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Development of a lab-on-chip electrochemical biosensor for water quality analysis based on microalgal photosynthesis

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Abstract

The present work was dedicated to the development of a lab-on-chip device for water toxicity analysis and more particularly herbicide detection in water. It consists in a portable system for on-site detection composed of three-electrode electrochemical microcells, integrated on a fluidic platform constructed on a glass substrate. The final goal is to yield a system that gives the possibility of conducting double, complementary detection: electrochemical and optical and therefore all materials used for the fabrication of the lab-on-chip platform were selected in order to obtain a device compatible with optical technology. The basic detection principle consisted in electrochemically monitoring disturbances in metabolic photosynthetic activities of algae induced by the presence of Diuron herbicide. Algal response, evaluated through oxygen (O₂) monitoring through photosynthesis was different for each herbicide concentration in the examined sample. A concentration-dependent inhibition effect of the herbicide on photosynthesis was demonstrated. Herbicide detection was achieved through a range (blank – 1 μM Diuron herbicide solution) covering the limit of maximum acceptable concentration imposed by Canadian government (0.64 μM), using a halogen white light source for the stimulation of algal photosynthetic apparatus. Superior sensitivity results (limit of detection of around 0.1 μM) were obtained with an organic light emitting diode (OLED), having an emission spectrum adapted to algal absorption spectrum and assembled on the final system.

Keywords

Herbicide detection, algal metabolism, electrochemical sensor, ultramicroelectrodes, fluidic platform, OLED

1. Introduction

Assessment of water quality has been generating major interest over the past few years as there is an essential need to preserve freshwater sources such as lakes, rivers, water reservoirs and ground waters. Several factors can be responsible for water quality degradation such as the presence of heavy metals, organic contaminants, pathogenic micro-organisms as well as an excess in nutrients leading to eutrophication. ~~More particularly, organic contaminants such as pesticides, mainly deriving from the increase in industrial and agricultural activities, need to be controlled in water as they represent a major source of pollution.~~ A particular interest has been placed on the detection of pesticides due to their ever-growing use but also the lack of instructions for their proper application and control of the post-application phase.

Herbicides represent a category of pesticides that are used to protect crops and non-crop areas and prevent growth of undesired weeds. Herbicides can easily penetrate the soil, be transported to rivers through groundwater paths and be often detected in different water bodies such as lakes and rivers. ~~In this way, they can potentially affect non-targeted living organisms and pose possible threats on the environment and human health.~~ Diuron, or 3-(3,4-dichlorophenyl)-1,1-dimethylurea is an urea-based herbicide, widely employed for total, non-selective vegetation control (Fedtke and Duke, 2004). It is mainly used upon non-crop areas, in irrigation or drainage canals but has also found applications in paints to protect from fouling. According to conducted surveys, Diuron has been found in 70% of European rivers and is also ranked high upon water contaminants for Australian, Canadian and U.S agencies, posing considerable threats to aquatic microorganisms. ~~Governments establish guidelines and implement strict legislations aiming at the sustainability of freshwater resources.~~

Herbicide determination and detection are most commonly performed in laboratories. Conventional methods include advanced instrumental techniques such as gas and liquid chromatography coupled with different detection techniques as mass spectrometry, chemiluminescence or electrochemical detection. These techniques are highly sensitive, selective and they include controlled and validated protocols. However, it is still essential to meet the ever-growing need for systems appropriate for rapid, on site analysis. ~~Such demands triggered increasing research efforts centered on the development of biosensors.~~ Biosensors are analytical detection devices that convert a biochemical phenomenon into a detectable and measurable signal, which can be amplified and treated. ~~The fabricated devices can be low cost and easy to use, offering the possibility of conducting on site analysis.~~ These devices meet the requirements of an application that demands a low-cost portable system for on-site detection, providing an early indication by sorting the samples needed to be further analyzed by conventional techniques. Biosensors consist of two principal parts, the biological sensing element, the so-called bioreceptor, and the physical transducer. ~~Different types of biosensors can be found, depending on the bioreceptor (i.e. enzymes (Clark and Lyons, 1962), whole cells (Karube et al., 1977), DNA (Wang et al., 1997), antibodies and antigens (Karube and Suzuki, 1986)) and the transduction system (i.e. piezoelectric (Shons et al., 1972), thermal (Mattiasson et al., 1977), optical (Peterson et al., 1980), electrochemical (Clark and Lyons, 1962) used.~~

