher scan rates could result in porous layers and electroactive species trapped between pores induce changes in reference electrode's potential (Christophe et al., 2013).

2.4 Electrochemical characterization and calibration

All electrochemical experiments were conducted using a potentiostat (VMP3, BioLogic)

with a low-current option controlled by EC-Lab software, at room temperature.

Cyclic voltammetry in a zero oxygen 0.5 M H₂SO₄ solution was conducted to characterize the quality of fabricated Pt and Pt BI electrodes. Potential range scanned was -0.2 to +1.1 V vs Ag/AgCl/KCl_{sat} at a scan rate of 100 mV. Material properties were tested in order to validate that all thin layers developed by microfabrication technology gave results similar to the ones obtained with bulk materials.

For Pt and Pt BI working electrodes, active surface area was determined by linear sweep voltammetry in solutions of potassium hexacyanoferrate (II) trihydrate (10 mM) in 0.1 M KCl used as a supporting electrolyte.

Calibration curves for H₂O₂ measurements were then obtained by recording current responses to different concentrations. pH sensors calibration was performed by monitoring the potential of the two metal oxide electrodes in solutions of different pH through open circuit potential (OCP) mode. O₂ sensors performance was pre-validated in saturated in oxygen and zero oxygen CaCl₂ (0.1 M) solutions by comparing current values obtained.

2.5 Bioassays

As far as bioassays are concerned, green micro algae *Chlamydomonas reinhardtii* (CC-125) were cultivated at 25°C with time regulated light/dark (16h/8h) cycles in High Salt Medium (HSM) (N Sueoka, 1967). The same solution was used through all our tests. The main goal of this study being to determine the relation between the change in metabolic activities of algae and the presence of herbicides, measurements were conducted in model HSM solution in order to eliminate possible interferences found in real water samples. Nevertheless, for final application, we plan to validate this concept in consumption water obtained from groundwater, rivers or lakes). Growth parameters were controlled so that algae's growth rate is constantly kept at the exponential phase. Considering that the quantity of species produced or consumed depends on the number of cells in the solution, initial algal solutions were centrifuged to obtain a 5-fold

density increase, in order to yield the maximum possible signal. Concentration of cells per mL of solution was always kept constant through different experiments and was measured by Multisizer Coulter Counter (Beckman Coulter). Bioassays using the fabricated electrochemical microsensors were performed in the dense algal solutions and current evolution was observed during light and dark periods in the presence and absence of Diuron (3(3,4-dichlorophenyl)-1,1-dimethylurea) herbicide, the toxic chemical to be detected. Chronoamperometry was conducted and the potential applied corresponds to the O₂ reduction potential which was estimated through cyclic voltammetry at -0.7 V vs integrated Ag/AgCl.

All reagents were purchased from Sigma-Aldrich.

3. Results and discussion

3.1 Electrochemical characterization - calibration of ultramicroelectrodes

The voltammograms obtained for fabricated Pt and Pt BI thin film electrodes in 0.5 M H₂SO₄ (voltammograms not shown) presented peaks related to the formation and reduction of platinum oxides as well as the electrosorption and electrodesorption of protons indicating a behaviour similar to the one of Pt polycrystalline bulk material (Tilak et al., 1973). The radius of fabricated single and array Pt and Pt BI electrodes was then evaluated in Fe(CN)₆⁴⁻ solution and compared to the dimensions initially designed. The value was determined through the theoretical equation relating I_{lim} current (value of the oxidation plateau) to the radius of the electrode (Equations 1 and 2) (Saito, 1968). Equation 2 is given for an array of microdisk electrodes widely spaced where there is no overlapping of diffusion layers and I_{lim} represents the sum of currents obtained for each ultramicroelectrode as they are connected in parallel. As expected, platinum black porous structure yielded well-defined oxidation plateaus and steeper waves indicating rapid electron transfer reactions. Platinization induces a 6-fold increase in electrode's I_{lim} value.

$$I_{lim} = 4nFDCr \quad \text{Equation 1}$$

$$I_{lim} = 4nFDCr z \quad \text{Equation 2}$$

Where F is the Faraday constant ($F = 96500 \text{ C.mol}^{-1}$), n is the number of electrons exchanged per mole ($n = 1$), C is $\text{Fe}(\text{CN})_6^{4-}$ ion concentration (0.01 M), D represents the diffusion coefficient ($6.3 \times 10^{-6} \text{ cm}^2.\text{s}^{-1}$) (Konopka and McDuffie, 1970) and z is the number of electrodes in the array.

