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Antoine Gosset

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**Evaluation de l'écotoxicité des Rejets
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Développement d'une batterie de
bioessais et application à la conception
de biocapteurs**

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Résumé

Evaluation de l'écotoxicité des Rejets Urbains par Temps de Pluie : Développement d'une batterie de bioessais et application à la conception de biocapteurs

Les Rejets Urbains par Temps de Pluie (RUTP) représentent une pollution très complexe et variable de par la diversité des événements pluvieux et des bassins versants lessivés. Les RUTP sont, dans la majorité des cas, rejetés dans des milieux récepteurs aquatiques péri-urbains tels que les lacs, rivières ou eaux souterraines, sans traitement d'épuration. La pollution déversée, qui peut être très diluée, est le plus souvent liée à des événements relativement courts et difficiles à prévoir. L'impact écotoxique des RUTP peut donc s'avérer difficile à évaluer, en particulier par des mesures directes *in situ*.

Parmi les organismes utilisés en écotoxicologie, les microalgues sont extrêmement intéressantes. En effet, elles constituent la base des réseaux trophiques, sont sensibles à une large gamme de polluants et sont très sensibles à la présence de substances exogènes.

C'est la raison pour laquelle nous avons développé dans un premier temps une batterie de biomarqueurs cellulaires sur microalgue (perturbation de la physiologie (comme la photosynthèse) de *Chlorella vulgaris*), afin de montrer leur intérêt pour détecter rapidement et sensiblement l'impact toxique d'échantillons de RUTP collectés sur la région Lyonnaise. La réponse de ces biomarqueurs a été comparée en laboratoire à une batterie de bioessais écotoxicologiques monospécifiques classiques sur microalgues et microcrustacés (e.g. essais de croissance, reproduction).

Dans un second temps, nous avons travaillé à l'adaptation de ces biomarqueurs afin de créer des outils de détection *in situ*. Des biocapteurs à cellules entières, basés sur une mesure de la perturbation de la photosynthèse (fluorescence chlorophyllienne) de microalgues, ont été développés. Pour leur création, deux techniques de mise en contact bio-récepteur/transducteur ont été testées : (i) la double encapsulation des microalgues dans des hydrogels alginate/silice utilisant un procédé sol-gel, et (ii) l'inclusion des microalgues dans des puces microfluidiques fabriquées par xurographie. Une station portable autonome de terrain a été élaborée et testée avec efficacité pour effectuer des mesures *in situ* de la toxicité des RUTP, et des milieux aquatiques urbains contaminés.

Ce travail de thèse présente de nombreuses perspectives concernant une meilleure connaissance de l'impact des RUTP sur les organismes aquatiques. Il apporte également des réponses à la problématique du développement des biocapteurs à cellules algales entières pour la surveillance environnementale.

Mots-clefs : Rejet Urbain par Temps de Pluie, Microalgue, Bioessai, Biocapteur, Fluorescence chlorophyllienne, Hydrogel, Microfluidique.



Abstract

Ecotoxicological evaluation of Urban Wet-Weather Discharges: Development of a bioassay battery and application to the biosensor design

Urban Wet-Weather Discharges (UWWD) represent a very complex and variable pollution due to the diversity of the rainfall events and leached watersheds. In most cases, UWWD are released without, or after very low treatments only, in suburban aquatic environments such as lakes, rivers or groundwaters. Spilled pollution is often linked to relatively short and difficult to predict events, and can be very diluted. Thus, it may be complicated to assess the ecotoxic impact of UWWD, and particularly *in situ*.

Among the organisms used in ecotoxicology, microalgae are particularly interesting because they are the basis of trophic networks, are sensitive to a wide range of pollutants found in UWWD and can react very quickly to their presence.

It's why, first, we developed and used a battery of microalgae (*Chlorella vulgaris*) cellular biomarkers (e.g. photosynthesis disturbance) to demonstrate their utility in detecting the toxic impact of UWWD samples collected in the Lyon city area in a rapid and sensitive way, in comparison with a battery of monospecific bioassays on microalgae and microcrustaceans (e.g. growth or reproduction inhibition assays).

