DEVELOPMENT OF AN ALGAL BIOSENSOR FOR TOXICITY ASSESSMENT OF URBAN WET WEATHER EFFLUENTS

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Abstract
Stormwater runoff from urban areas generates various pollutants (suspended solids, heavy metals, polycyclic aromatic hydrocarbons, nutrients...) This cocktail of pollutants can contribute to adverse quality of receiving water bodies (urban streams, ground water). In order to preserve aquatic ecosystems it is therefore necessary to evaluate quality and toxicity of wet weather effluents.
In this work dry and wet weather effluents were sampled and different ecotoxicological assays based on enzymatic activity measurementwere performed. Results of bioassays with free algae were compared to results obtained with a conductometric biosensor. Differences of results between both toxicity evaluation methods are linked to the chemical composition of effluents and the sensibility of the chosen test. This study shows the ability of the sensor to evaluate toxicity of urban effluents and its interest in environmental pollution monitoring.

Keywords
biosensors; environmental monitoring; storm water

INTRODUCTION

Urban wet weather effluents (UWWE) consist of all water discharged from sewage treatment plants, sewer overflows and stormwater outfalls during a storm event and the time period that follows it, the sewerage sanitation have not yet found a nominal operation of dry weather (Chocat, Bertrand-Krajewski, et al., 2007). Many studies have shown the contamination of these effluents by various compounds: heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), pesticides, organotins, volatile organic compounds, chlorobenzenes, phthalates and alkylphenols (Eriksson, Baun, et al., 2007; Gasperi, Garnaud, et al., 2008; Jartun and Pettersen, 2010; Karlsson, Viklander, et al., 2010). These periodic discharges contribute to the chronic pollution of urban stream and ground water.

Although today contamination of these effluents is well known, few studies have been undertaken to evaluate their impact on aquatic ecosystems/receiving water bodies. Environmental monitoring of pollutants with automatic systems, applied online and allowing rapid response is one of the best ways to control the quality of the environment. Real time analysis offers the advantage of detecting rapidly the presence of pollutants before they cause any damages. Such a strategy is only possible through biosensors.

In this work, algal whole cell based biosensor has been used to assess toxicity of urban wet or dry weather effluents, which flow in urban river or retention tanks. Samples came from an overflow device and two retention/infiltration tanks located near Lyon (France). At the same time, free algae bioassays have been carried out and results were compared.

METHOD AND MATERIALS

Algal cultures

Experiments were carried out with *Chlorella vulgaris*, a freshwater strain. *C. vulgaris* (Chlorophycea) is a non motile unicellular green algae common in fresh water. This strain is often used in ecotoxicology thanks its ecological advantages, in particular *C. vulgaris* can accumulate large quantities of pollutants. The *C. vulgaris* strain (CCAP 211/12) was purchased from The Culture Collection of Algae and Protozoa at Cumbria, United Kingdom. The algal strain was grown in the culture medium (AFNOR T90-304, 1980) with a photoperiod of 16h light/8h dark.

Sampling sites
Three different sampling sites have been chosen according to the occupation of watershed.

- The first site, located in Ecully at the west of Lyon is an overflow device which discharges effluents in a suburban stream. The sewer system is combined; the area occupation is mainly residential, covering a surface of 245 ha of about 7,000 people equivalent and impervious to 45%;

- The second site, located in Chassieu at the east of Lyon and nicknamed "Django Reinhardt", is a retention/infiltration tank which collects water from an area of primarily industrial land use. It covers a surface of 185 ha with a total impervious about 72%;

- The last site, located in Bron at the east of Lyon, is a retention/infiltration tank which collects water from a lorry park of 3.5 ha.

Samples were collected by wet weather and dry weather for Chassieu and Ecully and only by wet weather on Bron site.

Table 1:characteristics of sampling campaigns

Site	Chassieu	Bron		Ecully
Occupation of watershed	Industrial	Lorry park		Residential
Dry weather samples	sample (1) 03/03/2011	/		sample (5) 03/03/2011
Analyses	Ions, metals & organics	/		Ions, metals & organics
Wet weather samples	sample (2) 13/07/2011	Sample (3) 13/07/2011	Sample (4) 19/10/2011	sample (6) 24/02/2011
Analyses	Ions & metals	Ions & metals	Ions, metals & organics	Ions & metals

Analytical methods
Ions: levels of NO^{3-};PO_4^{2-}; $SO_4^{2-}$and K^+ were determined by ionic chromatography (IC, Thermo Scientific Dionex DX-100);
Metals: levels of Pb, Ni, Cu and Zn were determined by Atomic absorption spectroscopy (AAS, Hitachi Z-8200);
Organics: Gas chromatography associated to time-of-flight mass spectrometry (GC-ToF, AGILENT 6890N) and high performance liquid chromatography associated to tandem mass spectrometry (LC-MS/MS, AGILENT 100/1200 for LC and ABSciex/3200 QTRAP for MS).