Regarding Ag electrode it is important to note that the obtained Ag/AgCl is a quasi-reference electrode as it is in direct contact with the examined solution without any internal electrolyte (Simonis et al., 2004). Quasi-reference electrode's stability was evaluated in 0.01 M KCl solution. Ag/AgCl electrode's potential compared to external Ag/AgCl/KCl_{sat} electrode was plotted versus time (results not shown). Potential deviation in time was estimated around $0.16 \mu\text{V}.\text{s}^{-1}$, value that is an acceptable for the present application given the fact that short-time measurements are conducted.

Following validation of electrochemical performance of different electrode materials, micro-electrochemical cells were used to detect electroactive species of interest (H_2O_2 , O_2 , pH related ions $\text{H}_3\text{O}^+/\text{OH}^-$).

H_2O_2 detection was firstly performed by following current increase after successive injections of H_2O_2 in PBS (10 mM) solution (Figure 2a). Concentration range tested was between 10nM and 10 μ M so that it can be verified that the detection of low H_2O_2 quantities (range of interest in biological applications) is feasible. An anodic potential of 0.6 V and 0.3 V vs Ag/AgCl/KCl_{sat} was applied to initiate the oxidation of H_2O_2 present in the solution for Pt and Pt B1 electrodes respectively. The potential applied corresponds to the plateau potential of the oxidation wave determined by cyclic voltammetry in 10 mM H_2O_2 solution in PBS. The lower

potential value applied for Pt BI compared to the one applied for Pt electrodes is ascribed to electrocatalytic activity of platinum black towards H_2O_2 reactions (Arbault et al., 1995). Calibration curves for H_2O_2 microsensors were obtained by plotting current increase recorded for each injection (Figure 2b). Platinum and platinum black single electrodes and arrays were compared in terms of sensitivity and limits of detection (LOD).

Linear response was observed through the entire concentration range tested ($R^2 \geq 0.99$) for all Pt and Pt BI single electrodes and arrays. Black platinum electrode arrays exhibited the greatest sensitivity for H_2O_2 detection with a slope of $17 \text{ pA}\cdot\text{mm}^{-2}\cdot\text{nM}^{-1}$ and a limit of detection of 10 nM. As a matter of fact, arrays hold the advantages of microelectrodes while generating a higher current signal. Their high signal-to-noise ratio combined with the high electrocatalytic activity of platinum black surface lead to enhanced electrode performance regarding detection properties, detection limits and sensitivities. Pt BI single electrodes also yielded higher sensitivity than Pt electrodes with a slope of $12.4 \text{ pA}\cdot\text{mm}^{-2}\cdot\text{nM}^{-1}$ but 1.4 times lower than the one provided by Pt BI arrays.

Prior to validation of O_2 microsensors in algal solutions it was important to verify that the sensor yields a response correlated to O_2 concentration. The electrode potential was therefore set at $-0.7 \text{ V vs Ag/AgCl/KCl}_{\text{sat}}$, (potential corresponding to the plateau value of oxygen reduction current) and output current was recorded for a saturated and a zero oxygen CaCl_2 (0.1 M) solution at $22 \text{ }^\circ\text{C}$. As expected, O_2 concentration variation induces a variation in current intensity generated by the O_2 reduction reaction on platinum or platinum black electrodes (results not shown).