In a second phase, we worked on the adaptation of these biomarkers for the creation of ecotoxicological field tools, biosensors. We developed two types of whole-cell biosensors based on the microalgal photosynthesis (chlorophyll fluorescence) disturbance. We particularly worked on the microalgal immobilization processes in the aim to design biosensors, using mainly two techniques: a double-encapsulation in alginate/silica hydrogels using a sol-gel process and an inclusion in microfluidic chips created by xurography. Finally, we were able to create an autonomous portative biosensor able to conduct *in situ* measurements of UWWDs toxicity and contaminated urban aquatic environments in general.

This thesis project presents many perspectives concerning the deeper understanding of the UWWDs impact on aquatic organisms, and also on the development of whole-cell biosensors for the environmental monitoring.

Key-words: Urban Wet-Weather Discharge, Ecotoxicology, Microcrustacean, Microalgae, Bioassay, Biomarker, Biosensor, Chlorophyll fluorescence, Hydrogel, Microfluidic, Xurography.



Liste des publications et communications

Communication dans des journaux internationaux à comité de lecture

Durrieu, C., Ferro, Y., Perullini, M., **Gosset, A.**, Jobbágy, M., Bilmes, S. A. (2016). Feasibility of using a translucent inorganic hydrogel to build a biosensor using immobilized algal cells. *Environmental Science and Pollution Research*, 23(1), 9-13.

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Wigh, A., Devaux, A., Brosselin, V., Gonzalez-Ospina, A., Domenjoud, B., Aït-Aïssa, Creusot, N., **Gosset, A.**, Bazin, C., Bony, S. (2016). Proposal to optimize ecotoxicological evaluation of wastewater treated by conventional biological and ozonation processes. *Environmental Science and Pollution Research*, 23(4), 3008-3017.

Gosset, A., Durrieu, C., Orias, F., Bayard, R., Perrodin, Y. (2017) Identification and assessment of ecotoxicological hazards attributable to pollutants in urban wet weather discharges. *Environmental science: Processes & Impacts*, 19, 1150–1168.

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Liste des abréviations

ADN : Acide désoxyribonucléique
AFC : Antifouling compound
APX : Ascorbate peroxydase
ARN : Acide ribonucléique
ATP : Adénosine triphosphate
BSA : Bovine serum albumine
CAT : Catalase
CSO : Combined sewer overflow
DBO : Demande biologique en oxygène
EC_x : Effect concentration (Concentration leading to x % of effect on the studied organism)
EDCs : Endocrine disruptive compounds
ELISA : Enzyme-linked immuno assay
HC : Herbicides
HM : Heavy metals
HQ : Hazard quotient
IPE : Impact des polluants sur les écosystèmes
LEHNA : Laboratoire d'écologie des hydrosystèmes naturels et anthropisés
LOEC : Lowest observed effect concentration
MDA : Malondialdehyde
MEC : Measured environmental concentration
NOEC : No observed effect concentration
OLED : Organic light-emitting diode
OPD : Organic photodetector
OTHU : Observatoire de terrain en hydrologie urbaine
PDMS : Polydiméthylsiloxane
PEHD : Polyéthylène haute densité
PNEC : Predicted no effect concentration
PSI : Photosystème I
PSII : Photosystème II
ROS : Reactive oxygen species
RQ : Risk quotient
RUTP : Rejets urbains par temps de pluie
RUTS : Rejets urbains par temps sec
SAMs : Self-assembled monolayers

SOD : Superoxyde dismutase

TBA : Thiobarbituric acid

TCA : Trichloroethanoic acid

Introduction générale

Les écosystèmes aquatiques sont des milieux fragiles. Ils sont soumis à de fortes pressions anthropiques, et plus particulièrement en milieu urbain où la forte densité des populations génère une source majeure de pollution. Dans ce contexte de forte pression, la Directive Cadre sur l'Eau (Directive n° 2000/60/CE) fixe comme objectif la gestion et la protection des eaux superficielles (eaux dulçaquicoles et eaux côtières) et souterraines. Pour cela, elle prévoit une surveillance du bon état écologique et chimique des masses d'eaux.