In order to determine the biological detection element and physical transducer to be used for the detection of herbicides, it is essential to study the mode of action of each herbicide on the targeted vegetation. They can inhibit cell growth, fluorescence

and photosynthesis depending on their molecular structure and site of action (Ross and Childs DJ, 1996). More particularly, Diuron, similarly to 50% of herbicides used today, inhibits photosynthesis, acting at vital systems of the photosynthetic apparatus. As stated by Davison, given the fact that algal physiology resembles to the one of the targeted vegetation, microalgae are directly affected by herbicides (Davison, 1991). They can thus be successfully used as biological recognition elements among herbicide biosensors (Brayner et al., 2011). Furthermore, they integrate several other advantages related to the use of whole cells such as their robustness, stability as well as the simple procedures related to their cultivation, isolation and manipulation (Giardi and Piletska, 2006). Based on previously reported ecotoxicological studies on monitoring the effect of herbicides on living organisms (Schubnell et al., 1999), *Chlamydomonas reinhardtii*, wild type microalgae were selected as biological recognition elements as they are extensively studied and characterized.

As a matter of fact, the presence of Diuron herbicide has a visible impact on the photosynthetic oxygen production and the emitted algal fluorescence (see Supplementary Information 1). The majority of microalgal biosensors that aim at detecting Diuron are therefore either based on fluorescence or photosynthetic oxygen production monitoring (Brayner et al., 2011). These two approaches are effective alternatives to the conventional method which is the standard growth test, where the inhibition of algal growth is measured (Ma et al., 2002). As a matter of fact, although this method yields good results in terms of limit of detection and sensitivity, long assay duration is an important issue when rapid results are desired. Concerning fluorescence biosensors, they are based on optical transduction system in order to detect the photons emitted by algae, while the transduction for oxygen monitoring is performed through electrochemical measurements. It is demonstrated in literature that fluorescence-based biosensors employed for the detection of Diuron have often high performances with low limits of detection (Naessens et al., 2000). However, they often demand high stabilization times and can only be effective when optically clear, not turbid samples are examined (Haigh-Flórez et al., 2014). Consequently, a complementary electrochemical biosensor can be beneficial to the determination of pollution level as this type of sensor can yield solid and stable systems that are easily miniaturized and simple to use.

Among previous experimental works based on algal photosynthetic activity as an indicator of the presence of Diuron, amperometric monitoring is reported several times as the detection technique (Shitanda et al., 2009) (Koblížek et al., 2002). The inhibiting effect on photosynthetic activity of algae is evaluated by monitoring electrochemically the photosynthetically produced oxygen and the concentration inhibiting 50% of the activity (IC_{50}) is estimated.

The aim of this study is to develop a lab-on-chip system with integrated electrochemical and fluorescence sensors enabling double complementary detection. The present work therefore reports the development of the electrochemical detection system integrated on a fluidic platform for the detection of toxicants based on algal physiology. The system uses small sample quantities due to the incorporation of microfluidic structures, is easily implemented and simple to use for on-site measurements. The three-electrode electrochemical system, integrating an ultramicroelectrode (UME) array of platinum black (Pt-BI) could effectively follow modifications in photosynthetic oxygen production rates due to pollutants. The design of the electrochemical device is compatible with optical technology in order to further integrate light source and fluorescence detection in the same substrate. To study the effects of illumination on algae photosynthesis two light sources will be

evaluated and compared: a classical halogen light and an organic light emitting diode (OLED) with a specifically selected wavelength. The development of the second option has been considered in order to obtain a final sensing lab-on-chip with integrated light source.

2. Materials and methods

2.1 Fabrication procedure

The lab-on-chip platform was comprised of the electrochemical sensors as well as the fluidic structure with channels and measurement chambers for sample testing through the bioassays (see Supplementary Fig S1 and Fig S2). Six independent detection chambers were designed on each platform enabling the simultaneous processing of different assays. The complete electrochemical cells were integrated on the three chambers while the other ones were dedicated to the further work involving fluorescence-based optical detection. In this way, it is possible to increase analysis frequency by conducting parallel analysis of several samples in order to reduce false alarms. Moreover, this matrix configuration gives the possibility of calibrating the sensor by using one of the chambers for control measurement with a non-polluted sample and compare with the values obtained for the polluted samples. It also enables future integration of different algal species in order to increase sensor selectivity as each algae species will be sensitive to different pollutant giving the possibility of conducting multi-analysis. Concerning the light source for algal excitation, the fabrication of a blue OLED was considered.

