Contribution of biosensors based on unicellular organisms to evaluate the ecological impact of stormwater

Contribution de biocapteurs à base d'organismes unicellulaires à l'évaluation de l'impact écologique des eaux pluviales

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RÉSUMÉ

Cet article présente des méthodes d'évaluation de la toxicité des eaux pluviales grâce à des algues unicellulaires. Les microalgues sont des organismes utilisés fréquemment dans les études écotoxicologiques pour leur capacité à réagir rapidement face à divers type de polluants : pesticides, métaux lourds, hydrocarbures et pour leur grande représentativité écologique. Des tests normalisés et non normalisés, globaux et spécifiques ont été menés sur de nombreux rejets urbains de temps pluie prélevés dans l'agglomération lyonnaise. La nature de ces tests et certains résultats sont ici présentés. En parallèle un outil de terrain basé sur l'étude de réactions enzymatiques des microalgues a pu être développé. Il s'agit d'un biocapteur conductimétrique capable de fonctionner en semi autonomie. Dans ce travail le fonctionnement de cet appareil est décrit, accompagné des premières mesures réalisées *in situ*.

ABSTRACT

This paper presents methods for assessing the toxicity of stormwater using unicellular algae. Microalgae are organisms commonly used in ecotoxicological studies as they are ecologically representative and react quickly to various types of pollutants such as pesticides, heavy metals and hydrocarbons. Standardized and non-standardized, comprehensive and specific bioassays were carried out on many urban wet weather effluents collected in Lyon. The nature of these tests and the results obtained from them are presented. Also presented is a field-tool under development that uses the enzymatic reactions of microalgae to detect contamination. It is a conductometric biosensor able to operate semi-autonomously. A description is given of how this device operates and the first *in situ* measurements obtained from it are presented.

KEYWORDS

Algae, Bioassays, Biosensors, Ecosystems, Impacts

1 INTRODUCTION

Runoff generated during rain episodes contains contaminants leached from the catchment concerned. It therefore presents a potential hazard for host ecosystems. A large number of works have highlighted the toxic nature of runoff (Angerville 2009; Boisson and Perrodin 2006; Parent-Raoult and Boisson 2007). Bioassays performed with target organisms highlight possible harmful effects on the biocenosis. Consequently, the interest of using unicellular algae as an indicator of the impact of stormwater was shown in a previous study (Durrieu *et al.* 2010) aimed at assessing the impact of road runoff.

Microalgae are the base component of trophic chains and can accumulate large quantities of pollutants. They are therefore pertinent toxicity indicators useful for studying the response of ecosystems exposed to emissions of cocktails of pollutants present in stormwater. The latter frequently contains pesticides, heavy metals, hydrocarbons and a quantity of what are known as emerging substances whose effects are as yet poorly understood (Joshi and Balasubramanian 2010; Jartun and Pettersen 2010; Aryal et al. 2010).

Different types of bioassays can be performed on the compartment of unicellular algae: global tests based on growth inhibition, a standardised test exhibiting the global impact on the algal compartment, and other non-standardised but more selective bioassays that permit more precise identification of the types of pollutant present. Thus the modification of chlorophyll fluorescence emission indicates the presence of compounds that disturb photosynthesis, while the exploration of particular metabolic channels by monitoring enzymatic activities may lead to identifying the major classes of chemical compounds (heavy metals, pesticides, PAHs, etc.). We are more specifically interested in certain membrane enzymes whose activity can be measured directly on the cell without prior extraction. Tests can be carried out *in vivo* in the natural environment of the enzyme, providing genuine ecological realism. Enzymatic activities include alkaline phosphatase activity (APA) vital for phosphorous metabolism, and esterase activity (EA) which is involved in photosynthetic activity. These enzymatic activities are most usually disturbed before the occurrence of more general effects such as growth inhibition; they therefore constitute early indicators of toxicity, making it possible to obtain information on impacts to the ecosystem from the lowest concentrations of contaminants. Previous studies showed the specific characteristics of the response of these activities to certain families of pollutants, especially the high sensitivity of phosphatase to the presence of heavy metals (Chouteau *et al*. 2005; Berezhetskyy, 2007) whereas esterase activity appears more selective for organic compounds, such as organophosphorous pesticides in particular (Guedri 2010).