Les eaux pluviales sont l'une des sources majeures de pollution urbaine. En temps de pluie, ces eaux vont ruisseler sur les différentes surfaces urbaines (routes, toitures, sols enherbés, ...) et emporter la pollution déposée sur ces surfaces. Ainsi, toutes les eaux qui, par temps de pluie, rejoignent les milieux aquatiques récepteurs sont communément appelées Rejets Urbains par Temps de Pluie (RUTP). Cette pollution est à la fois complexe à gérer et son impact toxique difficile à évaluer, du fait de son caractère épisodique, de sa variabilité spatio-temporelle (surface lessivée, volume rejeté, concentration finale des polluants) et de ses traitements relativement limités. Bien qu'il s'agisse d'une pollution épisodique et ponctuelle, elle peut induire des perturbations importantes dans le fonctionnement des écosystèmes.

Il est donc nécessaire de trouver des méthodes de détection de l'impact toxique des RUTP qui permettent d'apporter une réponse sensible et rapide pour ce type de pollution. Parmi les organismes de référence utilisés dans les études écotoxicologiques, les microalgues sont couramment mises en œuvre du fait de leur grande sensibilité (réponse à de faibles concentrations en polluants et rapidité de réponse) à une large gamme de polluants présents dans les RUTP. De plus, elles se situent à la base des chaînes trophiques et elles présentent l'avantage d'être très faciles à cultiver. Les différents bioessais algaux permettent de détecter précocement des perturbations pouvant potentiellement se répercuter sur l'ensemble de la chaîne alimentaire. De plus, il est crucial de pouvoir développer des méthodes de suivis *in situ* des RUTP de manière à surveiller la qualité des cours d'eaux récepteurs. Les biocapteurs, initialement dédiés au domaine biomédical, apportent une réponse satisfaisante car ils ont depuis une vingtaine d'années été appliqués avec succès aux études environnementales. Cela a permis d'obtenir des outils sensibles pour la détection rapide, et à faible coût, de polluants. Parmi les différentes familles de biocapteurs, les biocapteurs à cellules entières permettent à la fois la détection de contaminants et l'observation d'un impact écotoxique sur les organismes utilisés. Cela en fait de très bons outils d'alerte précoce. En résumé, les biocapteurs à cellules entières, et notamment ceux utilisant des microalgues, permettent d'adapter des bioessais développés en laboratoire à la création d'outils écotoxicologiques de terrain.

L'objectif de cette thèse s'inscrit dans une démarche d'amélioration de la connaissance des effets écotoxicologiques des RUTP, et de prévention des dangers liés au déversement de polluants

contenus dans ces rejets. Les attendus scientifiques sont : (1) le développement et l'application de bioessais sur microalgues (e.g. perturbation de la photosynthèse ou d'activités enzymatiques) permettant de détecter de manière précoce un impact des RUTP, (2) le développement de biocapteurs sensibles pour la détection de la toxicité des RUTP *in situ*, à l'aide des biomarqueurs algaux développés en laboratoire (e.g. suivi de la perturbation de la photosynthèse de microalgues immobilisées). Ce travail s'attache ainsi à proposer un outil prédictif pour les gestionnaires du milieu aquatique (collectivités territoriales ou entreprises privées devant gérer les eaux pluviales).

Le présent manuscrit se compose de quatre parties. La première partie (divisée en 3 sous-parties) est constituée d'un état de l'art concernant l'impact écotoxique des RUTP et leurs méthodes d'étude :

- (i) Description des méthodes de caractérisation du danger écotoxique des micropolluants présents dans les RUTP, prenant en compte la concentration et la toxicité des molécules en jeu ;
- (ii) Description des méthodes (e.g. indices biologiques, bioessais, modélisation, SIG, biocapteurs) actuellement employées pour étudier l'impact des RUTP sur la biocénose des écosystèmes aquatiques récepteurs ;
- (iii) Présentation de l'état de l'art des différents biocapteurs (organismes, méthode de détection, polluants/effets toxiques détectés) utilisés en écotoxicologie afin d'évaluer *in situ* l'effet des RUTP.

La deuxième partie de ce manuscrit présente la méthodologie générale employée au cours de ce travail de thèse afin de répondre à la question posée : sites d'étude, rejets prélevés, bioessais et biomarqueurs développés, principe général et conception des biocapteurs utilisés au cours de ce travail.

La troisième partie est dédiée à la présentation des résultats obtenus aux cours des expérimentations menées. Cette dernière est subdivisée en 2 sous-parties.