Toxicity measurements
<u>Bioassays</u>
Bioassays were carried out in 48 wells microplates with free algae; they were based on fluorescence measurements (Fluostar BMG) linked to the enzymatic activity. Methylumbelliferyl Phosphate (MUP), dissolved at different concentrations (between 2 and 18 µM) in a Tris– HCl (0.1M, pH 8.4) buffer solution was used as substrate of the alkaline phosphatase enzymatic reactions. Methylumbelliferone (MUF), the fluorescent product of the reaction, was detected within fluorescent parameters (λexcitation =365nm and λemission =460 nm) (Durrieu, Badreddine, et al., 2003). For each substrate concentration, three replicates were carried out.

For bioassays, 48 wells microplates were filled with algal solution. After sedimentation, culture medium could be removed and replaced by test solution (or algal culture medium for control). Exposures last 2h, 24h and 48h. After removing the test solution and resuspending algae in distilled water, fluorescence measurements were carried out. K_m and V_m values were determined using

Lineweaver-Burk linearization (Cornish-Bowden, Jamin, et al., 2005). The ratio K_m/V_m, specific time of enzyme, is used as a parameter representative of enzymatic activity (the specific time is the time required to consume all the substrate). Inhibition rate of APA is defined as:

$$I(\%) = \frac{\frac{K_m}{V_m}_{test} - \frac{K_m}{V_m}_{control}}{\frac{K_m}{V_m}_{test}} * 100 \quad (1)$$

Biosensors

The conductometric transducers were fabricated at the Institute of Chemo-and Biosensors (Munster Germany) (Trebbe, Niggemann, et al., 2001). Two pairs of Au (150 nm thick) interdigitated electrodes were made by the lift-off process on the Pyrex glass substrate. The Cr intermediate layer of 50 nm thick was used to improve adhesion of Au to substrate. Central part of the sensor chip was passivated by AlO_3 layer to define the electrodes working area. Both the digits width and interdigital distance were 20 nm and their length was about 1mm. Thus, the sensitive part of each electrode was about 1,5 mm^2. Measurements are based on the detection of solution conductivity variation inside algal cells immobilized. The technique of immobilization consists in the formation of a self-assembled monolayer of algae (SAM) (Guedri and Durrieu, 2008).
Measurements were carried out under daylight at room temperature for Alkaline Phosphatase Activity (APA) in a 2 ml glass cell filled with buffer (Tris-HCl, 0.1 M, pH 8.4). The biosensor was preincubated in a test solution (or in algal culture medium for control) for 2h, 24h and 48h. After washingbiosensors were immersed in this vigorously stirred solution. After stabilisation of the output signal, 10 µl of the substrat (paranitrophenyl phosphate) were added into the vessel. The differential output signal (dS) was registered using a "home made" conductometric laboratory;dS control and dS test were compared and the inhibition rate was calculated as following:

$$I(\%) = \frac{dS_{control} - dS_{test}}{dS_{control}} * 100 \quad (2)$$

RESULTS AND DISCUSSION

Results are expressed as percent of control before exposure. They are expressed in terms of percentage of inhibition, i.e. the percentage of activity compared to a control treated under the same conditions of the test, but with the sample replaced by algal culture medium.
Annual average environmental quality standards (AA-EQS) are definite according to Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008.

Samples from Chassieu

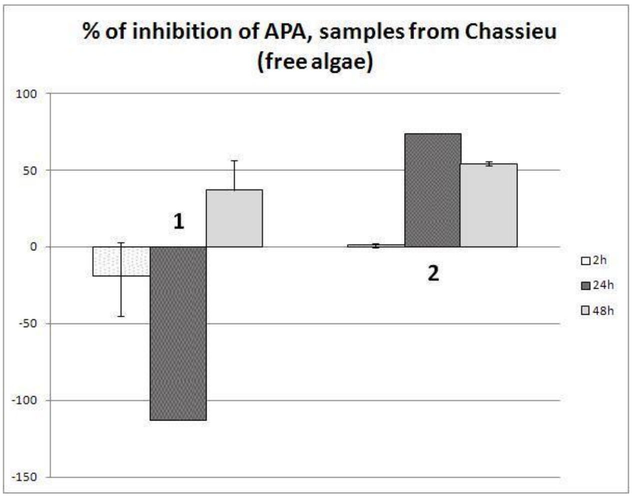

Figure 1: results of bioassay after exposure to sample (1) and (2)

