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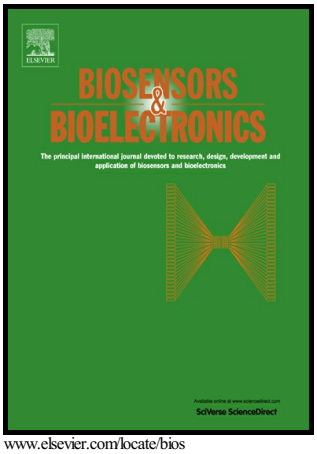

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Xurograghy-based microfluidic algal biosensor and dedicated portable measurement station for online monitoring of urban polluted samples.

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Abstract

A critical need exists to develop rapid, *in situ*, and real-time tools to monitor the impact of pollution discharge toxicity on aquatic ecosystems. The present paper deals with the development of a novel, simple-to-use, low-cost, portable, and user-friendly algal biosensor. In this study, a complete and autonomous portable fluorimeter was developed to assess the A-chlorophyll fluorescence of microalgae, inserted by capillarity into low-cost and disposable xurography-based microfluidic chips. Three microalgae populations were used to develop the biosensor: *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, and *Chlamydomonas reinhardtii*. Biosensor feasibility and sensitivity parameters, such as algal concentration and light intensity, were optimized beforehand to calibrate the biosensor sensitivity with Diuron, a pesticide known to be very toxic for microalgae. Finally, the biosensor was employed in 10 aqueous urban polluted samples (7 urban wet-weather discharges and 3 wastewater) in order to prove its reliability, reproducibility, and performance in the detection of toxic discharges in the field.

Keywords: Microfluidic biosensor, Microalgal A-chlorophyll fluorescence, Xurography, Portable fluorimeter, Pesticide, Urban polluted water.

1. Introduction

A wide range of anthropic activities are responsible for the release of micropollutants through the production of polluted runoffs. Among them, urban wet weather discharges (Zgheib et al., 2011; Gasperi et al., 2012), agricultural runoffs (Willis and McDowell, 1982), industrial, urban (Rosal et al, 2010) or hospital (Verlicchi et al., 2010) effluents can lead to substantial amounts of contaminant mixtures in the environment: tap, underground, coastal and surface waters (Masner et al, 2017).

In Europe, the Water Framework Directive (WFD, 2000/60/EC) aims to maintain the ecological status of bodies of water exposed to such contamination. The chemical analysis of bodies of water quantifies the extent of contamination by complex chemical substances. Yet, sample collection, preparation, and chemical analyses are time consuming, expensive, and provide no information about the biological impact (toxicity) of these substances (Axelrod et al., 2016). Moreover, common lab-scale ecotoxicological bioassays, such as algal growth inhibition assays, are labor intensive, time consuming, require skillful personnel, and use a considerable amount of reagents (Zheng et al., 2013, Gao et al., 2016, Yang et al., 2016). Therefore, these methods do not provide a realistic toxic impact quantification of polluted samples, as sample transportation, storage, and conservation before bioassay may affect outcomes. For this reason, new tools are highly anticipated for real-time monitoring of the toxicity of water-contaminated matrices (Rogers, 1995, Axelrod et al., 2016, Yang et al., 2016). To this end, whole-cell biosensors are very promising for the development of *in situ*, sensitive, and fast-response pollution monitoring tools (as early warning systems).

Among microorganisms, microalgae are commonly used to design whole-cell biosensors. Microalgae represent primary producers, at the bottom of the food chain. Consequently, an impact on these organisms can affect all higher trophic levels; accordingly, they provide essential information for predicting environmental impact (Zheng et al., 2012, 2013). Additionally, microalgae are highly sensitive to numerous pollutants commonly detected in suburban aquatic ecosystems and their use does not pose an ethical dilemma in the scientific community. Among the toxicity effects observed in microalgae, photosynthesis disturbance is the most widely studied, which has led to the development

of highly sensitive tools (Védrine et al., 2003; Peña-Vázquez et al., 2009; Ferro et al., 2012; Lefevre et al., 2012; Wong et al., 2013; Tsopela et al., 2016). Photosynthesis is a simple, fast, and sensitive toxicity parameter for the detection of a wide range of micropollutants such as Polycyclic Aromatic Hydrocarbons (Aksmann and Tukaj, 2008), pesticides (Védrine et al., 2003), heavy metals (Suresh Kumar et al., 2014), and pharmaceuticals (Liu et al., 2011). Different methods have been developed to measure disturbances in photosynthesis (e.g. fluorescence or electrochemistry). Optical A-Chlorophyll fluorescence modification detection is the most widely used for (micro-)sensing systems, due to its high selectivity and sensitivity (Kuswandi et al., 2007).

Cell entrapment (or immobilization) is the most critical element in the design of a microalgal biosensors. Sensor sensitivity can potentially decrease by an order of magnitude compared to non-entrapped cells (Avramescu et al., 1999, Zheng et al., 2012, Wong et al., 2013). This phenomenon has also been observed with hydrogel entrapment, mainly due to the diffusion barrier created between the microalgae and their environment (Ferro et al., 2012). To overcome the problem of cell immobilization, microfluidic techniques provide the advantage of direct contact in micrometer-sized channels between microorganisms and pollutants, without any physical barriers. Other important advantages are the low sample and reagent consumption, low cost, and flexibility in design; these pave the way for portable microscale systems for field monitoring and mobile sensing (Jokerst et al., 2012a; Zheng et al. 2013). Consequently, several microfluidic biosensors for pollutant detection and toxicity assessment have been developed over last decade. Biosensors are principally calibrated on various microorganisms such as microalgae (Lefevre et al., 2012; Tsopela et al., 2016; Tahirbegi et al., 2017), bacteria (Buffi et al., 2011; Roda et al., 2013), and yeast (García-Alonso et al., 2009). Most microfluidic devices are built with “soft-lithography” using polymers, mainly polydimethylsiloxane (PDMS), to replicate small-scale features (micro-channels) from a master mold. Despite the simplicity of this design process, it requires specific clean room facilities (Renaud et al., 2015) and skilled personal. It is time consuming, and not scalable to an industrial level for large-scale production. Moreover, many of the existing microfluidic devices/biosensors failed to be implemented *in situ* because of the equipment supply needed (signal acquisition instruments, pumps, electronic, and optic

components) (Jokerst et al., 2012a), which cancel out the advantages of microfluidic systems (e.g. miniaturization).