Overall, Pt BI ultra-microelectrode arrays present the greatest sensitivity for O_2 and H_2O_2 reactions due to the electrocatalytic activity of platinum black towards these reactions (Arbault et al., 1995; Ben-Amor et al., 2014). Indeed, Pt BI electrodes exhibit increased electro-active surface area and porosity compared to platinum electrodes and therefore enhanced mass

transport of species towards electrode surface. Their nanoscale structure (Vaddiraju et al., 2010) and large number of electroactive sites enhance electron transfer (Li et al., 2013).

pH measurements were conducted by the open circuit potential technique for calibration of W/WO₃ and Pt/IrO₂ electrodes. Equilibrium potential was recorded for different pH solutions. This potential is given by Nernst equation and for metal/metal oxide electrodes depends only on the pH of the solution. As pointed out by the graph in Figure 3a, the Pt/IrO₂ pH sensor presented no hysteresis. Both electrodes demonstrated an almost linear response over a wide pH range (2.0–12.0). The sensitivity of Pt/IrO₂ electrode (60 mV/pH) was superior to that of W/WO₃ electrode (42 mV/pH) (Figure 3b). Pt/IrO₂ exhibits a supra-Nernstian response due to the oxidation states present on the surface as already reported in literature (Kim and Yang, 2014; Olthuis et al., 1990). On the other hand, W/WO₃ electrodes demonstrate a sub-Nernstian response that can be due to defects in the metal-metal oxide interface produced through oxidation of the tungsten layer. What is more, if the oxidation of the tungsten layer deposited through PVD is not optimized, native tungsten oxides such as WO₂ or W₂O₅ (Pasti et al., 2012) can be also created on the surface along with WO₃ which leads to a lower sensitivity of the final layer. In order to improve characteristics of the W/WO₃ sensors, optimization of the electro-oxidation procedure is in progress.

3.2 Bioassays

Special emphasis was placed on O₂ detection as it was considered to be the main pollution indicator, given the fact that O₂ evolution reflects photosynthetic activity of algae and that it is largely altered by pollutants' existence. Photosystem II (PSII) is a complex located in the thylakoid membranes of algae where light energy is used to produce oxygen from water oxidation through a series of electron transfer reactions during photosynthesis (Varsamis et al., 2008; Giardi and Pace, 2005). Urea-based pollutants, such as Diuron, disrupt the electron flow in PSII because of their high-affinity binding to the PSII complex (Pfister et al., 1981). They bind

to the D1 protein of PSII within its plastoquinone QB binding pocket blocking photosynthetic electron transport and therefore inhibiting O₂ production (Tischer and Strotmann, 1977).

At first, the performance of the sensor for O₂ detection was evaluated with algae in the absence of toxicants in order to demonstrate the relationship between the photosynthetic activity and the current signal measured. Light and dark periods were therefore altered to obtain equal periods of daylight illumination and darkness and current variations were recorded through chronoamperometry. Under illumination, amperometric responses recorded, exhibited an increase in the oxygen reduction current due to photosynthesis. The decrease was evident when light was turned off as a result of respiration process during which algae consume oxygen and produce CO₂ (Figure 4). Initial duration of light and dark cycles was six minutes. We then reduced cycle duration to two minutes in order to validate that similar response is obtained even by applying shorter time intervals. It is important to notice that the change in current evolution is immediately evident when light is switched on and off.

Variations in photosynthetic activity of algae were then determined in presence of Diuron herbicide (Figure 5). As ethanol 70% is the solvent used to prepare Diuron solutions, its volume was kept constant through different injections to prevent solvent influence on algal response. Current evolution through respiration and photosynthesis with and without Diuron is presented in Figure 5a. The impact of the herbicide on electron flow and therefore photosynthetic O₂ production is confirmed by comparing the steep slope corresponding to oxygen production rate (nA/min) during photosynthesis obtained for the blank algal solution with the smoother one obtained for Diuron contaminated algal solution. It is yet demonstrated that there is no toxicant-induced inhibition on respiration procedure as the two slopes corresponding to oxygen consumption for blank and contaminated algal solution are identical.

Oxygen production rates for different pollutant concentrations (0.2-1 µM) were compared in order to demonstrate concentration dependent inhibition effect of the herbicide on

photosynthesis (Figure 5b). The response of a blank algae solution was first compared to the one of an algal solution with ethanol to validate that ethanol has no inhibitory effect on O₂ production. Oxygen production rate obtained with our Pt BI ultramicroelectrode array (25 disks of diameter d=10 μm) for a blank algae solution (given strain: *Chlamydomonas reinhardtii* with a concentration 6 *10⁶ cells/mL) is 10nA/min. We then compared the latter rate to the rate obtained for algal solution containing different concentrations of Diuron herbicide. The rate corresponding to the limit of maximum acceptable concentration of this pollutant implemented by governments (0.6 μM of Diuron) was finally calculated at 4 nA/min.