- (i) Bioessais sur microalgues développés et utilisés pour étudier la toxicité des RUTP prélevés ;
- (ii) Développement et validation de biocapteurs optiques en laboratoire et leur application pour le suivi de la toxicité des RUTP (biocapteurs à hydrogels et systèmes microfluidiques pour application à des biocapteurs).

Pour conclure ce manuscrit, une quatrième partie propose une discussion générale des résultats obtenus, les avantages et limites des méthodes choisies, ainsi que les perspectives qui s'en dégagent.

Une part substantielle de ce travail est présentée sous forme d'articles scientifiques publiés, soumis ou en préparation. Dans l'objectif de faciliter la lecture, une introduction et une conclusion générale reprenant les principaux résultats sont dans ce cas insérées en début et fin des parties concernées.

CONTEXTE SCIENTIFIQUE ET FINANCIER DE LA THESE

Cette thèse s'inscrit dans une des thématiques de l'équipe IPE (Impact des Polluants sur les Ecosystèmes) du laboratoire LEHNA (Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés). Son objectif vise à caractériser l'impact des polluants urbains/des matrices complexes polluées sur les écosystèmes receveurs telles que les rivières, les nappes ou les lacs.

Cette étude s'inscrit dans la démarche des actions menées par l'OTHU (Observatoire de Terrain en Hydrologie Urbaine), et a donc été en partie financée par cet organisme. Cet observatoire a pour but d'observer les rejets urbains, leurs qualités physico-chimiques et leurs impacts sur les milieux récepteurs de la région Lyonnaise, afin de réfléchir à des méthodes visant à l'amélioration de la gestion de ces effluents.

Ce travail de thèse a été financé par :

- Le Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation,
- L'Observatoire de Terrain en Hydrologie Urbaine (OTHU) (pour l'aide à la collecte et à l'analyse physico-chimique et biologique des RUTP),
- Le CNRS via Projet Exploratoire Premier Soutien (PEPS) « Algues-On-Chip » (pour la conception des biocapteurs microfluidiques),
- Le programme ECOS Sud (pour la conception des biocapteurs à hydrogels).



1 Etude Bibliographique

Cette première partie est un état de l'art de l'impact écotoxicologique des Rejets Urbains par Temps de pluie. Outre la description des dangers encourus, les méthodes d'étude des impacts observés sont détaillées. La première sous-partie dresse un état des lieux des polluants analysés dans ces rejets et détermine leurs dangers écotoxicologiques (basés sur leur toxicité et sur les concentrations retrouvées dans les RUTP) pour les organismes aquatiques d'eau douce à l'aide d'une méthode de calcul de quotients de danger. La seconde sous-partie présente une étude critique des méthodes de détermination de l'impact des RUTP sur les écosystèmes aquatiques. Il s'agit d'identifier les problématiques actuelles, ainsi que les perspectives de recherche (e.g. le développement de biocapteurs) pour détecter de manière sensible et réaliste leur toxicité. La troisième sous-partie est un état des lieux sur le développement de biocapteurs dédiés à la détection des polluants et aux mesures d'écotoxicité.

1.1 Danger écotoxicologique des rejets urbains par temps de pluie

1.1.1 Contexte et enjeux

Dans un premier temps, nous avons caractérisé et quantifié le danger représenté par les RUTP sur la base de l'identification et la quantification des principaux polluants présents dans ces rejets. Les RUTP sont des rejets urbains déversés massivement dans le milieu naturel, généralement sans traitement préalable. De plus, comme ils sont de nature variable en termes de type de polluants et de concentration dans le temps et l'espace, il est donc d'autant plus important de caractériser le danger qu'ils représentent pour l'écosystème. Pour cela, nous avons réalisé une synthèse bibliographique des connaissances sur les polluants présents dans les RUTP. Nous avons analysé séparément les données des RUTP issus des réseaux d'eaux pluviales strictes des villes et ceux issus des réseaux unitaires (déversoirs d'orage) mélangeant eaux pluviales et eaux usées. Dans un second temps, nous avons recherché les données d'écotoxicité disponibles dans les bases de données scientifiques sur les polluants majoritaires (Valeurs de PNEC : Predicted No effect Concentration). En l'absence de données sur certains polluants, les valeurs de PNEC ont été déterminées. Pour cela, nous avons utilisé les valeurs d'écotoxicité référencées dans la littérature et avons appliqué un facteur de sécurité à la concentration la plus faible (e.g. NOEC, LOEC, EC_{10,50}). Cette détermination s'est basée sur différents paramètres tels que le nombre d'espèces étudiées ou la chronicité des essais écotoxicologiques réalisés, ceci en nous référant aux lignes directrices européennes. Nous avons créé un quotient de danger (HQ) pour chacune de ces valeurs liant PNEC et concentration la plus élevée de chaque polluant (cas du pire scénario, basé sur le principe de précaution), afin d'en déterminer leurs dangers écotoxicologiques respectifs. Dans ce travail, les