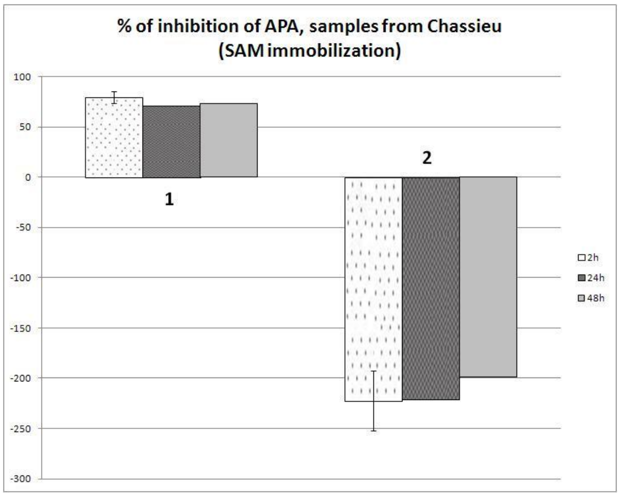

Figure 2: results of biosensors after exposure to sample (1) and (2)

Table 2: ions and metals concentration for dissolved fraction of sample (1) and (2)-comparison with AA-EQS

References	**UDWE Chassieu (1)**	**UWWE Chassieu (2)**	**AA-EQS**
Date	03/03/2011	13/07/2011	
Ions	Dissolved fraction		
NO_3^- (mg/l)	16,1	6,5	
PO_4^{2-} (mg/l)	0,5	<0,1	
SO_4^{2-} (mg/l)	38	3,3	
K^+ (mg/l)	5,7	1,3	
Metals	Dissolved fraction		
Pb (µg/l)	< 2	< 2	7,2
Ni (µg/l)	20,13	2,89	20
Cu (µg/l)	3,22	9,41	
Zn (µg/l)	220	< 2	

Tableau 3: organics concentration for dissolved fraction and suspend solids (SS) of sample (1) - comparison with AA-EQS

References	**UDWE Chassieu (1)**		**AA-EQS**
Date	03/03/2011		
Organics	Dissolved (ng/l)	Particulate (ng/g) (SS = 1,63g/l)	
Atrazine	19,9	<38	600
Diuron	833	109,1	200
Isoproturon	82,9	6,1	300
Pentachlorophénol	12,1	22,6	400
Simazine	44,2	3,6	1000
Anthracène	70,90	<174	100
Fluoranthène	54,30	2394,90	100
Naphtalène	78,90	42,30	2400
Benzo (a) pyrène	<2,9	32,00	50
Benzo (b) fluoranthène	<1,7	94,00	Σ = 30
Benzo (k) fluoranthène	<1,7	24,40	
4-nonylphénol	21,30	396,70	300
Acenaphtene	101,50	195,10	
Fluorene	204,40	97,40	
Phenanthrene	440,60	6993,20	
Pyrène	30,80	1460,90	
Benzo (a) anthracène	<0,9	163,00	
Chrysène	<0,7	97,70	
Benzo (g,h,i) perylène	<10	58,50	Σ = 2

Bioassays

With free algae assays, inhibition of APA is around 50% with both samples after 48h of exposure.Chemical analyses show an important organic contamination of suspended solids by PAHs and dissolved matters by pesticides.

Biosensors

With dry weather effluent (sample 1) activity of the biosensor is strongly inhibited (more than 50%) and this, from 2h to 48h of exposure. This inhibition can be related to the presence of Ni (220 µg/l), pesticides (833 µg/l) and PAHs in suspended solids (up to 7 ppm). With wet weather effluent (sample 2) APA is strongly enhanced. In previous works we have shown that small amount of heavy metal ions (< 100 µg/l) enhance algal APA (Chouteau, Dzyadevich, et al., 2005). In the sample 2 metal contamination is very small (inf. to EQS) and could explain the enhancement of APA.

With the biosensors there are important differences about enzymatic activities between algae exposed to sample 1 and algae exposed to sample 2 which not exist with bioassays. Biosensors are more sensitive than bioassays and could integrate chemical differences between the two samples.