In this work, we study the interest of performing bioassays with unicellular algae to assess the impact on a river receiving the discharges of a stormwater spillway located at Ecully, west of Lyon, and two retention/infiltration basins installed at Chassieu and at Bron, respectively, both east of Lyon.

In addition, we emphasise the fact that improving the environmental quality requires controls of toxicity *in situ* and continuous monitoring of sources of pollution and contaminated environments, using automated systems with fast response times. This entails using biosensors that permit performing bioassays on immobilised organisms placed at the sites to be monitored. These systems provide responses in real-time and could, in the long term, be used to quickly stop the discharge of a toxic substance, for example, and prevent accidental pollution. These tools have a considerable advantage over conventional test methods that only permit episodic toxicity controls in the laboratory, performed under static conditions relatively unrepresentative of dynamic conditions (Lagarde and Jaffrezic-Renault 2011). This is why, in addition to the bioassays on algae, we study the feasibility of a biosensor designed with immobilised algae and intended to operate autonomously. This article presents and compares the results obtained from the bioassays and biosensors.

2 MATERIAL AND METHODS

2.1 Cell cultures

Strains of the microalgae *Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* came from the Culture Collection of Algae and Protozoa in the United Kingdom. They were maintained by weekly transfer in the culture medium Lefebvre/Czarda (AFNOR 1980) for *C. vulgaris* and *P. subcapitata* and in Tris-Acetate Phosphate (TAP) medium for *C. reinhardtii* (Gorman *et al.* 1965) previously sterilised by autoclaving (20 minutes, 130°C, 1.3 bars). The strains were maintained in a nyctohemeral cycle of 16 hours light at 5000 lux and 8 hours darkness.

2.2 Urban discharges

Three sampling sites were selected in the city of Lyon (Figure 1). The first was a stormwater spillway located on an urban stream in Ecully, west Lyon. The second site was a retention/infiltration basin located in east Lyon, downstream of an industrial area. They both belong to a network of sites instrumented by the *Observatoire de Terrain en Hydrologie Urbaine* (OTHU). The last site was a retention/infiltration basin also located in east Lyon, downstream of a heavy truck park next to Bron airport. This provided us with stormwater from three different types of catchment, namely residential, industrial and roadway, typical of an outer urban environment. The characteristics of the catchments are given in the table 1.