The inhibition on the oxygen production rate percent induced by herbicides was estimated from the oxygen production rate calculated before and after the herbicide injection and it is defined as followed:

$$\text{Inhibition ratio}\% = \frac{\text{rate}_{\text{blank}} - \text{rate}_i}{\text{rate}_{\text{blank}}}$$

Where $\text{rate}_{\text{blank}}$ represents oxygen production rate during photosynthesis for blank algal solution and rate_i represents the rate for different concentrations of Diuron injected in algal solutions.

The Diuron calibration graph, given in Figure 6, demonstrates that inhibition increases along with Diuron concentration. Variation in production rate obtained was 65% inhibition per μM of Diuron with LOD of around 0.2 μM lower than maximum acceptable concentration in drinking water implemented by European governments (0.64 μM). It is therefore demonstrated that the fabricated electrochemical biosensor can be an efficient indicator of water pollution.

Although aforementioned fabricated systems aim for environmental applications that require disposable devices in compliance with regulations, the characteristics of our prototypes were tested in order to examine their reproducibility. The devices were used through several tests with algae and different concentrations of Diuron pollutant without significant variation in their response (around 60 tests with one device). A cleaning step in 0.5 M H₂SO₄ before each series of

measurements was applied in order to reactivate the electrode surface and recycle the system. The stability of the insulation layer that defines active areas as well as the stability of different active electrode layers can thus be validated. We can therefore also target other applications that require repetitive and/or online measurements.

4. CONCLUSION

We have presented above the development of a micro-biosensor for the detection of environmental pollution. Electrochemical microcells were fabricated for the detection of O_2 , H_2O_2 and H_3O^+/OH^- ions, species taking part in metabolic activities of algae. Electrochemical microcells consist of three integrated electrodes (working, reference, and counter) on silicon chip. Electrode materials were chosen according to the species to be detected: Pt and black Pt electrodes were therefore used for O_2 and H_2O_2 detection while W/ WO_3 and Pt/ IrO_2 electrodes were selected for pH monitoring. Electrodes were characterized, calibrated and further used in bioassays in algal solutions. Given the fact that this paper aims at demonstrating that pollutants' detection can be simply carried out by monitoring changes in algal metabolism induced by presence of herbicides, we firstly focused on O_2 monitoring (i.e photosynthetic activity) as indicator of pollution as it is the element the most representative of variations in metabolic activities. O_2 evolution was therefore measured in algal solutions and Diuron herbicide detection was achieved with a sufficiently low LOD of 0.2 μM . However, we also plan to determine in future work the increase in H_2O_2 and OH^- production caused by presence of herbicides. To the best of our knowledge, the safety level of H_2O_2 and OH^- in *Chlamydomonas reinhardtii* algae cells in non-contaminated water is not already determined but it is clearly mentioned that herbicides cause metabolic disruptions resulting in significant increase in the production of ROS (Fedtke and Duke, 2004). Similar to O_2 monitoring, the measurement will be conducted by comparing the production of these two species for a blank solution and a herbicide contaminated solution using the H_2O_2 and pH sensors we fabricated that exhibit high sensitivity and

satisfactory LOD.

These aforesaid results may form the basis for the integration of the three independent μ electrochemical sensors (O_2 , H_2O_2 , pH) on the same fluidic platform by creating a matrix of channels and measurement chambers for multianalysis. Monitoring of each species can indicate the presence of different herbicides type. Indeed, herbicides like Diuron and Atrazine target Photosystem II, with a direct impact on O_2 production, while herbicides like Paraquat target Photosystem I by causing an increase in H_2O_2 and OH^- production. The detection of the three species can be performed at the same time by integrating each sensor in one independent chamber. A different fluidic chamber should be dedicated for the detection of each species as detection protocols are different: extracellular O_2 variation measurement can be directly performed after adding algae cells whereas intracellular H_2O_2 and pH variation monitoring calls for cell lysis prior to measurement. Moreover, dissociation of measurement chambers is important as not only electrochemical methods conducted for detection are different (chronoamperometry or potentiometry) but also detection of one species can produce elements that interfere with the measurement of the other species (i.e O_2 reduction produces H_2O_2).