Samples from Bron

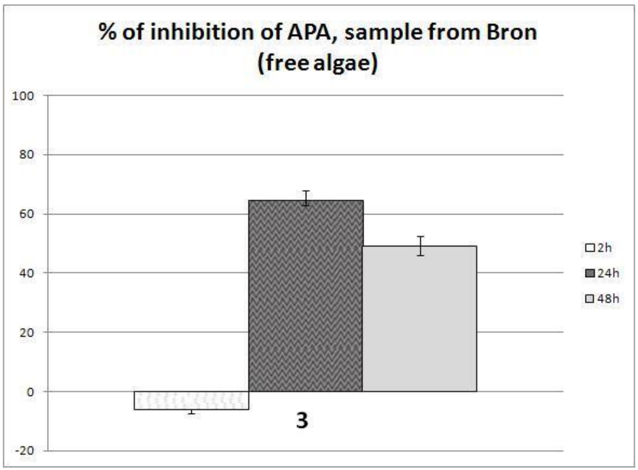

Figure 3: results of bioassays after exposure to sample (3)

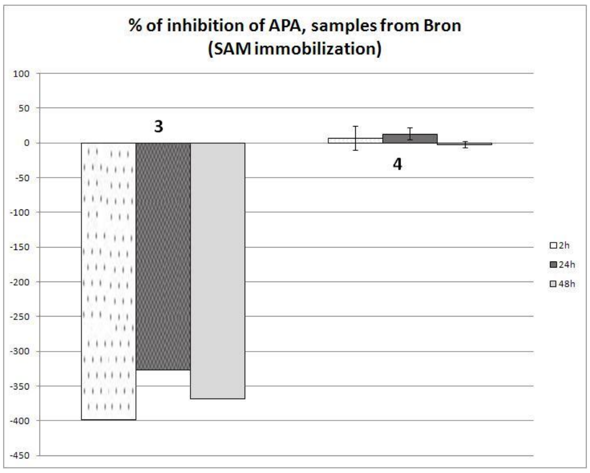

Figure 4: results of biosensors after exposure to sample (3) and (4)

Table 4:ions and metals concentration for dissolved fraction of sample (3) and (4)-comparison with AA-EQS

Reference	**UWWE Bron (3)**	**UWWE Bron (4)**	**AA-EQS**
Date	13/07/2011	19/10/2011	
Ions	Dissolved fraction		
NO_3^- (mg/l)	<0,1	/	
PO_4^{2-} (mg/l)	<0,1	/	
SO_4^{2-} (mg/l)	1,6	/	
K^+ (mg/l)	6,5	/	
Metals	Dissolved fraction		
Pb (µg/l)	< 2	< 2	7,2
Ni (µg/l)	< 2	< 2	20
Cu (µg/l)	4,04	15,42	
Zn (µg/l)	< 2	< 2	

Table 5:organics concentration for dissolved fraction and suspend solids (SS) of sample (4) - comparison with AA-EQS

Reference	**UWWE Bron (4)**		**AA-EQS**
Date	19/10/2011		
Organics	Dissolved (ng/l)	Particulate (ng/g) SS=0,023g/l	
Fluoranthène	13	12290	100
Naphtalène	16	780	2400
Benzo (a) pyrène	<2,9	5660	50
Benzo (b) fluoranthène	<1,7	16565	Σ = 30
Benzo (k) fluoranthène	<1,7	7110	
Indéno (1, 2, 3) pyrène	<10	6252	Σ = 2
4-nonylphénol	<0,1	2864	300
Para-ter-octylphénol	<4,3	1049	100
Phenanthrène	8,3	3250	
Pyrène	12,3	11321	
Benzo (a) anthracène	<0,9	6574	
Chrysène	<0,7	8846	
Benzo (g,h,i) perylène	<10	4563	

<u>Bioassays</u>

With sample 3, free algae assays show an inhibition of APA around 50% after 24h and 48h of exposure.

<u>Biosensors</u>

Contrary to bioassays, when they are exposed to sample 3, biosensors respond as previously (with sample 2). The sample 3 enhances APA with biosensors, probably because of the small metal concentrations. With sample 4 APA of biosensors is almost the same as the control. According to the land occupation, suspended solids of the sample 4 are strongly contaminated by PAH's (more than 10 ppm) but suspended solids concentration is very small (23 mg/l) and the dissolved fraction presents only few contamination. That's why APA is not influenced by the sample 4.