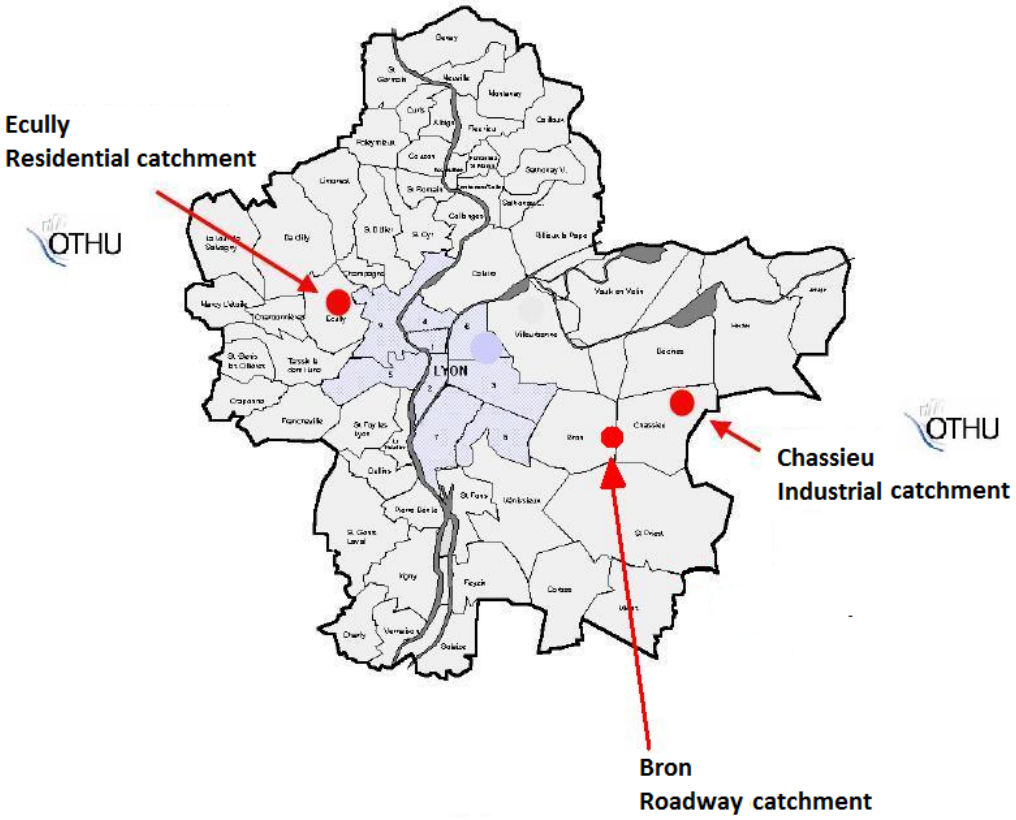

Figure 1: Localisation of the study sites

Table 1: Characteristics of the catchments studied

Site		Chassieu	Ecully	Bron
Total surface area (Ha)		185	245	4
Land use of catchment (% of total surface area)		Industrial	Residential	Heavy truck park
	Surface sealing	75	42	100
	Green spaces	20	60	0
	Farm areas	4	5	0
	Natural areas	4	1	0

More than 20 samples were taken between 2011 and 2012. For each sample we analysed the quantity of nitrates (NO_3^-), phosphates (PO_4^{2-}), sulphate (SO_4^{2-}) and potassium (K^+) (by ion chromatography, Thermo Scientific Dionex DX-100) as well as 5 heavy metals (lead, cadmium, nickel, copper and chrome, by atomic absorption spectroscopy, Hitachi Z-8200). In parallel, for certain samples, more thorough analyses were performed by the *Service Central d'Analyse* of the CNRS in order to dose the organic compounds featuring on the list of priority substances of the DCE (European Parliament and the Council of the European Union. 2000). The latter were dosed by gas phase chromatography coupled with mass spectrometry (GC-ToF, AGILENT 6890N) and by high performance liquid chromatography coupled with mass spectrometry (AGILENT 100/1200 and ABSciex/3200 QTRAP).

2.3 Bioassays on free algae

The different activities are measured following exposure of the algae to discharges and compared to the results of experiments performed under the same conditions with ultrapure water (control value).

2.3.1 Alkaline phosphatase

This activity is measured with MethylUmbelliferyl Phosphate (MUP, Sigma), used as a substrate in buffer medium (TRIS-HCl, pH 8.4) in 96 well microplates. The degradation of the substrate by the enzyme generates a fluorescent product, MethylUmbelliFerone (MUF) that can be dosed spectrofluorescence for an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The kinetics is studied at 20°C for 20 minutes for different concentrations of substrate. The maximum speeds (V_m) of the reaction and the affinity of the enzyme for its substrate (K_m) are considered and compared with each other (parameters calculated by linearization). Attention is given to the K_m/V_m ratio, the specific reaction time which, expressed in minutes, gives the time the enzyme takes to consume all the substrate available at maximum speed (Cornish-Bowden 2005).

2.3.2 Esterase

This activity is measured with Fluoresceine Diacetate (FDA, sigma) used as a substrate in 96 well microplates. The degradation of the substrate generates fluorescence linked to the production of fluoresceine that can be dosed in spectrofluorescence for an excitation wavelength of 480 nm and an emission wavelength of 538 nm. As with the phosphatase the kinetics is studied at 20°C and the K_m/V_m ratio is considered to interpret the results.

2.3.3 Chlorophyll fluorescence

Chlorophyll fluorescence is measured with the excitation / emission (469nm/682nm) parameters of algae exposed beforehand to the samples. We analyse variations of intensity expressed by a change of the height of the emission peak. They are compared to the increase of fluorescence caused by DCMU that completely blocks photosynthesis and therefore maximum fluorescence. The results are expressed in terms of percentage of residual photosynthetic efficiency (E) according to the following formula: $E\ (\%) = \Delta F_{\ assay} / \Delta F_{\ control} * 100$, with $\Delta F = F_u - F_o$, F_u being the fluorescence before contamination and F_o the fluorescence after contamination by 4mg/l DCMU. The test entails exposing the microalgae to urban discharges for a given time whereas the control entails placing the algae under the same conditions but using ultrapure water instead of the discharge (Rebillard 1989).