The entire fabrication procedure (lab-on-chip platform and OLED) is detailed in Supplementary Information, section 2.

2.2 Bioassays

2.2.1 O₂ measurement in control algal solutions.

Green algal cells were used through the bioassays and the cultivation procedure is explicated in Supplementary information 3. The response of the sensor was firstly evaluated in control algal solutions that do not contain any herbicide. **Given the fact that the detection principle is based on following the algal photosynthetic activity, the electroactive species to generate the electrical signal was oxygen (O₂) which is electrochemically reduced on the PtBI working electrode surface. The recorded reduction current was proportional to the concentration of dissolved oxygen in algal solution.** Oxygen evolution was followed through photosynthesis and respiration process during light and dark cycles. Experiments were carried out in a dark Faraday cage using an external, halogen white light source or a blue OLED as excitation sources for algal photosynthesis. The potentiostat used was Bio-Logic SP-200 equipped with a low current option. The centrifuged algal cells, re-suspended either in HSM medium or lake water samples was injected in the detection chamber by simply using a syringe. Chronoamperometry was conducted and the potential applied corresponds to the O₂ reduction potential which was estimated before through cyclic voltammetry and is -0.7 V versus integrated Ag/AgCl.

2.2.2 Herbicide detection.

Ethanol solutions containing Diuron herbicide were mixed in an Eppendorf with algal test solutions re-suspended in either HSM culture medium or lake water in order to prepare final solutions of various Diuron concentrations. Final solutions were injected in the detection chamber with a syringe. Calibration tests were conducted by mixing algal solutions of identical cell concentration with different concentrations of Diuron (control -1 μ M Diuron algal solution). Working electrode potential was determined through cyclic voltammetry in a potential range of 0 to -0.9 V and was eventually set at -0.7 V vs Ag/AgCl integrated pseudo reference electrode for the following chronoamperometric detection. Temperature variations were not taken into account through measurements and a constant value of 22°C was estimated.

3. Results and discussion

3.1 O₂ measurements in control algal solutions.

Dark and light periods were altered so that changes in O₂ level can be registered as shown in Figure 1, that presents the evolution of the recorded current through time, reflecting oxygen concentration variation in the solution. It was first verified that changes in recorded current (increase-decrease) were not related to light interferences but only caused by algal photosynthesis (results not shown). Algae were first left in dark (not presented in graph). The onset of photosynthesis is indicated by a cathodic current increase which represents the oxygen production when light is on. On the other hand, when light is off, algae are consuming oxygen for the respiration procedure. The saturation effect illustrated in the graph of Figure 1 is detailed in supplementary information, section 4.

3.2 Herbicide detection using halogen white light source.

The optimal algal cells concentration needed first to be estimated. Concentration of algal cells has an effect on the oxygen production rate and therefore the slope of the current versus time graph. When the concentration is high, the total production of O₂ and therefore the slope is more prominent. A sufficiently high concentration of algae (Shitanda et al., 2009) is required in order to yield a high and measurable O₂ production rate and obtain a well-defined difference between respiration and photosynthesis slopes. On the other hand, cell concentration should not be too high so that the signal-to-noise ratio will be optimal, the signal being the variation in oxygen production rate induced by a particular herbicide concentration and the noise being the continuous O₂ production rate component resulting from all algal cells, even those that are not affected by the herbicide. Indeed, Deblois et al. reported that the effect of atrazine, a herbicide that targets PSII in the same way as Diuron, on algal metabolism is less visible when the availability of total binding sites is high compared to the number of sites that can actually be blocked by the herbicide (Deblois et al., 2013a). A high cell concentration can therefore relatively reduce the apparent toxic effect of a specific quantity of the herbicide as the slope corresponding to O₂ production is important and therefore the slope variation related to presence of Diuron appears to be negligible. In this study, a concentration of 13×10^6 cells.ml⁻¹ was considered optimal as this concentration yielded the optimal signal-to-noise ratio.