However, Bartholomeusz et al. (2005) developed a new process for designing microfluidic channels and chips, called xurography, as an alternative to classical methods. Xurography is based on the use of thin and flexible films (in general a pressure-sensitive double-sided adhesive) and a cutter plotter. This technique provides many advantages, as it does not require special clean room facilities and is extremely low-cost. The design of a microfluidic device is reduced to the use of a CAD tool, and production takes just a few minutes (Greer et al., 2007, Islam et al., 2015, Renaud et al., 2015). Moreover, is it possible to design microfluidic chips using only capillarity properties to insert and transport any aqueous liquid (such as algal solutions) without an external pump, avoiding cumbersome gear deployment *in situ*. Recent advanced xurography-based microfluidic systems have been published; for example, a rapid immunosensing platform (Kim et al., 2014). Nonetheless, despite these advantages, to the best of our knowledge, no xurography-based whole-cell biosensors have been described in the literature.

Consequently, we report here a new, low-cost, compact, and stand-alone portable algal toxicity biosensor incorporating algae inserted in simple and disposable xurography-based microfluidic chips. A dedicated portable fluorimeter using LED Light Emitting Diode (LED) as a light source was also developed to read algal chlorophyll fluorescence. LEDs provide many advantages for portable microfluidic systems: long lifetime, low cost, small dimensions, and low power consumption (Novak et al.,2007; Jokerst et al., 2012a). Dedicated software was developed to provide a user-friendly interface with full control of fluorescence acquisition parameters. . As recommended previously by Tsopela et al. (2016), since each alga is specifically sensitive to different pollutants, we carried out experimental tests with three different algae strains to develop the biosensor. Finally, in a first step in this study, the reliability and optimization of biosensor parameters were studied, including excitation light intensity and algal concentration, which play an essential role in biosensor sensitivity (Védrine et al., 2003; El-Ali et al., 2006). The performance of this system was demonstrated in a second step, using Diuron as a model pesticide. Finally, this biosensor was tested on real

environmental polluted matrices, i.e. urban and hospital wastewaters, urban wet weather discharges, in order to prove its relevance and sensitivity as an early-warning system for suburban ecosystems.

2. Materials and methods

2.1 Chemical products and solution preparations

For the preparation of the pesticide solution, absolute ethanol (≥99.5 %) was obtained from VWR (Radnor, USA). Diuron (≥98 %) was purchased from Sigma–Aldrich (Saint-Louis, USA). The Diuron stock solution was prepared in a pure ethanol solution at 10 g/L (1 g of pesticide in 100 mL of absolute ethanol). Just before the experiment, 100 µL of the ethanol solution of Diuron was mixed in a final 100 mL of ultra-pure water to create the 10 mg/L Diuron solution used for experiments.

2.2 Algal cultures

Three microalgal strains were used in this experiment: *Chlorella vulgaris* (*C. vulgaris*), *Pseudokirchneriella subcapitata* (*P. subcapitata*), and *Chlamydomonas reinhardtii* (*C. reinhardtii*). These algal strains were purchased from the French National Museum of Natural History (Paris, France). *C. vulgaris* and *P. subcapitata* were grown in Lefebvre-Czarda medium (Ionescu et al., 2006). *Chlamydomonas reinhardtii* were cultured in Tris–acetate–phosphate (TAP) medium (Gorman and Levine 1965). These strains were transplanted weekly in sterile conditions, using sterilized glassware and medium (autoclaved for 20 min, at 130 °C, under 1.3 bar), in order to cultivate fresh algae in growth phase. Algae were cultivated in a nychthemeral 16 h/8 h light/dark cycle using 5000 lux lighting in special culture room maintained at 21 °C. The algal cell concentration was determined with a Thoma cell counter.

2.3 Microfluidic chips fabrication and algal insertion procedure

Xurography-based technology, extensively described in Renaud et al. (2015), Yuan et al. (2015), Neuville et al. (2017), was used to construct the disposable microfluidic chips, designed to take a A-chl fluorescence readings with a dedicated, portable fluorimeter built for this purpose (see Figure 1A). The microfluidic channels and analysis chambers for algae entrapment and fluorescence measurement were cut into a 100 µm-thick double-sided pressure adhesive (DSPA) film (Plusform; Germany; Tesa, Germany) with a cutter plotter (Graphtec-CE-5000-40, Ankersmit, France). Each microfluidic device contains two independent chambers (5 mm diameter, 100 µm, and thick, 1.96 µL) fed by microfluidic channels (100 µm thick, 90 mm long, and 1 mm wide). The first one is the control chamber with algae in the reference medium, while the second one is filled with algae exposed to pollutants. After cutting, the DSPA is taped onto a glass slide (25x75x1 mm, Fisher Scientific, Illkirch, France) and used as a substrate. Then, a glass coverslip (24x60x0.15mm) is taped to the second adhesive side of the DSPA to create the hood. As the length of the coverslip is shorter than the substrate, the remaining uncovered area is used to create the inlets where the user can deposit droplets of the algae suspension. No external pump is required. Capillary force generates the flow from each inlet into the corresponding chamber. In summary, in all experiments the algae were immobilized in the microfluidic chips by adding a drop of around 15 µL at the inlets, which was drawn into the reading chamber by capillarity before the fluorescence reading. A representation of an algae-filled microfluidic chip is presented in Figure 1B.

- Figure 1 -

2.4 Portable fluorimeter description

A portable fluorimeter was developed to read the A-chlorophyll fluorescence (ex. 460-480 nm/em. 680 nm) of microalgae immobilized in the microfluidic chips previously described (Figure 2). The fluorimeter is composed of two parts: an optical column for microfluidic chip illumination and fluorescence measurement, and an electronic circuit for system control, data acquisition, and USB computer communication (Figure 2A, B, C).

The optical column (see Figure 2D) is composed of a single blue LED (470 nm central wavelength, (MA470D2, Thorlabs) for photosystem II (PSII) excitation. The LED beam is collimated (50 mm diameter Fresnel lens, FRP232, Thorlabs) before passing through a diffuser (DG20-120, Thorlabs) for an equal illumination of the two chambers and adequate light incidence for subsequent filters. Because the fluorescence detectors face the excitation source, a set of filters are necessary to discriminate the emission light (675 nm) from the excitation light (470 nm). Before reaching the chambers, the spectrum of the excitation light is cut beyond 500 nm (interferential low pass filter, OD > 5, FESH0500, Thorlabs), and polarized using a plastic polarizer (LPVISE2X2, Thorlabs). The light illuminates the algae inside the microfluidic chambers, and then passes through a second polarizer at a crossed orientation with respect to the first. This pair of polarizing filters enhances the rejection of excitation light. Finally, the light passes through a band-pass filter (680 nm, 10nm bandwidth, OD > 4, ref 88-571, Edmunds) to isolate the fluorescence emission. This signal is then detected with two silicon photodetectors (3.6mm x 3.6 mm, FDS100, Thorlabs) delivering a signal for each chamber, amplified by transimpedance amplifiers (10^9 A/V, modified "multiboard" amplifier from Roithner LaserTechnik). The second part of the portable fluorimeter is composed of a LED power control and a digital acquisition system (NI USB-6002, National Instrument) to control algal exposure to light intensity and to collect the fluorescent signal emitted by the algae (see part 2.5). All the optical column and acquisition parts are protected by a plastic polymer case constructed with a 3D printer (Formlabs1+, Formlabs, USA). This case includes Eppendorf holders for convenient field experiments. Software for fluorimeter control, data acquisition, and processing was developed (LabWindows CVI environment) and is hosted on a laptop computer dedicated to field experiments (see figure 2.5). Finally, the laptop was equipped with an USB GPS (BU-353-S4 USB GPS, US Global Sat Incorporated©) to save the position of the sample collection.