2.3.4 Algal growth

The cell growth inhibition tests were performed in conformity with the standard in force (AFNOR 2012). The algae *P. subcapitata* was transferred at 10^4 cells per ml in the test medium and in the control medium. The total test volume was 5 ml (4.5 ml of discharge or ultrapure water and 0.5 ml of culture medium). Variable parts of the discharge were introduced in the test, i.e. 90%, 81%, 63%, 45%, 27% and 9% of the total volume. After 72 hours light (5000 lux) the cells were counted under a microscope. The results are expressed as a percentage of inhibition of cell growth rate obtained compared to that of the control performed under the test conditions.

2.4 Measurements of biosensor activity

The biosensor used is composed of a conductimetric transducer with two identical pairs of gold interdigited electrodes marketed by the Institute of Semiconductor Physics, Kiev, Ukraine. The interdigited electrodes are 150 nm thick and deposited on a ceramic substrate. An intermediate layer of Ti 50 nm thick was used to improve the bonding of the gold on the substrate. The digits composing the interdigited electrodes are 10 µm wide, which is also the distance between them, and they are about 1 mm long, providing a sensitive zone on each of the electrodes of about 1 mm^2. The central part is covered with epoxide resin to distinguish the sensitive part of the sensor (Figure 2). The measurements are based on the detection of variations of conductivity in the sensitive zones linked to the movements of ions between the anode and the cathode. The alkaline phosphatase and esterase activity of *C. vulgaris* cause catalytic reactions generating ionic species that result in measurable changes of conductivity.

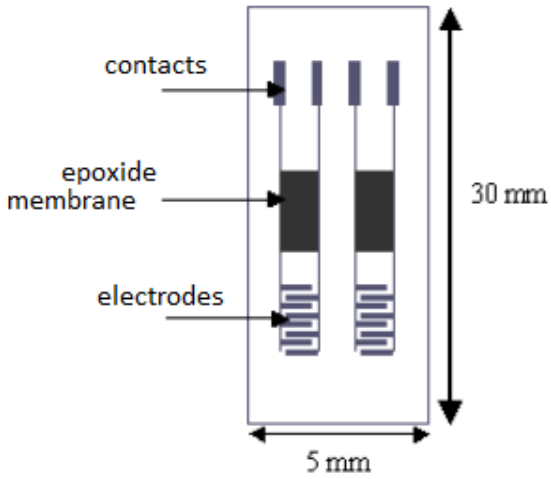

Figure 2: Diagram of interdigited electrodes (Durrieu et al. 2006)

3 RESULTS

The different bioassays performed are presented first. They provide information on the degree of disturbances caused (global when they affect growth or more specific to a metabolic channel when they affect one or more enzymatic activities). These bioassays were then used to validate the responses obtained from the biosensors presenting the highest variability. In order to simplify, results obtained from a sample picked up in the Bron retention basin are presented.