The characteristics of our prototypes were tested in order to examine their stability. Indeed, the stability of the electrochemical signal during following bioassays was assured by validating the robustness of the fluidic structure, passivation layer and

electrode materials. The devices were used through several tests with algae and different concentrations of Diuron pollutant without significant variation in their response (around 100 tests with one device). Focusing on the microfluidic structure, in contrast to classical PDMS microfluidic devices that sometimes present poor adhesion, the optimized procedure (described in Supplementary Information, section 2) for the fabrication of SU-8 chambers and channels yielded stable structures that didn't demonstrated any leakage after extensive use.

Figure 2-a presents algal response to Diuron concentrations varying from control to 1 μ M illustrated through the current versus time graph. Light-induced oxygen evolution was measured for approximately two minutes and changes in oxygen production rates point out the toxicant effect. Oxygen production rate corresponds to the slope obtained for reduction current through time during illumination. Oxygen production rate decreased upon the addition of Diuron and varies in a concentration-dependent way. In particular, when increasing Diuron concentration, the slope and consequently the rate are decreasing confirming the inhibition in algal photosynthetic activity induced by the toxicant. Compared to other detection methods that demand long stabilization times, in our case, the diminution in the rate of O₂ production was evident immediately after injection of herbicide.

However, similarly to previous observations with control algal solutions, respiration slopes registered are not identical for various pollutant concentrations, while normally Diuron is a herbicide targeting only photosynthetic activity (Fedtke and Duke, 2004), so the effect on respiration should be negligible. It is difficult to determine the causes of this variability but certain assumptions have been made. Firstly, an heterogeneity in sample activity is often observed when biological organisms are used. Another important parameter inducing this variability in slope values is the fact that algae are not yet integrated on the device but are introduced in the detection chamber by a syringe for each measurement. The protocol followed to load algal solutions can inevitably introduce variations in number of cells injected each time. Moreover, an important cause of this variability is the biofouling on the porous Pt-BI surface, which can eventually be avoided by integrating a membrane (Wu et al., 2010). As a matter of fact, when one sensor is used to conduct several measurements, algal cells get attached and then detached from the porous surface of Pt-BI electrodes through consecutive measurements in a random way that cannot be controlled.

Therefore, a correction step needs to be conducted in order to compare results obtained regarding photosynthetic activity and eliminate this variability (see Supplementary Information 5). The corrected photosynthetic slopes are presented in Figure 2-b and the effect of Diuron on photosynthetic activity of different algal test solutions was evaluated for all measurements by comparing corrected O₂ production rates.

Compared to the classic approach used in toxicology to determine the inhibition effect on photosynthetic activity (IC₅₀ value; see Supplementary Information 6), through the approach proposed here, the sensitivity of the electrochemical sensor is evaluated through the comparison of the rates of oxygen production for different Diuron concentrations. By plotting the corrected oxygen production rate versus Diuron concentration the concentration-response curve was obtained (Figure 3). Calibration curves presenting O₂ production rates versus Diuron concentration were compared for two different light intensities. A concentration-dependent decrease in the rate was found for both light intensities tested and the sensitivity of the

fabricated sensor was evaluated. It is important to precise that the selection of the Diuron concentration range tested (control-1 μ M) was based on the maximum acceptable concentration value implied by Canadian government (0.64 μ M). As shown in figure 3, photosynthetic activity for control algal solutions was higher for light intensity of 600 μ E.m⁻².s⁻¹ (red square points) compared to the one obtained for 1800 μ E.m⁻².s⁻¹ (black circular points), demonstrating that photosynthetic apparatus is more efficient in the first case. This result is in accordance with the results presented by Deblois et al. on *Chlamydomonas* indicating that there is a light intensity for maximal growth rate (Deblois et al., 2013b). It is important to precise that this optimal value shown in figure 3 is given for a halogen white source, the spectrum of which is presented later. It can therefore be deduced that for this particular algal strain and its physiological state, a light intensity of 1800 μ E.m⁻².s⁻¹ yielded by a white halogen source, introduces an additional stress that has an impact on algal physiology and consequently on the sensitivity of the device regarding herbicide detection. As a matter of fact, light intensity has an important role in photosynthesis procedure as explained in supplementary information, section 7. Sensitivity was determined by estimating the variation in the O₂ production rates between control and 1 μ M Diuron solutions. A value of 0.26 nA.s⁻¹. μ M⁻¹ was calculated for the measurement performed at 600 μ E.m⁻².s⁻¹ compared to the value of 0.1 nA.s⁻¹. μ M⁻¹ at 1800 μ E.m⁻².s⁻¹. This result demonstrates that for a more adapted light intensity, the photosynthetic activity is enhanced and the sensitivity is greater and outlines the strong contribution of light conditions to photosynthetic activity. However, further study should be conducted in order to determine the optimal light intensity for the final device configuration.