- Figure 2 -

2.5 Software presentation

The software developed to control the portable station parameters and record fluorescence signals is illustrated in Figure 3. Its different elements and operating mode are presented below.

Before each algal fluorescence measurement, offset (without light excitation) and gain (with light excitation) correction of amplifiers was carried out for each empty microfluidic chip (Figure 3F) to insure a consistent response. A command was created to control and adjust the emitted light intensity (Figure 3C) from the LED in the direction of the immobilized algae in chambers. The parts A and H on the laptop screen control the number of measurements done during the assays, the number of samples taken into account for each measurement, and the acquisition time associated. Raw signals during acquisition are displayed in parts E and G of the software interface (see Figure 3) and calculate relative difference in fluorescence between control and exposed algae, displayed in real time in parts D and E. GPS coordinates of sample locations are displayed in part B. Location and raw differential signals were saved automatically (storage path shown in part I of the software).

- Figure 3 -

2.6 Algal reliability, repeatability and optimization of sensitivity parameters

All experiments were performed at room temperature (around 23 °C, in air-conditioned room). In our study and for all assays, algae were exposed continuously to 3 minutes of LED excitation light and fluorescence assessment was done every 2 seconds (2000 measurements each time (400 ms acquisition time) for mean and standard deviation computation), i.e. 90 fluorescence measurements for each assay. All assays were done in triplicate (three independent microfluidic chips) in this study.

2.6.1 Characterization of algal filling in microfluidic chips

The algal filling in the microfluidic chip reading chambers was studied to insure a repeatable measurement of A-chlorophyll fluorescence. After chips were filled with algal solutions (15 µL), photographs of the fluorescence lecture chambers (“control” and “exposed”) were taken using a camera linked to a binocular microscope (Zeiss® Stemi 2000, Germany), under 4.5x magnification, and the images were analyzed using ImageJ free software (Abràmoff et al., 2004). Following a similar principle (Jokerst et al., 2012b), average grey level was used to assess the control and exposed reading chambers for an equal algal filling and thus concentration of the three algal strains (See Figure S1 A).

2.6.2 Algal concentration

Optimal algal concentration was assessed to optimize the sensitivity of the response. 50 mL of algal suspension in growth phase at a pH adjusted to 7 was centrifuged at 3000 g and resuspended in a culture medium to concentrate the algae and to obtain a range of concentrations for each one. After centrifugation, 160 µL of the algae solution was put in contact with 40 µL of Diuron at a final concentration of 100 µg/L, or with ultra-pure water for control (Naessens et al., 2000). According to the literature (Tsopela et al., 2016) and after short preliminary optimization, A-chl fluorescence of microalgae inserted into the microfluidic chip was read (with an arbitrary light-excitation intensity corresponding to a LED current set to 100 mA) after 5 min (*C. vulgaris)*, 20 min (*P. subcapitata*) and 10 min (*C. reinhardtii*) of exposure to Diuron into Eppendorf tubes respectively. Exposed algae fluorescence enhancement was calculated using the following equation:

$$FE\ (\%) = \frac{Fs - Fc}{Fc} * 100$$

Were FE is the fluorescence enhancement (in %), Fs the maximum fluorescence signal acquired for algae exposed to toxicants (in Volts), Fc the maximum fluorescence signal for control algae (in Volts).

2.6.3 Light intensity

After selection of the optimum algal concentration, the optimal light intensity for algal fluorescence measurement was determined. For this purpose, 50 mL of algae in growth phase and at a pH adjusted to 7 was centrifuged at 3000 g to concentrate the algae at the optimum concentration assessed previously. Following the same procedure, algae were exposed to Diuron (100 µg/L) or to pure water and fluorescence response was observed at different levels of continuous light excitation intensity (LED current set to 40, 60, 80 and 100 mA).

2.7. Performance of the biosensor

Using the optimized parameters for light excitation and algal concentration, biosensor performance and reliability were assessed by exposing microalgae strains to a dilution range of Diuron

pesticide (1, 10, 100 and 1000 µg/L). The response of control algae was compared with algae exposed to the maximum ethanol concentration used in the study (0.01%) to ensure that the ethanol did not impact the fluorescence disturbance observed with the Diuron. Limits Of Detection (LOD) values were obtained as commonly done in algal biosensor studies (Védrine et al. (2003); Shitanda et al. (2009); Tsopela et al. (2014,2016)) and EC_{50} values were calculated using the Hill probit model (Regtox®, E. Vindimian, http://normalesup.org/~vindimian/). Pesticide effects on microalgae are well established and dose-response curves obtained in this study have been compared to the data available from the scientific literature to validate the sensitivity of our apparatus (e.g. in term of detection limits and EC_{50} values).

2.8 Application to real urban polluted samples

The biosensor was used with urban polluted samples to validate its reliability and exploitability. Four types of urban polluted matrices were tested to evaluate biosensor relevance and performance: storm waters (3 samples) combined sewer overflows (CSO) (2), associated contaminated downstream creek waters (2), urban (1), and hospital effluents (2). For all real sample exposures, 100 µL of the microalgae suspensions (with final optimal concentration determined before) were mixed with 100 µL of the 10 samples and remained in contact with them during the algal-specific time previously described. To avoid clogging issues, all real samples were decanted for 10 minutes before algal exposure to remove any large particles. Physicochemical analyzes (classical parameters such as pH, conductivity, heavy metals, and organic micropollutants) were performed in order to explain A-chlorophyll fluorescence disturbance (enhancement or reduction) observed in the real polluted samples. Data are provided in the supplementary Tables S1 and S2.

3. Results and discussion

3.1 Construction and characterization of the properties of the portable station

The development of this algal biosensor required the construction of a special fluorimeter dedicated to the reading of algae inserted into disposable microfluidic chips. The first step was to

ensure the correct calibration and stability of the fluorescence signal without algae for different light intensities. Light intensity was controlled by the software which can deliver a current range between 0 and 100 mA. These data are presented in Figures S2 and S3. Figure S2 shows that although the excitation light is filtered, a small background signal was still detected. A slight decrease of the signal over time was also observed (3% over 3 min) for the highest excitation intensity (100 mA). This variation is probably due to the warming of the LED, but it has not been further investigated. Effectively, because of the differential nature of the measurement, this spurious signal and its variations are negligible. Figure S3 shows that when an empty microfluidic chip is inserted, the spurious signal increases slightly. As the slope of the signal also increases with the LED intensity, one can hypothesize that this phenomenon is due to a small amount of fluorescence coming from the chip (probably from the tape). Nevertheless, because of the differential measurement protocol, this effect is negligible.