3.1 Algal growth

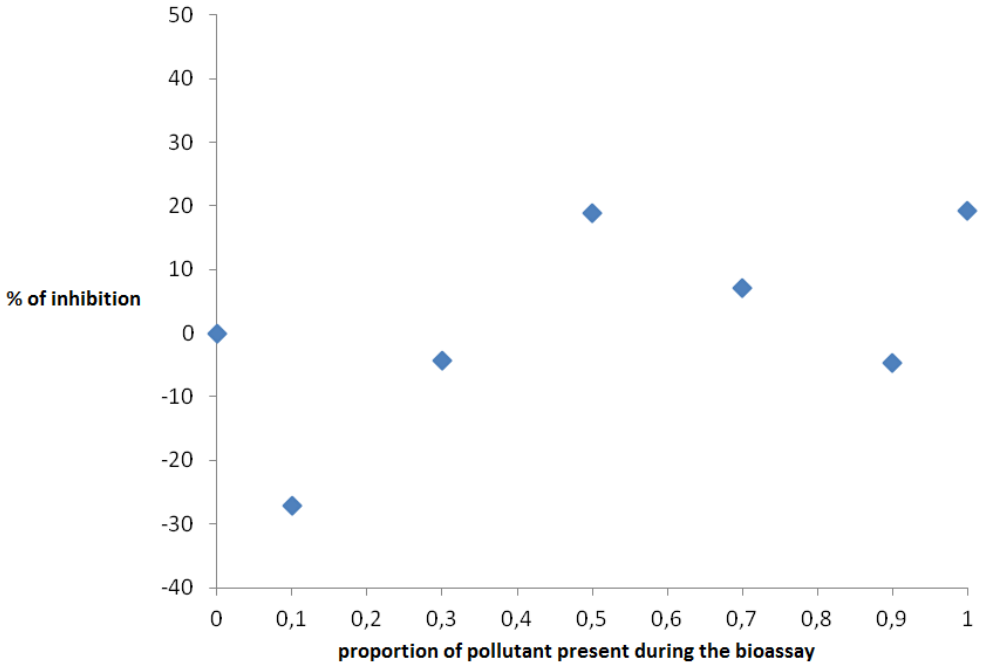

Figure 3: % of algal growth inhibition as a function of the proportion of pollutant present during the bioassay. In this case the pollutant is a discharge of a wet period from a stormwater retention basin in Bron sampled on 02/01/2012.

From this discharge from the retention basin of Bron we obtained stimulation of growth at low concentrations (up to 30% pollutant) and then low growth inhibition (20% max.).

The physicochemical analyses (see table 2) show the presence of several PAHs, phenols, metals and pesticides. This cocktail of pollutants inhibits the growth of *P.subcapitata.*

Table 2: Physicochemical analyses of the discharge sampled at Bron on 02/01/2012
(ND = not detected; WWUR = wet weather urban runoff)

Reference	WWUR Bron 02/01/2012
Ions (mg/l)	Dissolved
NO_3^-	8.5
PO_4^{2-}	0.36
SO_4^{2-}	1.8
K^+	0.5
Metals (µg/l)	Dissolved
Pb	ND
Cd	ND
Cr	ND
Ni	ND
Cu	5.36
Zn	ND
Organics (ng/l)	Dissolved
Isoproturon	1.9
Fluoranthene	28
Naphtalene	104
Benzo (b) fluoranthene	14
4-nonylphenol	10
Acenaphtene	4.7
Fluorene	9.8
Phenanthrene	116
Pyrene	23
Benzo (a) anthracene	8.6
Chrysene	8.7

3.2 Esterase activity (EA)

The measurements were performed after 2h, 24h and 48h exposure to discharges to assess possible kinetic action on the activity studied (figure 4)

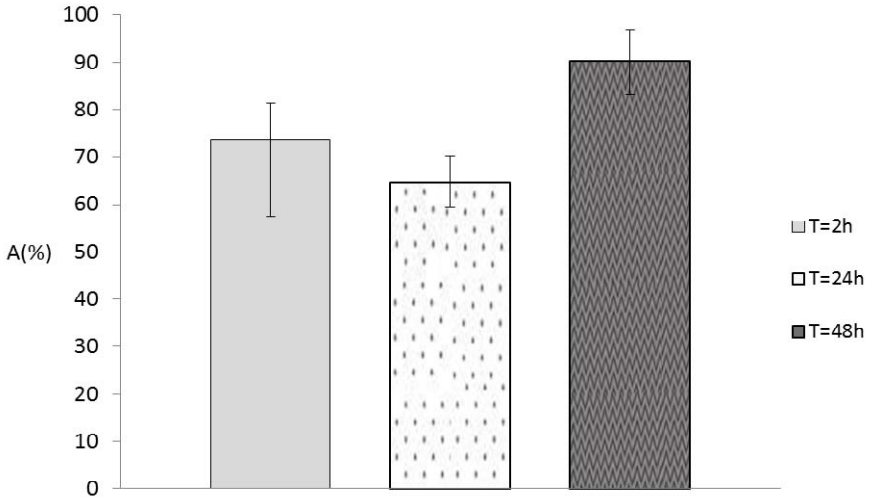

Figure 4: % of residual activity of EA of free algae in bioassays after 2h, 24h and 48h exposure to the WWUR of Bron sampled on 02/01/2012

The results shown in figure 4 highlight inhibition of 25% of activity after 2 hours contact. We consider any inhibition above 10% as significant (this value was determined on the basis of the mean of standard deviations obtained for all the bioassays performed). The inhibition obtained here after 2 hours is therefore significant. The evolution observed between 2 and 24 hours appears logical. The result obtained after 48 is, however, surprising. Nonetheless, after 48 hours contact, it was possible to observe the result of several phenomena (toxicity, reaction of cell protection by biosynthesis of stress proteins, etc.). The results obtained after 48 hours were often difficult to interpret.