données issues des rejets d'eaux pluviales strictes sont séparées de celles des surverses de déversoirs d'orage, compte tenu des origines et natures différentes des sources de pollution.

1.1.2 Article 1 – *Identification and assessment of ecotoxicological hazards attributable to pollutants in urban wet weather discharges.*

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PAPER



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Identification and assessment of ecotoxicological hazards attributable to pollutants in urban wet weather discharges†

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Urbanization has led to considerable pressure on urban/suburban aquatic ecosystems. Urban Wet Weather Discharges (UWWD) during rainfall events are a major source of pollutants leached onto and into urban surfaces and sewers, which in turn affect aquatic ecosystems. We assessed the ecotoxicity of the different compounds identified in UWWD and identified the hazard represented by each of them. To this end, hazard quotient (HQ) values were calculated for each compound detected in UWWD based on their predicted no effect concentration (PNEC) values and their maximum measured effluent concentrations (MEC_{max}) found in the dissolved part of UWWD. For the 207 compounds identified in UWWD, sufficient data existed for 165 of them to calculate their PNEC. The ecotoxicity of these compounds varied greatly. Pesticides represented a high proportion of the wide variety of hazardous compounds whose HQ values were calculated (94 HQ values), and they were among the most hazardous pollutants (HQ > 1000) transported by stormwater. The hazard of combined sewer overflows (CSO) was linked mainly to heavy metals and pharmaceutical compounds. Consequently, the monitoring of these pollutants should be a priority in the future. The hazard level of certain pollutants could have been underestimated due to their adsorption onto particles, leading to their low concentration in the dissolved phase of UWWD. Hence, an in-depth study of these pollutants will be required to clarify their effects on aquatic organisms.

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Environmental impact

Urban Wet Weather Discharges (UWWD) constitute an important source of emerging pollutants and, thus, a risk for the quality of surface water, groundwater and seawater. It is, therefore, important to optimize their management. Our study concerns the conception of a ranking method of the hazards of pollutants in UWWD to identify those that need to be managed first. We used international data on chemical concentrations and ecotoxicity data (values of predictive no effect concentration) to calculate the hazard quotient linked to each pollutant detected in UWWD. Using this method, we demonstrated that some pollutants among pesticides (*e.g.*, pyrethroids), heavy metals and pharmaceutical compounds in UWWD must be managed first to avoid degradation of the receiving aquatic ecosystems.

1. Introduction

Urban Wet Weather Discharges (UWWD) are complex and variable polluted matrices, notably due to the different types of catchments leached by the rain, and the variable flows for different events.¹ Many “micropollutants” have been detected in these effluents as a function of population density and anthropogenic activities. The most important among them are: (i) European-priority substances such as polycyclic aromatic

hydrocarbons (PAH),^{2–5} diethylexylphthalate,^{4,6} 4-nonylphenol,^{4,6} bisphenol A and alkylphenoethoxylate;⁷ (ii) other micropollutants such as heavy metals (copper, lead, zinc), polychlorobiphenyls (PCB), pesticides, and phthalates (PHT);^{3,5,8–10} (iii) pharmaceutical compounds (PC) in the particular case of the combined sewer overflow (CSO).^{11,12} These micropollutants have also already been detected in receiving river water that has received these discharges.^{13,14} Many of these micropollutants are potentially hazardous for aquatic organisms. That is why UWWD have toxic effects on aquatic species,^{15–17} and can thus modify biological communities.^{15,18–21} Treatment to diminish UWWD pollutants through abatement is lacking or very limited depending on municipal sewer systems, and “raw” UWWD are often released into (sub)urban aquatic ecosystems. It is, therefore, necessary to optimize their management. This optimization can be based on: (i) better monitoring of toxic pollutants

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and their effects, including continuous monitoring with biosensors;^{22,23} (ii) specific treatment of pollutants;²⁴ (iii) the suppression or substitution at the origin of these pollutants (in particular, certain detergents, disinfectants and pharmaceuticals) by less toxic (but equally efficient) products. For optimization to succeed, it is necessary to identify and prioritize the hazardous pollutants present in UWWD.