3.2 Characterization and validation of xurography-based microfluidic chips for algal entrapment

Grey level determination was used to evaluate the reproducibility of microfluidic chips in terms of algal concentration and filling for each A-chlorophyll fluorescence reading chamber (control and exposed). As described in Figure S1B, no difference of grey value was observed between the two microfluidic chambers for each alga. The amount of algae that would be excited by the LED might be the same. Consequently, the difference in fluorescence between the two channels cannot be due to a difference in capacity of the reading chambers, but only to an effect of tested herbicide/wastewaters. This leads to the conclusion of good reliability of xurography for the design of reproducible chips for algal toxicity assessment.

3.3 Algal response, concentration and light intensity parameter optimization

Algal autofluorescence response during the observation period, depending on the algal concentration in the microfluidic chips, is shown in Figure 4A. Our data are in agreement with those obtained by Naessens et al. (2000) with *Chlorella vulgaris*. As for biosensor optimization, linearity between the algal concentration of the three strains and the emitted A-chlorophyll fluorescence was

established/verified beforehand to ensure optimal algal concentration and light intensity. Results are presented in Figure 4B. As already discussed, algal concentrations and intensity of light incident on the algae can strongly impact the sensitivity of the algal biosensors (El-Ali et al., 2006, Tsopela et al., 2016). To assess the best conditions for each of the two parameters, we exposed algae to Diuron. This herbicide is known to inhibit the normal functioning of photosystem II (PSII) by binding with significant affinity to the Quinone B (Q_B) binding site of the D1 protein of the PSII complex, which arrests the electron flow transfer inside the photosynthetic complexes and chain (Haynes et al., 2000) of the microalgae. When the light adsorbed by A-chlorophyll is used for photosynthesis (with photochemistry processes including electron transfer inside the photosynthetic chain), excess energy is partly re-emitted by a light-chlorophyll fluorescence (Maxwell and Johnson, 2000). Finally, in the presence of Diuron, energy cannot be transferred into the photosynthetic chain. Therefore, a more significant energy dissipation by A-chlorophyll fluorescence appears compared to a "normal" situation (fluorescence enhancement). An example of the typical response observed (in this case with *P. subcapitata* exposed to 10 µg/L and 1 mg/L of Diuron) and quantified with the software is presented in Figure S4.

In addition to the experimental validation of xurography-based microfluidic chips for algal entrapment, algal concentration and light intensity ranges were studied by exposing or not exposing (control) algae, to the same concentrations of herbicide (100 µg/L) for each studied condition. As presented in Figure 4C and 4D, the optimum concentrations obtained for each algal suspension were respectively 3.10^8, 3.10^8 and 1.10^8 cell/mL for *P. subcapitata*, *C. vulgaris* and *C. reinhardtii*, leading to maximum fluorescence enhancement. At higher concentrations, a reduction of enhancement was seen. This decrease can be explained by two assumptions: first, a reabsorption of the light emitted by neighboring cells (Védrine et al., 2003) can decrease the signal measured by photodetectors; secondly, excessive algal cell concentrations can lead to an excessive number of possible binding sites for the pesticide compared to the number of sites potentially affected (Deblois et al., 2013; Tsopela et al., 2016), and consequently, the effect may be masked due to the already high fluorescent signal emitted by algae. Concerning algal lightening, the higher (100 mA) current powering the LED produced the best response for *C. reinhardtii*. However, a slight decrease of algal fluorescence enhancement at 100

mA was observed for *P. subcapitata* and *C. vulgaris* compared to the 80 mA condition. This result can be interpreted as a photoinhibition of the photosynthesis metabolism. When an excessive intensity was incident upon the algae, a saturation of the photosystems occurred and a decrease of photosynthetic activity was observed (Krause and Weis, 1991). Mechanistically, this decrease of activity is caused by a degradation of the photosystem proteins (e.g. D1 reaction center protein) (Rintamäki et al., 1996). This phenomenon can thus lead to a masking of toxic effects, as demonstrated by Camuel et al. (2017) with *C. vulgaris* exposed to Atrazine and Diuron.

- Figure 4 -

3.4 Biosensor sensitivity evaluation

To validate the sensitivity of our biosensor compared to others described in the literature, a Diuron dose-response curve was determined, using the optimum parameters for each alga previously defined. Results are presented in Figure 5. All three algae appeared to be sensitive to Diuron exposure, leading to important fluorescence increases at high concentration (100 µg/L and 1 mg/L). In this configuration, *C. reinhardtii* was the most sensitive alga comparing limits of detection (LOD) and EC_{50} data for Diuron (Table S3). It is important to specify that the variability observed in the dose-response curves with biosensors was due to different sources of errors: technical operating errors, xurography-based microfluidic chip construction precision, and a potential algal insertion problem inside the chip (negligible as observed before), and the insertion of the chip into the fluorimeter. Despite these potential sources of error, the variability was relatively limited and led to an accurate, repeatable and reliable biosensor.

Our results are compared with the literature in Table S3. Most of the recent microfluidic devices (Tahirbegi et al., 2017; Tsopela et al., 2014, 2016) and immobilization methods such as hydrogels (Nguyen-Ngoc and Tran-Minh, 2007) or serigraphic impression on electrodes (Shitanda et al., 2009) used to develop algal photosynthesis disturbance-based biosensors by Diuron (e.g. chlorophyll autofluorescence or oxygen-production disturbance) presented similar or lower sensitivity compared to our device. However, Lefevre et al. (2012) manufactured PDMS-based microfluidic

biosensor containing an integrated system of excitation/detection composed of organic light-emitting diode (OLED) and photodetectors (OPD) placed directly underneath and above the microfluidic channels. They obtained very low EC_{50} compared to this study. The employment of xurography in our study is not at the origin of this difference of sensitivity: the major difference between xurography and common microfluidic techniques is the fabrication time of a chip (2 minutes against 2 hours for a PDMS chip, and 2 days for silicium/glass chip). One plausible explanation is the reduced distance between the algae and the optical components (due to the flexibility of OLED and OPD) and the better sensitivity of OPD compared to commercial photodetectors. As already evocated in part 3.3, another explanation resides in a lower algal concentration used by Lefevre et al. (2012), leading to a greater number of possible pesticide binding sites compared to the present configuration, thus lowering EC_{50} values.