Previous works have shown that esterases are enzymes sensitive to organics pollutants (Guedri 2010). This sample contained a non negligible proportion of copper (5.36 µg/l), 9 different PAHs: Fluoranthene (28 ng/l), Naphtalene (104 ng/l), Benzo(b)fluoranthene (14 ng/l), Acenaphtene (4,7 ng/l), Fluorene (9.8 ng/l), Phenathrene (116 ng/l), Pyrene (23 ng/l), Benzo(a)anthracene (8,6 ng/l), and Chrysene (8.7 ng/l) as well as a pesticide (Isoproturon 1.9 ng/l) which may explain the inhibition observed.

3.3 Alkaline phosphatase activity (APA)

The same protocol was used to ensure contact with the samples of stormwater as for the measurement of esterase activity.

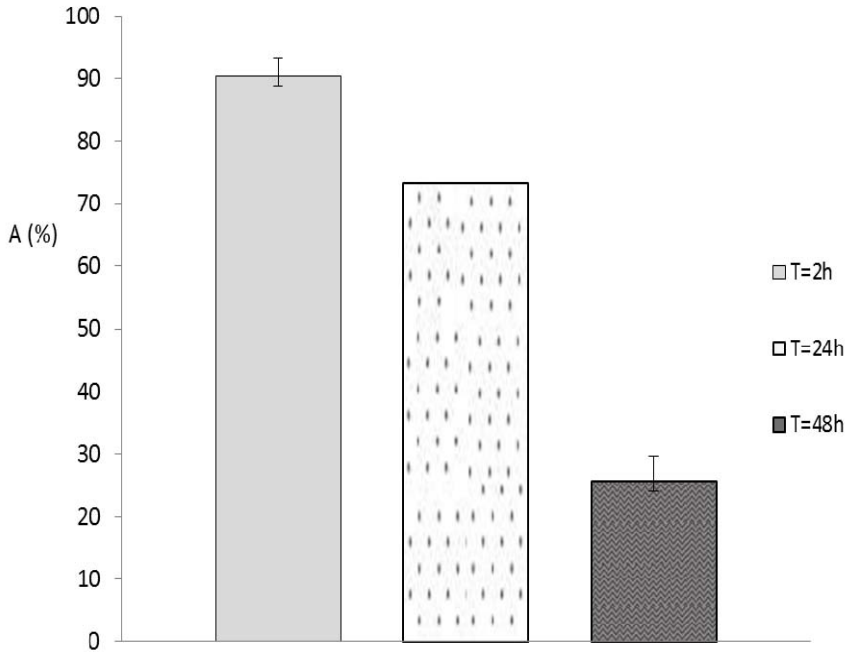

Figure 5: % of residual activity of APA of free algae in bioassays after 2h, 24h and 48h exposure to the WWUR of Bron sampled on 02/01/2012

The inhibition observed at 2h and which increased through time can be explained by the presence of phosphate ions in the medium (0.36 mg/l ≈ 3.79 µM). Previous works (Guedri *et al.* 2009) showed that APA is inhibited for concentrations in phosphate ions above 1µM (80% of residual activity after 30 minutes contact).

3.4 Chlorophyll fluorescence

Figure 6 presents the percentage of the photosynthetic efficiency inhibition (PEI) of *C. vulgaris* after 2h exposure to different discharges from the catchment of Bron. Each effluent was tested raw (NF for non filtered) and after filtration at 0.45 µm (F). Indeed, a large part of the pollution was often carried by particles, so it was important to test the soluble and particle fractions of the samples.