To establish which compounds can cause ecotoxicological hazards represented by a specific urban effluent, several authors have proposed formulation and application of a “hazard quotient” (HQ) based on the ratio of the concentration of the selected pollutant in filtered polluted matrices/predicted no effect concentration (PNEC) of the molecules selected.^{25,26} This HQ is used to distinguish the hazards associated with the different compounds/families of compounds found in UWWD. Orias and Perrodin²⁵ proposed comparing the maximum measured effluent concentrations (MEC_{max}) measured in hospital effluents with their PNEC value, which was the method used in the present study. This method has also been applied by several other authors for contaminated matrices such as rivers polluted by raw/treated municipal wastewaters contaminated by various pharmaceuticals²⁶ or pesticides,²⁷ or lakes polluted by antibiotics.²⁸ The choice of using the MEC_{max} is to be use the worst-case (extreme conditions) scenario^{25,27} and to use a precautionary principle. This method was applied here, for the first time, to the UWWD.

Consequently, the main objectives of our study were to: (i) conduct a review of the ecotoxicity data available for the different compounds commonly detected in UWWD; (ii) assess the concentrations of these pollutants in UWWD by performing a literature review of UWWD pollutant compositions; (iii) calculate the potential hazard of these pollutants for aquatic ecosystems. Several studies have already proposed to identify and prioritize hazardous pollutants in UWWD.^{29–32} Nevertheless, they generally considered only the stormwater (no studies have been reported for the CSO). Moreover, these hazard assessments of pollutants were based only on the intrinsic properties of the pollutants (bioaccumulation, biodegradability, physicochemistry, ecotoxicity) and did not take into account the concentrations found *in situ*.

We present the PNEC values inventoried for all the pollutants identified in UWWD, and the MEC_{max} found in those discharges. Lastly, we calculated the HQ for each pollutant found in UWWD. We also chose to focus on combined and separate stormwater systems (named here as “CSO” and “stormwater” systems) due to the very different sources and types of pollutants they contain.

2. Methodology

All the pollutants used and analyzed (on the dissolved phase and particles phase of UWWD) in the present study were classified into families of chemical compounds, as detailed in Table 1. Scientific research articles focusing on identification of UWWD pollutants were obtained using databases such as the Web of Knowledge, Science Direct and Google Scholar using the key search terms “stormwater”, “CSO”, “UWWD”, “pollutants”,

and “chemicals”. The bibliography and citations of an identified article were also checked to find other suitable studies. Articles used in the present study were restricted to peer-reviewed research articles published between 1995 and 2016. Other scientific productions (*e.g.*, conference papers, non-peer reviewed procedures) were not retained for our study.