- Figure 5 -

3.5 Application to real environmental samples

Our biosensor was applied to 10 real environmental samples (UWWD, receiving water, municipal and hospital wastewater) collected at different sites in the Rhône Valley (France). Algal strain responses are illustrated in Figure 6. Toxic effects were observed for a large portion of the samples, depending of the algae exposed, with percentage of fluorescence enhancement up to 29.22 % ± 2.83 for *P. subcapitata* exposed to the hospital wastewater A. Compared to previous experiments with pure pesticide molecules where only fluorescence increase was measured, enhancement or decrease of fluorescence was observed depending of the sample. Fluorescence decrease after pollutant exposure has been widely described in the literature. Several pollutant families impact sites of the photosynthetic chain other than Quinone B (Suresh Kumar et al., 2014), as Photosystem I (paraquat) (Fai et al., 2007) or ATPase proteins. These interactions with photosynthetic systems can thus lead to a decrease of algal A-chlorophyll fluorescence, as for example observed with algae (*P. subcapitata* and *C. vulgaris*) exposed to pharmaceutical pollutants detected in our environmental samples (Ciprofloxacin and Sulfamethoxazole) (Liu et al., 2011) or Glyphosate (Naessens et al., 2000). Consequently, the toxic effects observed result from algal exposure to very complex mixtures of

micropollutants with additives, synergic, or antagonism effects of the algal photosynthetic apparatus, producing chlorophyll fluorescence disturbance. For this reason, biosensors are particularly relevant for an early detection of toxic impact of urban samples, though they would not replace physico-chemical laboratory analyses of substances that are likely sources of toxicity.

The investigation also demonstrated the relevance of using three different algal strains. Indeed, algal response varied considerably, depending of the sample. For instance, the hospital wastewater B sample led to a 20.60 % ± 8.27 chlorophyll fluorescence increase of *P. subcapitata*, whereas it generated 26.00 % ± 2.53 of decrease of *C. reinhardtii* fluorescence compared to the control. The difference in the three algae strains' responses can be explained by their specific sensitivity to the different pollutants observed in the samples. This fact has been widely demonstrated for microalgae exposed to heavy metals, pesticides, and pharmaceuticals (Takamura et al., 1989; Magnusson et al., 2010; Brezovsek et al., 2014).

Finally, to our knowledge, only two others photosynthesis disturbance algal biosensors are currently used in environmental samples (Buckova et al., 2017; Durrieu et al., 2016). In these works, authors developed, respectively, an oxygen production disturbance with a Clark-type electrode biosensor to monitor the impact of roadside soil extracts, and a hydrogel chlorophyll fluorescence disturbance sensor to evaluate storm water sample toxicity. Algal sensors are sufficiently sensitive to detect the toxicity of polluted effluents. However, one limitation remains that should be highlighted in these two studies. These detected significant disturbances after 12 h and 24 h exposure. With the biosensor proposed in this study, a maximum delay of 20 minutes was necessary to detect a toxicity effect.

- Figure 6 -

These algal biosensors are not relevant for the identification of toxicity source due to the complex interaction between pollutants such as additivity, synergy, or antagonism (Moro et al., 2018), which require in-depth chemical analyses in laboratory. Algal biosensors constitute "early warning systems" for the detection of toxic conditions, but lack analytical capability. Nevertheless, these

biosensors present some advantages compared to conventional chemical analyses: (i) The ecotoxicological effects observed with biosensors take into account all pollutants present in discharges, whereas physico-chemical analyses can only identify what they are explicitly seeking and can therefore ignore hundreds of ecotoxic molecules; (ii) The bioavailibility of all micropollutants for the algae is considered (interactions between chemicals and particles sorption/desorption phenomenon) (Becouze-Lareure et al., 2016; Fahl et al., 1995).

4. Conclusion

An efficient, easy-to-use, and portable algal biosensor, based on algal photosynthesis disturbance analysis was developed for *in situ* ecotoxicological analyses of polluted effluents and contaminated freshwater. This device, apt for use *in situ*, was constructed using disposable microfluidic chips for algal immobilization (by insertion), and an electrically autonomous fluorimeter dedicated to algal photosynthesis disturbance analysis. Microfluidic chips were designed and created using a low-cost and low-constraint technique – xurography – and transparent and inert materials (glass slides). Simple two-entry (control and exposed) chips were constructed, using the capillarity phenomenon to insert microalgae. Three microalgae strains were studied (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamodomonas reinhardtii*) because of the extensive literature on them and their differences in sensitivity to environmental pollutants. Fluorimeter and microfluidic chips were calibrated to ensure correct responses. For the three algae, parameters such as algal concentration and light intensity delivered by the fluorimeter were optimized to obtain the most sensitive signal, using a well-referenced pesticide, Diuron, as a model. We demonstrated that variation in these two parameters could considerably change the algal response to pollutants. The sensitivity of our device was successfully demonstrated through dose-response curves with different Diuron concentrations and comparing data with the literature. Different sensitivities were observed depending of the algae, with highest accuracy for *C. reinhardtii* (lowest LOD and EC_{50}). Finally, the station was applied to real environmental samples, such as urban storm waters, effluents or contaminated rivers, and toxic effects were detected after short exposure times (≤ 20 min). The need to develop multi-species sensors was demonstrated by the fact that significant differences in response to urban samples

were observed depending on the algae species. Nevertheless, in our case, a high algal concentration could be the source of a decrease of sensitivity with low concentration of pollutants in samples. Future investigations are necessary to study lower algal concentration exposure. Further experiments are also required to develop multi-channel chips and detection systems that analyze replicates in parallel and decrease analysis time.

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Figure caption

Figure 1: Xurography-based microfluidic chip creation procedure (A) and photography (B) with algae inserted by capillarity, for the algal immobilization in portable station. DSPA: Double sided pressure adhesive film.

Figure 2: Image of the whole system (Computer, GPS, and portable station) (A), portable station (B), internal (D) and external (C) representation of the portable station.

Figure 3: Image of the algal A-chl fluorimeter user interface.

Figure 4**:** Algal chlorophyll fluorescence parameter optimization: Algal fluorescent response of the three algae over the time measured with the software (A), relation between chlorophyll fluorescence and algal concentration (B), relation between algal concentration and chlorophyll fluorescence enhancement after exposure to Diuron (100 µg/L) (C), relation between light intensity and chlorophyll fluorescence enhancement after exposure to Diuron (100 µg/L) (D). Ps : P. subcapitata ; Cv : C. vulgaris ; Cr : C. reinhardtii (mean ± S.D., N = 3).

Figure 5: Biosensor dose-response curves for algae exposed to the Diuron. *Ps* : *P. subcapitata* ; *Cv* : *C. vulgaris* ; *Cr* : *C. reinhardtii* (mean ± S.D., N = 3).