3.3 Herbicide detection using blue OLED excitation.

In order to demonstrate the possibility of integrating the light source on the microfluidic platform, a blue OLED fabricated in our lab was used for herbicide detection. Since double detection (electrochemical and optical) is envisaged for the final application, OLED development was performed to create a unique component used, at the same time, for excitation of algae for photosynthetic and fluorescence measurement.

The emission spectrum of the OLED shows a broad peak around 455 nm, which overlaps with the major absorption band of algae in the blue region (Figure 4). On the other hand, the emission of the halogen white light source performed with the same apparatus is mostly taking place in longer wavelengths that coincide with a less pronounced algal absorption peak in the red region. As a matter of fact, the overlap of the emission spectrum of the OLED with the absorption spectrum of algae can explain this sensitivity increase observed when using the blue OLED (see hereafter). The emission of the OLED is more centered on the absorption band of algae compared to the halogen white light source used through previous experiments and this can increase the efficiency of the device as more photons can be effectively captured by algae. Furthermore, different wavelengths were compared in order to determine the one that yields a more efficient photosynthetic activity (460 nm) and therefore a higher O₂ production rate (see Supplementary Information 8 - Table S1).

The matching between OLED emission and algae absorption/excitation spectra is vital for the performance of fluorescence sensor as the role played by OLED consists in algal excitation for algal fluorescence. **As far as the fluorescence mechanism is concerned, it is important to explicit where it occurs during the photosynthesis cycle. Indeed, light reactions are taking**

place in the thylakoids through photosynthetic pigments that are organized in photosystems. Light energy is collected by chlorophylls that are light-absorbing pigments present in the thylakoids. Photosynthetic pigments absorb light mainly in blue and red region (Taiz and Zeiger, 2006). When chlorophyll absorbs a high energy, blue photon, it gets into the excited, high energy, unstable state. In order to make the transition to an excited, lower energy state, chlorophyll transfers heat to its surroundings. The excess energy remaining after heat transfer or the energy gained after the absorption of a red photon needs still to be transferred so that chlorophyll will return to its initial stable state. This can occur through several pathways such as fluorescence, which consists in the emission of a photon of lower energy by chlorophyll.

Figure 5 gives UV-vis absorption and excitation spectra of micro algae *Chlamydomonas reinhardtii* in HSM solution. The excitation spectrum is determined by monitoring the variations of *Chlamydomonas reinhardtii* maximum fluorescence intensity (682 nm, not shown here) while algae are excited through consecutive wavelengths. The absorption and excitation spectra exhibit two large bands centered at 438 nm and 483 nm which helped to determine OLED active material. Hence, blue OLED were fabricated choosing PCAN, an anthracene derived molecular glass (Bergemann et al., 2012). OLED emission spectrum was collected using a JOBIN YVON HR1000 monochromator, equipped with a GaAs photocathode. Since the OLED electroluminescence spectrum is a broad band (Figure 4), it is reasonable to consider that it is a mix of both emissive layers, PCAN and Alq₃, used in the device which are respectively blue and green emitters. Thus the OLED seems to produce excitation light having desired specific spectral properties to algal absorption and excitation spectra.