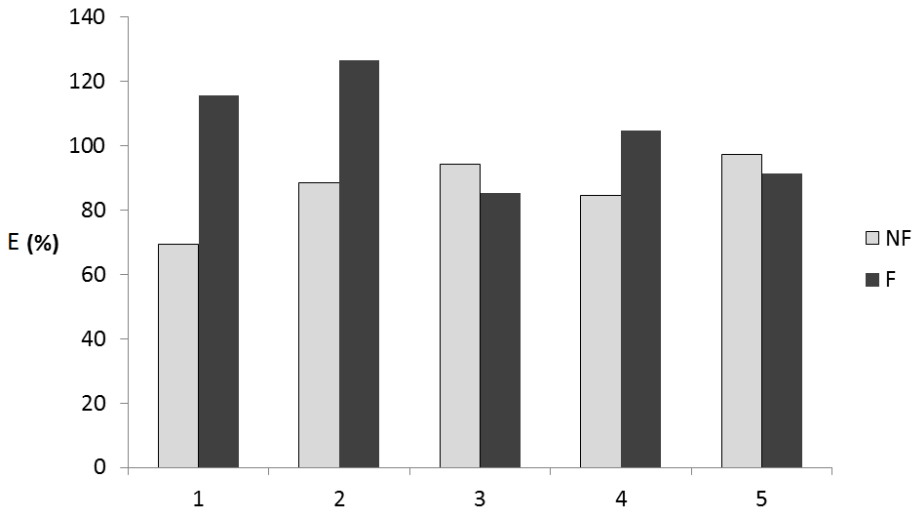

Figure 6: % of residual photosynthetic efficiency on different non filtered samples (NF) and samples filtered at 0.45 µM (F) from the retention basin of Bron (1=31/05/2011; 2=13/07/2011; 3=19/10/2011; 4=02/01/2012 and 5=22/05/2012)

The 5 non filtered samples led to inhibitions ranging from 5 to 30%. This inhibition was undoubtedly caused by considerable PAH particle pollution. For example, the sample taken on 31/05/2011 contained pyrene (10 µg/g) and fluoranthene (12 µg/g). On the contrary, filtering led to stimulations of photosynthetic efficiency for the discharges of 31/05/2011, 13/07/2011 and 02/01/2012 proving that the particle fraction is highly toxic for the microalgae. Filtration accentuated inhibition for the discharges of 19/10/2011 and 22/05/2012. This leads to the assumption that the particle fraction carried nutritive compounds that masked the toxicity of the dissolved fraction.

3.5 Biosensor tests

In parallel with the bioassays we worked with conductimetric biosensors. They have proved efficient for detecting heavy metals, herbicides or phosphate (Chouteau et al. 2005; Tekaya et al. 2013; Guedri and Durrieu 2009) but have not until now been used to monitor the quality of urban discharges. Our study compares the results obtained from bioassays with those obtained using biosensors.

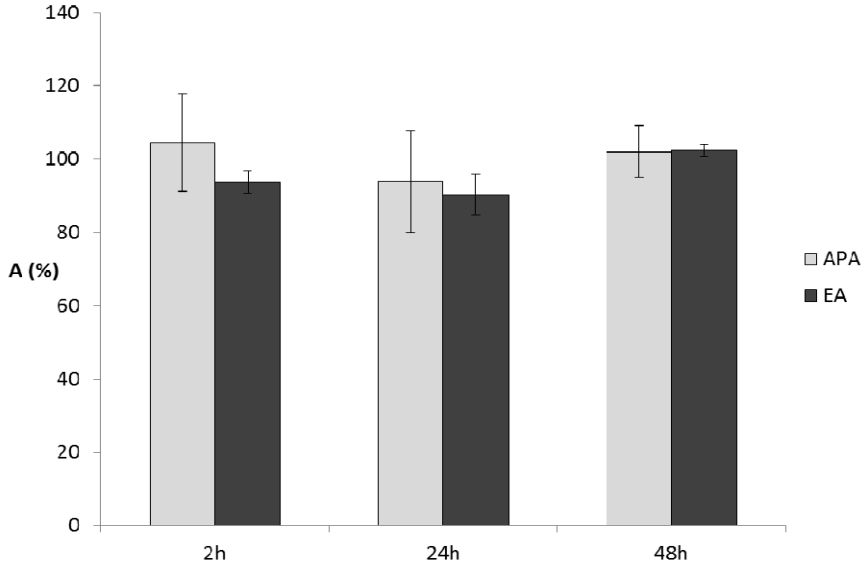

Figure 7: % of residual activity of APA and EA of algae immobilised on conductimetric biosensors after 2h, 24h and 48h exposure to the WWUR of Bron sampled on 02/01/2012

With APA the biosensor provided very variable results that tended to show that the toxicity of the discharge was low (Figure 7). This result contradicts the results obtained from the bioassays.
For the EA, the biosensor exhibited esterase inhibition after the first 2h. This inhibition increased after 24h and then decreased after 48h (Figure 8). These results agree with the results obtained from the bioassays.