Samples from Ecully

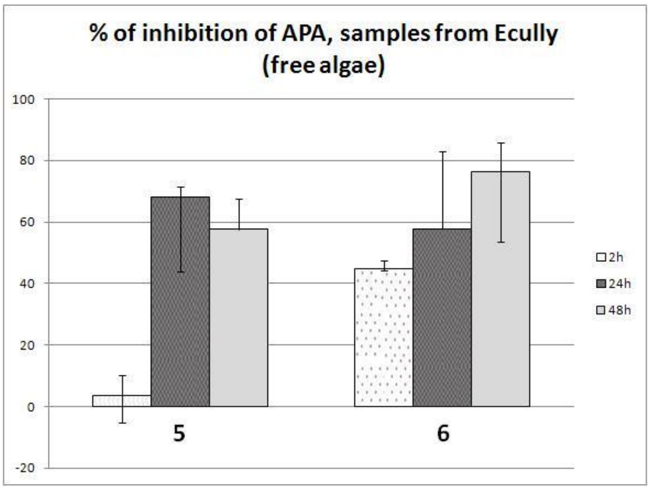

Figure 5: results of bioassay after exposure to sample (5) and (6)

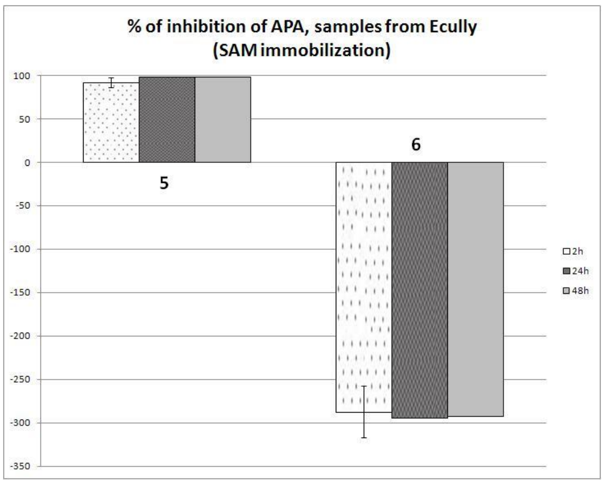

Figure 6: results of biosensors after exposure to sample (5) and (6)

Table 6:ions and metals concentration for dissolved fraction of sample (5) and (6)-comparison with AA-EQS

Reference	**UDWE Ecully (5)**	**UWWE Ecully (6)**	**AA-EQS**
Date	03/03/2011	24/02/2011	
Ions	Dissolved fraction		
NO_3^- (mg/l)	134	108,5	
PO_4^{2-} (mg/l)	10,7	6,5	
SO_4^{2-} (mg/l)	52,3	30,8	
K^+ (mg/l)	17,8	11,3	
Metals	Dissolved fraction		
Pb (µg/l)	< 2	< 2	7,2
Ni (µg/l)	< 2	<2	20
Cu (µg/l)	26,78	18,92	
Zn (µg/l)	< 2	< 2	

Table 7: organics concentration for dissolved fraction and suspend solids (SS) of sample (4) - comparison with AA-EQS

Reference	**UDWE Ecully (5)**		**AA-EQS**
Date	03/03/2011		
Organics	Dissolved (ng/l)	Particulate (ng/g) SS=0,36g/l	
Atrazine	2,9	<38	600
Diuron	25	<39	200
Simazine	2,8	<45	1000
Fluoranthène	<0,5	58,60	100
Naphtalène	35,00	48,60	2400
Phenanthrene	9,50	141,90	

<u>Bioassays</u>

Free algae assays are quite the same with sample 5 and sample 6. For both there is a strong inhibition of APA after 24h of exposure, and this from 2h for sample 6.

<u>Biosensors</u>

Results obtained with biosensors give the same results as those obtained with samples collected on Chassieu site, i.e. an inhibition of APA with dry weather sample and an enhancement with wet weather sample.

CONCLUSION

In this work, inhibition of enzymatic activities can be considered as efficient early toxicity signals of the presence of pollutants in samples. Bioassays with free algae already give us information about contamination of test sample and about its toxicity. However these results could be improved: the sensibility is low and assays could not be undertaken on the field. Responses of biosensors change relative to bioassays, these differences are linked to the chemical composition of effluent and the sensibility of the biosensor.

This study allowed us to test the reaction of whole cell conductometric biosensor with real complex effluent. To date this biosensor was able to detect heavy metal as cadmium with detection limit of 1 ppb and with good repeatability. Thanks to these experiments we have shown the capacities of the sensor to detect dangerousness of urban effluents and even more, to make the difference between dry weather effluents and wet weather effluents.

The next step is the implementation of these biosensors *in situ* for the monitoring of urban effluents and the development of early warning systems.

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