This final multi-detection structure will be completed by integrating an optical sensor on the same μ fluidic platform (Lefèvre et al., 2012). Thus, combining amperometric and optical detection, improved sensor's performance towards herbicide detection can be attained.

Acknowledgement

The authors would like to thank the French "Agence nationale de la Recherche" (ANR, project DOLFIN, n° ANR-13-JS03-0005-01). Special thanks should also be addressed to Céline Christophe (LAAS-CNRS), Stephane Arbault, Salem Ben Amor and Damien Quinton of the Institute of Molecular Sciences (ISM, University of Bordeaux) as well as Florent Lefèvre and Annie Chalifour (Department of biological science, UQAM). Furthermore, microfabrication procedure was partly supported by the French RENATECH network.

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Figures caption

Figure 1. (a) Schematic representation of all layers comprising the fabricated electrochemical sensor (b) Autonomous electrochemical micro-sensor.

Figure 2. (a) Current measurement after successive injections of H_2O_2 for a Pt BI array electrode. The potential applied is 0.3 V vs Ag/AgCl/ KCl_{sat} . Inset: Current response for 10 nM H_2O_2 injection. (b) H_2O_2 calibration curves obtained for Pt BI and Pt single electrodes and arrays.

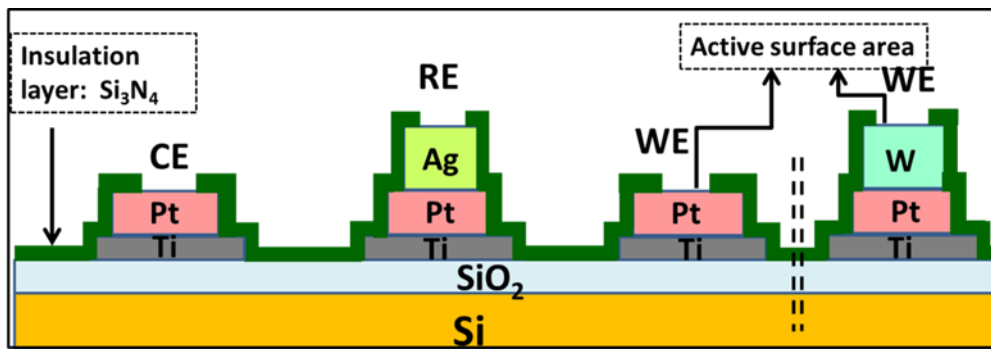
Figure 3. pH monitoring with OCP measurement. a) IrO_2 response for different pH values, b) Calibration curves for IrO_2 and W/WO_3 electrodes.

Figure 4. Current measurement through algal respiration and photosynthesis with Pt BI array electrode

Figure 5. Algal response to (a) 0.5 μM Diuron herbicide (b) various Diuron concentrations for Pt BI array electrode

Figure 6. Calibration curves (inhibition percent in oxygen production rate during photosynthesis versus Diuron concentration) for Pt BI array electrode.

a)



b)