Figure 6: Biosensor response for *P. subcapitata* (*Ps*), *C. vulgaris* (*Cv*) and *C. reinhardtii* (*Cr*) algae exposed to the 10 environmental samples (mean ± S.D., N = 3).

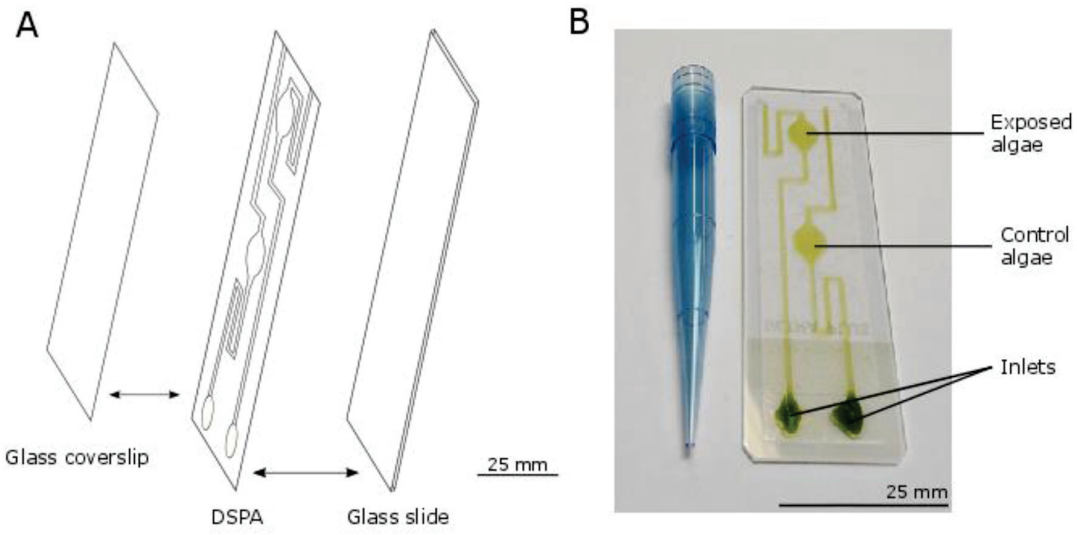

Figure 1

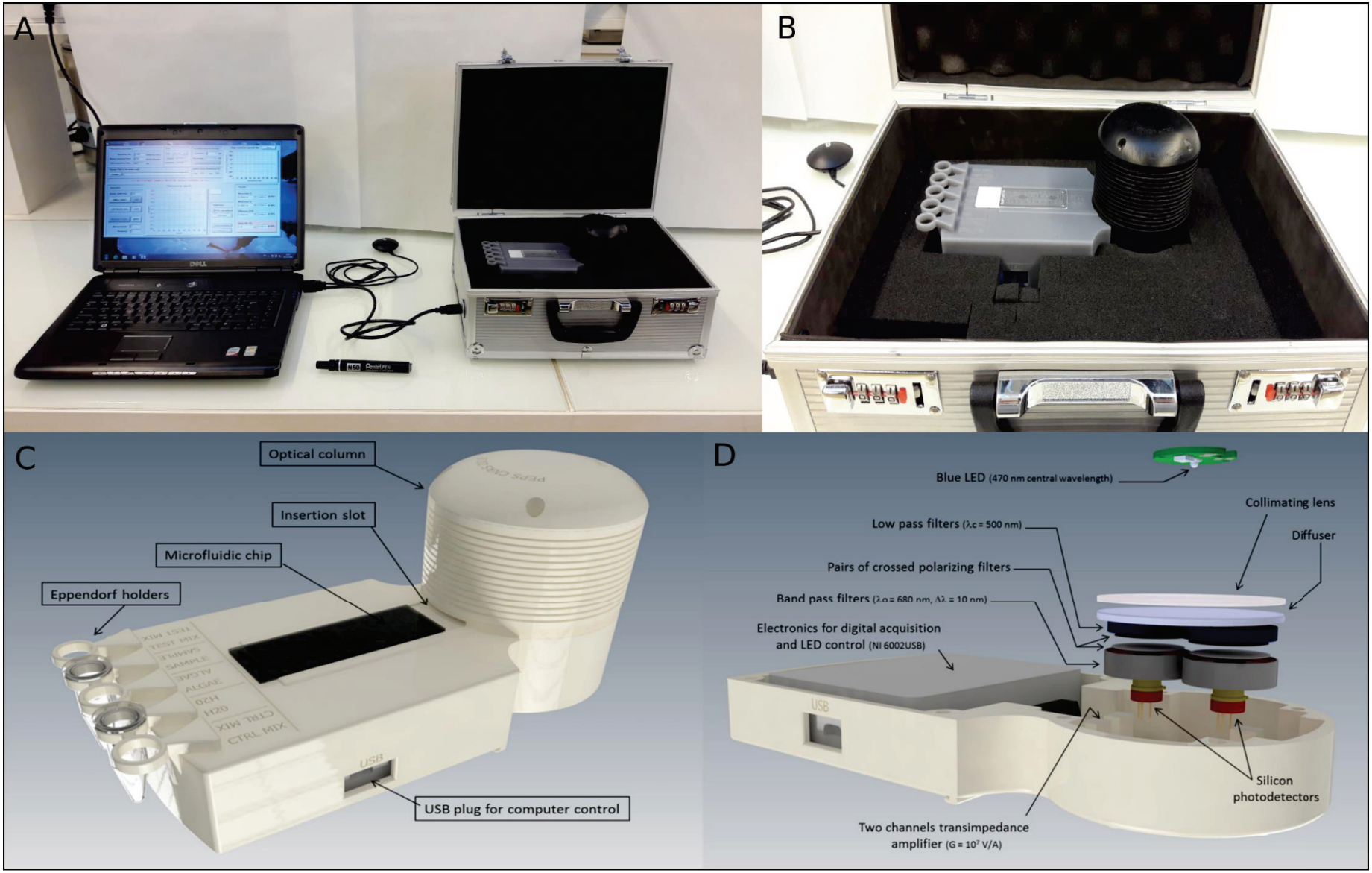

Figure 2