First, a control measurement of current recording through illumination and dark periods was conducted with a lake water sample in the absence of algal cells in order to examine if the OLED emission modifies the response of the sensor (Figure 6-a). In contrast to the control measurement carried out with the external, white light halogen source, a change in the reduction current was observed when the light was turned on. Given the fact that the sample contains no algal cells, this current increase could not be attributed to algal respiration but could be rather attributed to the temperature increase induced by the heat generated by the OLED and transferred to the test solution. Operation of high-brightness OLED can dissipate energy in the form of heat (Bergemann et al., 2012). As the OLED is in close contact with the microfluidic chamber, this heat can be transferred to the solution. Indeed, the temperature measured at the back side of the glass cover on which the OLED is stuck, after two minutes photosynthesis measurement with light on was 35°C. Temperature increase in the measurement solution induces an increase in chemical reaction rate. As a matter of fact, in a system limited by diffusion, temperature influences the diffusion coefficient and therefore enhances mass transport of electroactive species towards the electrode surface. Electrochemical signal variation induced by temperature increase after illumination should then be correctly compensated. This was achieved by subtracting the rate under illumination recorded for the non-algal control solution from O₂ production rates calculated for different Diuron concentrations.

Sensitivity graph was then plotted (Figure 6-b), presenting the response of the sensor to different Diuron concentrations using the OLED as light source (blue square points). The results obtained with the OLED were compared to the ones obtained in lake water samples using the halogen white light source of light intensity of 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. In this last case, it was verified that similar results and similar sensitivity (around 0.25 $\text{nA}\cdot\text{s}^{-1}\cdot\mu\text{M}^{-1}$) were obtained in real water samples and in HSM culture

solutions (see below). This demonstrates that the different properties (conductivity, CO₂ content) of fresh water and the possible biofouling of the electrode surface will not impede measurements. For the device that includes OLED, sensitivity obtained in the range of control-0.6 μM Diuron solutions was 0.48 nA.s⁻¹.μM⁻¹ that corresponds to almost double the value of 0.25 nA s⁻¹ μM⁻¹ obtained with the external halogen. Photosynthetic apparatus is more effective when OLED is used and this could be attributed to the wavelength used, more adapted to algal absorption spectrum (explained previously) and to the temperature increase of the test solution (35°C approximately) which influences the algae photosynthetic complexes. As far as temperature increase is concerned, photosynthetic rate is increasing with a short-term increase in temperature up to an optimal temperature, the value of which depends on the algal species used each time (Davison, 1991). Indeed, photosynthetic activity depends on temperature (Raven and Johnson, 2002) as it includes enzyme-catalyzed reactions (see Supplementary Information, Section 9).

It was therefore demonstrated that photosynthetic activity of each algal cell is more effective and therefore of greater amplitude when OLED is used. The dynamic range of photosynthetic activity is therefore larger, the variation induced by the herbicide more visible and the sensitivity improved. Temperature effect on sensor sensitivity and temperature variations through measurement duration should be further examined in order to determine the temperature at which algal photosynthetic activity is the most efficient and the sensor gives the greater sensitivity. Even though the temperature increase can be advantageous up to a certain point, it is necessary to minimize the dissipation of heat by the OLED by optimizing its fabrication procedure, in order to increase its lifetime and target more reproducible measurements.

4. Conclusion

A portable device for in-situ herbicide detection, based on algal physiology, was developed that provides an early indication system by sorting the samples needed to be further analyzed by conventional techniques. The fabricated lab-on-chip platform consists in three fluidic chambers integrating electrochemical sensors and three chambers dedicated to further optical fluorescence-based detection. The effect of Diuron herbicide was validated using the lab-on-chip devices with culture medium solutions. Illumination was first supplied through the halogen white light source and two different light intensities were tested: 1800 μE.m⁻².s⁻¹ and 600 μE.m⁻².s⁻¹. A Diuron concentration-dependent decrease in the oxygen production rate was demonstrated for both light intensities but the sensitivity of the sensor was higher for 600 μE.m⁻².s⁻¹ as in this case the light-related stress that can inhibit photosynthesis is minimized. Diuron detection was then conducted in real samples of fresh lake water similarly to final application using the lowest light intensity and it was verified that the different properties of fresh water compared to culture medium did not impede measurements. Finally, in order to obtain an autonomous system, the same experiments were successfully carried out with a blue OLED. It was demonstrated that photosynthetic apparatus was more effective when OLED is used compared to the halogen white light source. This can either be attributed to the fact that the OLED emission is more adapted to algal absorption spectrum or to the enhanced enzymatic activity due to the temperature increase. Finally, it is overall demonstrated that the fabricated lab-on-chip biosensor can effectively follow the change in

photosynthetic activity induced by Diuron herbicide and reflected through a modification in oxygen production rate. It can therefore be an efficient indicator of water pollution.

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