2.1 Obtaining and determining the PNEC values of micropollutants in UWWD

To define the hazard of the different pollutants identified in UWWD physicochemical studies, we first determined their ecotoxicity. Hence, PNEC values were found/calculated for all pollutants sought in physicochemical studies (in the particle phase and dissolved phase). In our study, PNEC values were obtained for all pollutants using a two-step approach. First, if they were available, PNEC values were obtained from free-to-use European databases^{33,34} (INERIS and AGRITOX) containing ecotoxicological data from the international scientific literature. Second, if PNEC values were not available in these databases, they were calculated using experimental ecotoxicological data found in the EPA ECOTOX database³⁵ following the method proposed by Orias and Perrodin,³⁶ and in agreement with the guidelines proposed by the EU (European Union) in the Technical Guidance Document (TGD)³⁷ (see Table 2). Using this method for each compound, bibliographical research using the EPA ECOTOX database allowed identification of all the bioassays performed, the trophic level present (decomposers, primary producers, primary consumers and secondary consumers) and the chronicity (or not) of the bioassay (*e.g.*, reproduction). Using all the ecotoxicity data, an extrapolation factor (EF; calculated following the criteria presented in Table S1†) was applied to the lowest toxicity values to establish the PNEC of a particular compound. No differentiation was made between the ecotoxicity data used (*e.g.* EC_{50} , EC_{10}) for the EF and PNEC calculation. However, several minor modifications in comparison with TGD were made. For example, ecotoxicity data on fungi were considered to be data on decomposers whereas data on periphyton were considered to originate from chronic bioassays on decomposers. If only acute ecotoxicity data were available, but not for all trophic levels, PNEC values were calculated using the ecological structure–activity relationship (ECOSAR) model by applying an EF to the lowest ecotoxicological value. In all cases, PNEC values were not calculated if at least one acute ecotoxicity value was available for each trophic level. The chronic algae and macrophyte bioassays did not permit lowering the EF if they were the only ecotoxicological data available. The EF was not reduced if rotifer (*Brachionus calyciflorus*) bioassays were the only tests performed on the trophic level of secondary consumers.³⁷ Finally, if PNEC and ecotoxicity data were not available in databases or the literature, PNEC values were estimated with modeled on ecotoxicological data obtained using the ECOSAR method.³⁸

Importantly, the authors of several studies did not indicate the speciation or isomer of the compounds measured. This was true for chromium, for which we chose to keep the two speciation forms (chromium III and chromium VI) because it has

Table 1 Families of compounds considered in this study and color codes associated in the various figures

Abbreviation	Family of pollutants	Figure colour code
ALK	Alkylphenols	Blue
FR	Flame retardants	Orange
M	Metals	Red
O	Others	Black
ORG	Organotins	Grey
P	Pesticides	Green
PAH	Polycyclic aromatic hydrocarbons and derivatives	Brown
PBDE	Polybrominated diphenyl ethers	White
PC	Pharmaceutical compounds	Cyan
PCB	Polychlorobiphenyls	Orange
PFAA	Perfluoroalkyl acids	Yellow
PHT	Phthalates	Grey
VOC	Volatile organic compounds	Purple

been reported that the annual concentrations of these two chromium species in rainwater samples are similar.³⁹ In the case of dichloroethane, we chose to keep the two isomers (1,1- and 1,2-dichloroethane), whereas the HQ was calculated twice for this pollutant using two PNEC values.

2.2 Obtaining the MEC_{max} of micropollutants

The second step for hazard assessment of UWWD pollutants was determination of the MEC_{max} for each of them. As described by Orias and Perrodin,²⁵ two approaches can be used to obtain the concentration of micropollutants in UWWD: (i) a theoretical method based on the calculation of the predicted effluent concentration (PEC) with the design of a scenario; (ii) an experimental approach based on analyses of the pollutants in the dissolved phase of the UWWD. The second method (MEC_{max} determination) is more realistic because it is based on real measured concentrations in UWWD, as opposed to the calculation of PEC values, which carry a high level of uncertainty due to the parameters used (*e.g.*, product consumption). Therefore, (ii) was selected (although not all the compounds were analyzed in the discharges).

We will discuss the different choices made to select concentration data (see Section 3.5). First, we considered studies providing concentration values for compounds only in the soluble phase (<1.2 μm). Indeed, ecotoxicity assays performed to calculate PNEC values for the different pollutants have relied only on pure liquid solutions. Moreover, evaluation of the bioavailability of the contaminants in the particulate phase for aquatic organisms was not possible. Additionally, taking into account the concentrations of particles could lead to overestimating the hazard (see Section 3.5). With regard to formulation of the HQ, we also chose to separate concentration values for separate and combined sewer systems, so we calculated the hazard for both cases. The hazards of these two systems differ because the source and type of pollutants (*i.e.*, the presence of pharmaceuticals in discharges from combined sewer overflows) are different.

For each compound sought in the dissolved part of the stormwater/CSO samples, the MEC of the events observed was reported for each study. The highest effluent concentration ever measured in UWWD (MEC_{max}) was determined by seeking the highest MEC reported previously with the aim to observe the worst-case scenario. In future studies, it would be interesting to undertake the same work using median and lowest values, but this approach would be limited because, for some compounds, only one concentration value was available in the scientific literature.