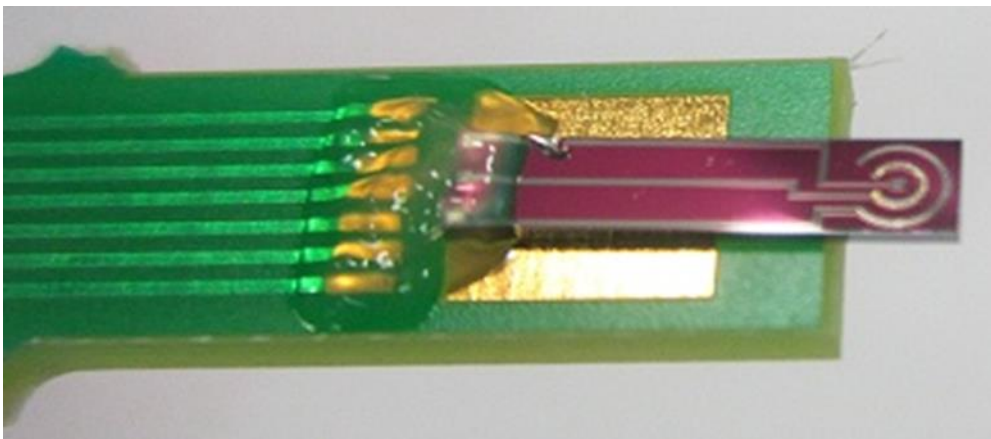
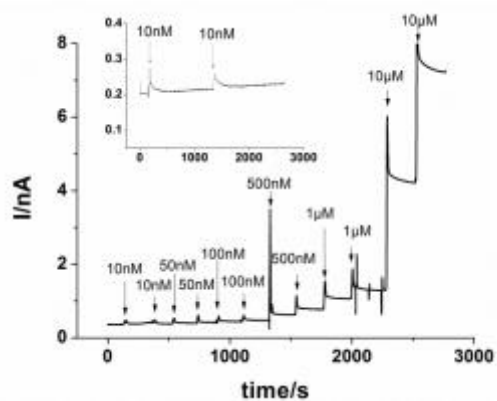


Figure 1. (a) Schematic representation of all layers comprising the fabricated electrochemical sensor
(b) Autonomous electrochemical micro-sensor.

a)



b)

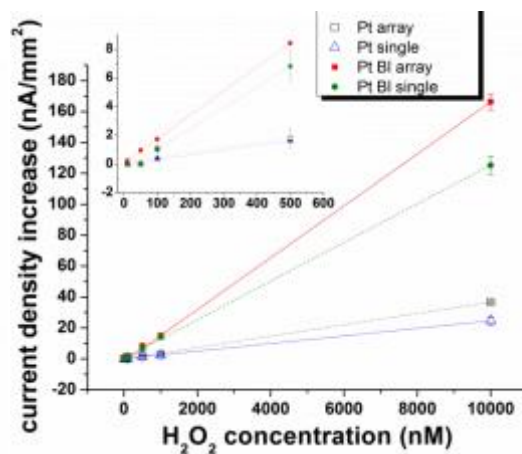
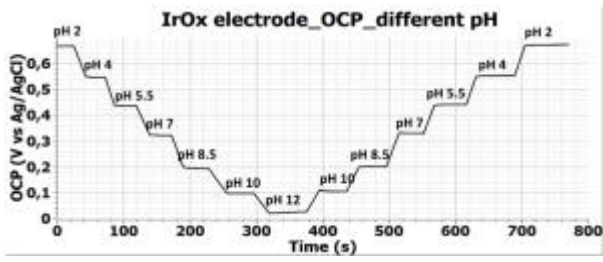


Figure 2. (a) Current measurement after successive injections of H_2O_2 for a Pt BI array electrode. The potential applied is 0.3 V vs Ag/AgCl/KCl_{sat}. Inset: Current response for 10 nM H_2O_2 injection. (b) H_2O_2 calibration curves obtained for Pt BI and Pt single electrodes and arrays.

a)



b)

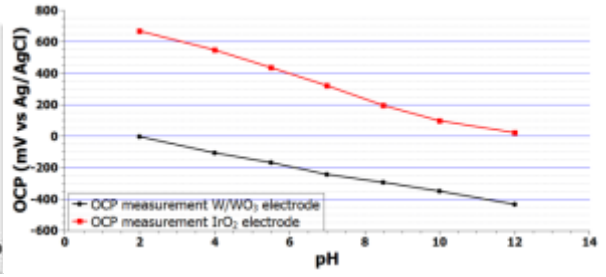


Figure 3. pH monitoring with OCP measurement. a) IrO₂ response for different pH values, b) Calibration curves for IrO₂ and W/WO₃ electrodes.