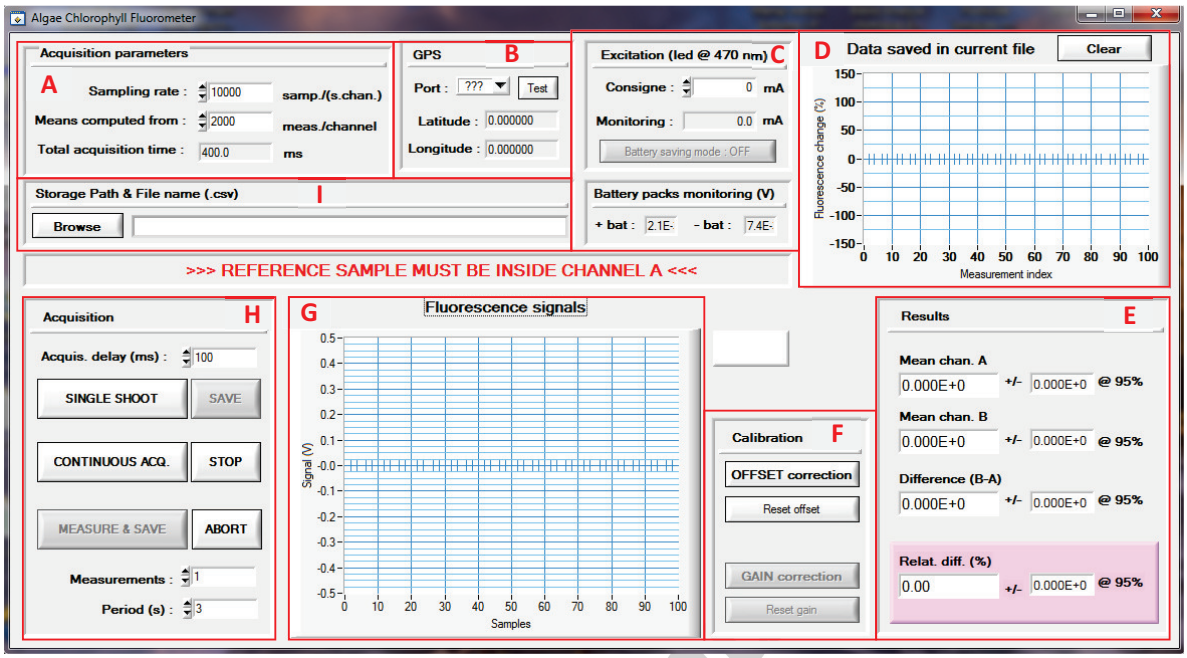

Figure 3

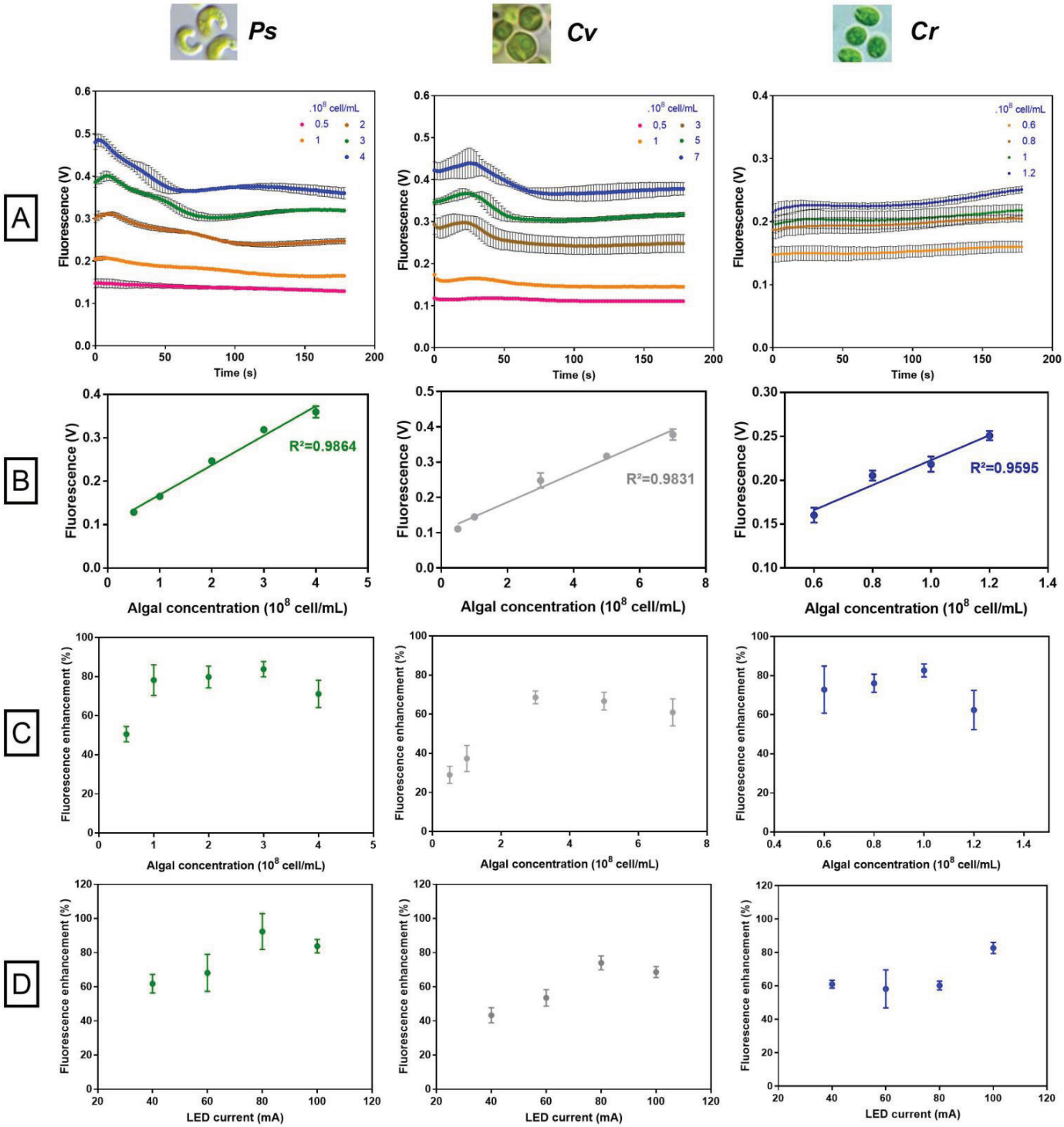
Ps
Cv
Cr
A
B
C
D
.10^8 cell/mL
0.5
1
2
3
4
0,5
5
7
0.6
0.8
1.2
Fluorescence (V)
Time (s)
R²=0.9864
R²=0.9831
R²=0.9595
Algal concentration (10^8 cell/mL)
Fluorescence enhancement (%)
LED current (mA)