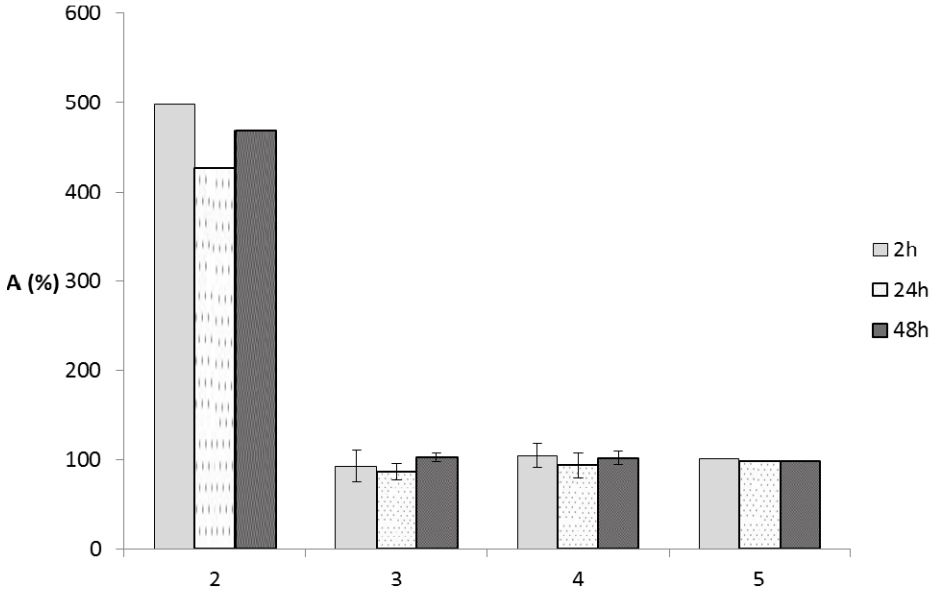

Figure 8: % residual activity of APA of algae immobilised on conductimetric biosensors after 2h, 24h and 48h exposure to the WWUR of Bron (2=13/07/2011; 3=19/10/2011; 4=02/01/2012 and 5=22/05/2012)

We can see here that the APA was considerably activated by the sample taken on 13/07/2011 which was not the case with the other samples. Unfortunately we did not dose the organic compounds of this sample but assumed that this enzyme was strongly stimulated by certain compounds that were absent in other samples.

4 DISCUSSION

The battery of bioassays implemented by us to assess wet weather urban runoff showed that it presents a major risk for the health of ecosystems. Indeed, most of the tests performed showed that, even when diluted, the samples taken led to modifications of the microalgae's metabolism. These modifications occurred within short times, after only 2h contact, and also after longer times, after 48h contact, with the organism setting up different detoxification protection mechanisms. These results demonstrate the complexity and the large number of effects on cellular metabolism and therefore the interest of performing ecotoxicological monitoring.

The physicochemical analyses carried out in parallel go a long way in explaining these results: organic and mineral pollutants were systematically detected in concentrations that often exceeded environmental quality thresholds (European Parliament and the Council of the European Union 2008). It is important here to examine the variability of the responses and therefore the information provided by the bioassays.

As shown by previous studies (Kafi *et al.* 2008; Hannouche *et al.* 2011) wet weather urban runoff presents very considerable inter-site and inter-event variability. This type of variability is also found (logically, given the pollution – toxicity relations involved) in ecotoxicological bioassays: according to the catchment studied, the same rainfall event does lead to the same effect, thereby emphasising the importance of land use in the catchment. In parallel, for the same site, two successive rainfall events may not have the same hazard level.

Lastly, it is essential to underline here that when carrying out bioassays, it is very difficult and even impossible to establish a direct link between a pollutant and such an effect on the organism. Chemical reactions take place all the time in cells and there are an infinite number of interactions between xenobiotics and biological material and above all between xenobiotics with each other. A large number of studies have highlighted the existence of synergies, antagonisms and additive effects when organisms are exposed to different pollutants.

CONCLUSION

The results obtained in this work confirm the interest of bioassays for detecting disturbances caused by discharges into the environments receiving them.
Nonetheless, as with physicochemical analyses, they fail to provide a real time assessment of the quality of the discharge in the site studied. Biosensors are alone in being able to satisfy the need to

monitor sites. Miniaturised, reliable and inexpensive, they show the path to be taken in the future for monitoring sites at risk.
However, although promising, improvements must still be made to these tools before they can be used for such monitoring.

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