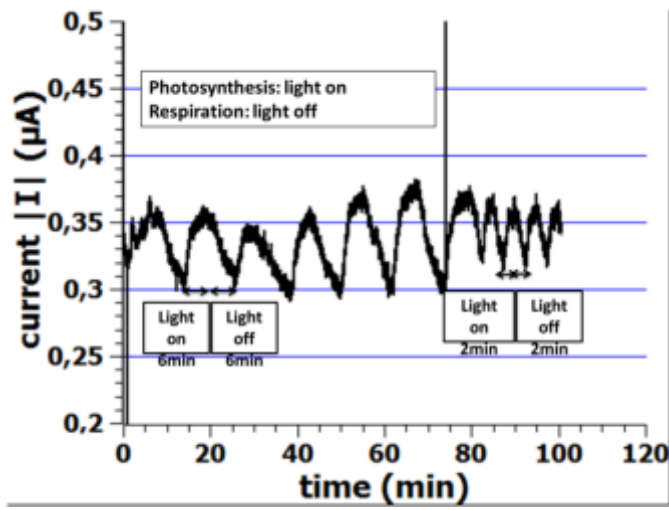
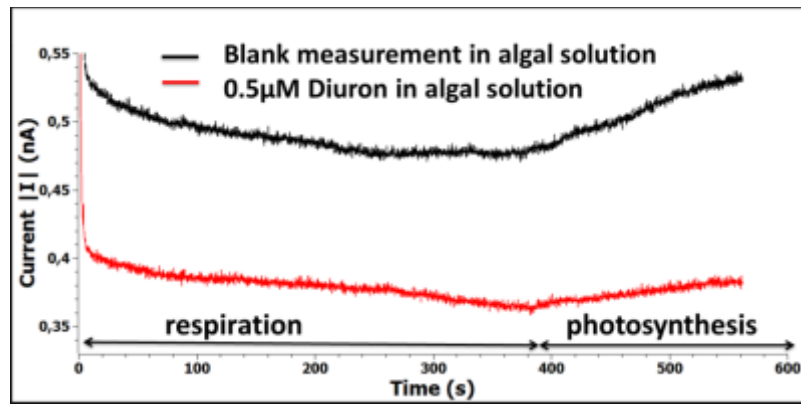


Figure 4. Current measurement through algal respiration and photosynthesis with Pt BI array electrode.

a)



b)

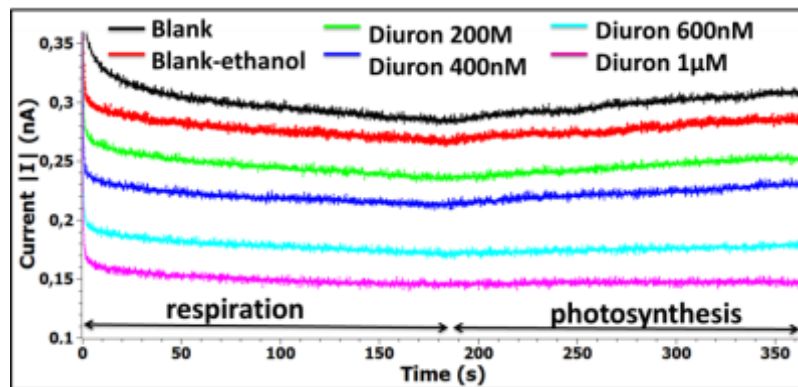


Figure 5. Algal response to (a) $0.5 \mu\text{M}$ Diuron herbicide (b) various Diuron concentrations for Pt BI array electrode.

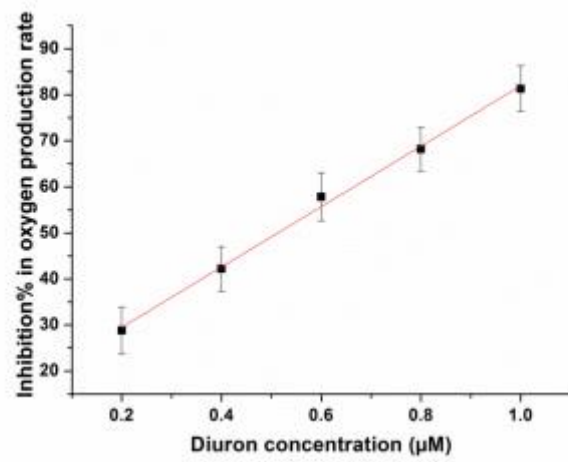


Figure 6. Calibration curves (inhibition percent in oxygen production rate during photosynthesis versus Diuron concentration) for Pt BI array electrode.