Figure 4

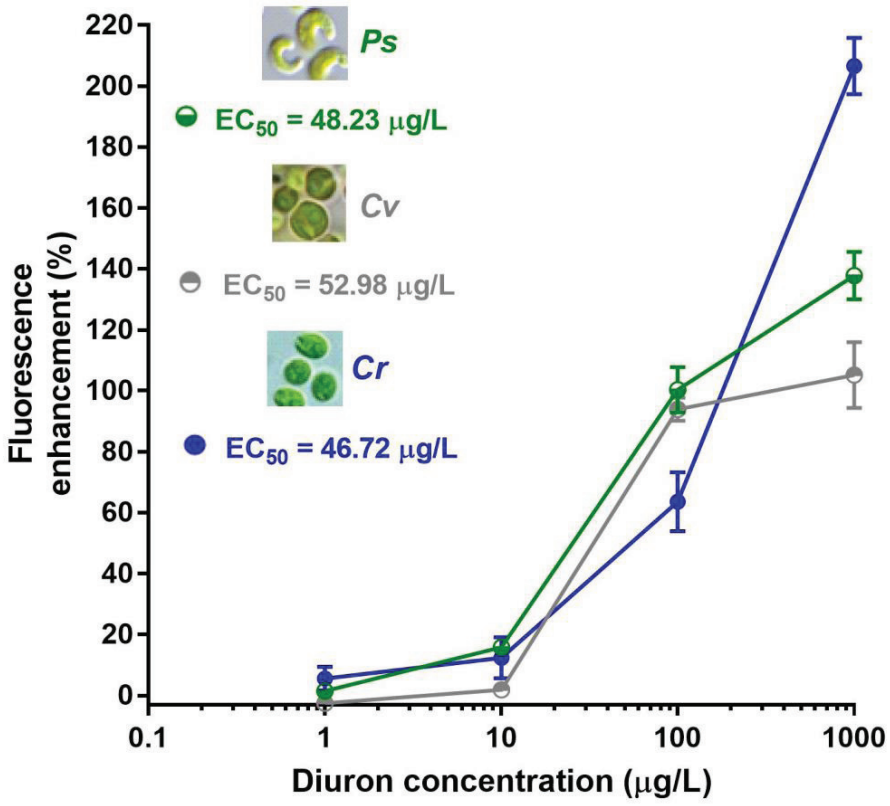

Figure 5

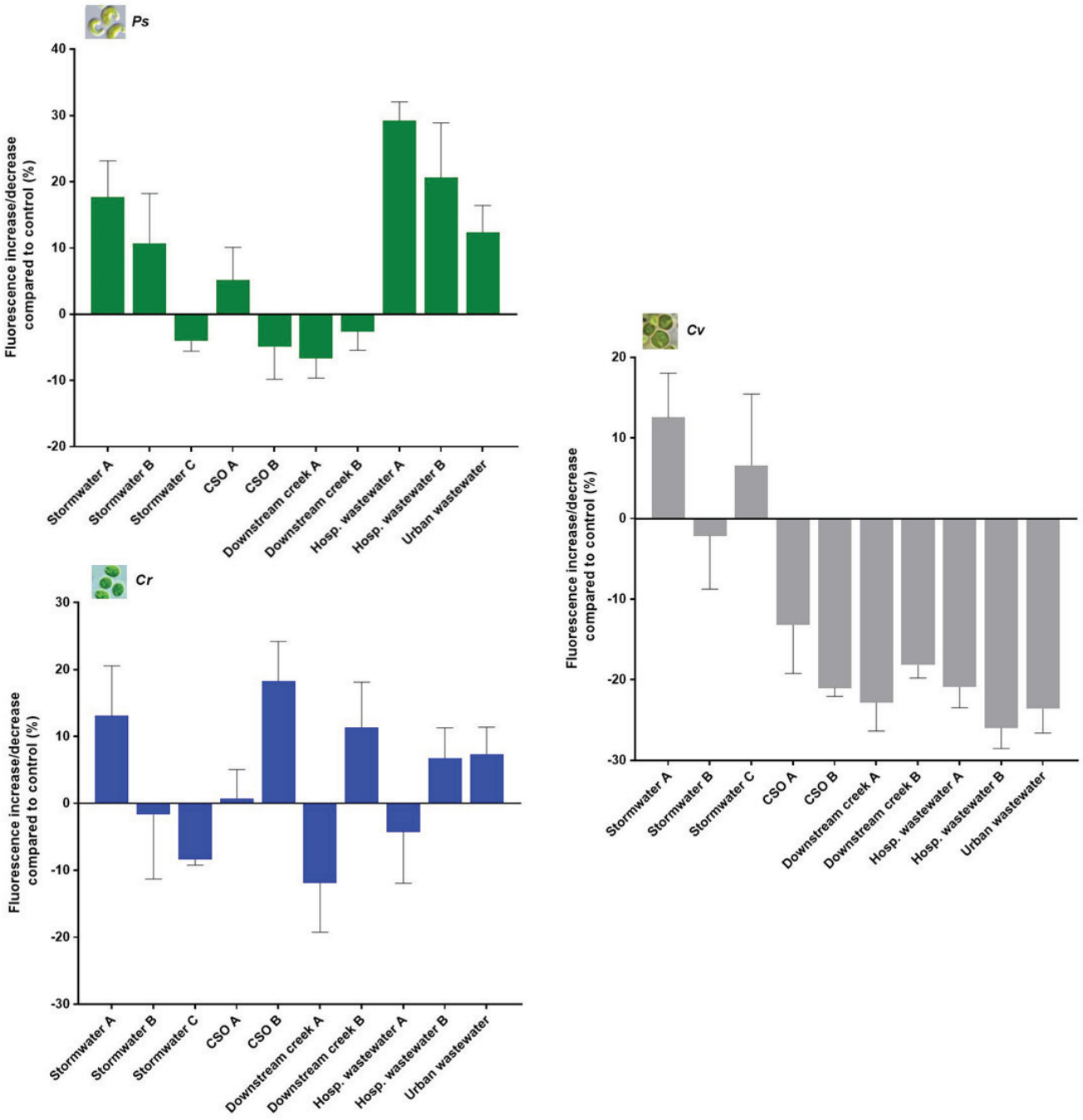
Ps
Fluorescence increase/decrease compared to control (%)
40
30
20
10
0
-10
-20
Stormwater A
Stormwater B
Stormwater C
CSO A
CSO B
Downstream creek A
Downstream creek B
Hosp. wastewater A
Hosp. wastewater B
Urban wastewater
Cr
Fluorescence increase/decrease compared to control (%)
30
20
10
0
-10
-20
-30
Stormwater A
Stormwater B
Stormwater C
CSO A
CSO B
Downstream creek A
Downstream creek B
Hosp. wastewater A
Hosp. wastewater B
Urban wastewater
Cv
Fluorescence increase/decrease compared to control (%)
20
10
0
-10
-20
-30
Stormwater A
Stormwater B
Stormwater C
CSO A
CSO B
Downstream creek A
Downstream creek B
Hosp. wastewater A
Hosp. wastewater B
Urban wastewater

Figure 6

Highlights

- We developed a portable microfluidic biosensor for *in situ* water toxicity analysis.
- Xurographic fabrication was used to create disposable and low-cost microfluidic chips.
- Algal biosensor sensitivity was tested with Diuron pesticide.
- The biosensor was applied to real urban polluted water samples.

Graphical abstract