2.3 HQ calculation

For each compound detected in discharges (Section 2.2) with an available PNEC value (Section 2.1), we calculated the HQ using eqn (1):

$$HQ = MEC_{max}/PNEC \quad (1)$$

with MEC_{max} measured in UWWD in μg L⁻¹ and PNEC measured in μg L⁻¹.

It was considered that compounds with an HQ ≤ 1 are potentially hazardous for the environment, and that compounds with the highest HQ are the most hazardous for aquatic ecosystems. Compounds with an HQ < 1 were considered to be slightly hazardous.

In the present study, 207 compounds were identified in UWWD. We found, calculated or modeled a PNEC value representing the ecotoxicity for 165 of them. By seeking the dissolved proportion analyzed in UWWD, we were able to determine a MEC_{max} for only 61 compounds in stormwater and 49 in CSO. Consequently, we calculated 94 HQ values using eqn (1) to prioritize their hazard for freshwater ecosystems.

3. Results and discussion

3.1 Pollutant classification and available data

Pollutants analyzed in UWWD were classified as shown in Table 1. All the PNECs, concentrations and HQ data were analyzed as per the pollutant ranking presented in Table 1.

Table 2 Summary of ecotoxicity data and PNEC values formulated by calculation or modeling for 60 pollutants sought in UWWD (legend: D: decomposers; P: primary producers; 1C: primary consumers; 2C: secondary consumers; C: ecotoxicity data available for organism community bioassays; Bc: *Brachionus calyciflorus*; EF: extrapolation factor)

No. CAS	Compound	Family	Test species	Experimental ecotoxicity												Endpoint	Value ($\mu\text{g L}^{-1}$)	EF	PNEC ($\mu\text{g L}^{-1}$)	Ref.
				Trophic level						Chronic test										
				D	P	1C	2C	D	P	1C	2C	1C	2C	Specie	Parameter					
104-40-5	4-(<i>para</i>)-Nonyl-phenol	ALK	77 + C	0	5	19	36 + 17	0	5	6	11 + 17	(Bc)	Reproduction	LOEC	3.31×10^{-2}	10	3.31×10^{-3}	35		
80-46-6	4- <i>tert</i> -Pentyl-phenol	ALK	4	0	0	1	3	0	0	1	2		Development	LOEC	3.20×10^1	50	6.40×10^{-1}	35		
78-51-3	Tris(2-butoxyethyl)phosphate	FR	3	0	0	0	3	0	0	0	0		Mortality	NOEC	4.00×10^3	—	—	35		
7439-89-6	Iron	M	6	0	1	2	3	0	1	0	0		Mortality	LC ₅₀	5.60×10^2	1000	5.60×10^{-1}	35		
86-74-8	Carbazole	O	4	0	1	2	1	0	1	1	0		Mortality	LC ₅₀	9.30×10^2	50	1.86×10^1	35		
789-02-6	2,4'-DDT	P	4	0	0	0	4	0	0	0	2		Reproduction (fertility)	LOEC	2.30×10^{-1}	100	2.30×10^{-3}	35		
50-29-3	4,4'-DDT	P	40	0	11	21	8	0	11	5	0		Mortality	LC ₅₀	7.00×10^{-2}	50	1.40×10^{-3}	35		
309-00-2	Aldrin	P	55	0	0	18	37	0	0	0	0		Mortality	LC ₅₀	9.70×10^{-2}	—	—	35		
218-01-9	Chrysene	P	3	0	0	2	1	0	0	0	0		Mortality	LC ₅₀	1.70×10^3	—	—	35		
1319-77-3	Cresol	P	6	0	1	2	3	0	1	0	0		Immobilization	EC ₅₀	7.00×10^3	1000	7.00×10^0	35		
52315-07-8	Cypermethrin	P	68	0	6	28	33 + 1	0	6	7	2 + 1	(Bc)	Mortality	LOEC	1.00×10^{-4}	10	1.00×10^{-5}	35		
6190-65-4	Desethylatrazine	P	11	0	8	3	0	0	7	1	0		Mortality	NOEC	3.30×10^{-1}	50	6.60×10^{-3}	35		
1007-28-9	Desethylisimazine	P	10	0	7	2	1	0	7	0	0		Growth	NOEC	6.00×10^{-1}	1000	6.00×10^{-4}	35		
333-41-5	Diazinon	P	131	0	9	53	69	0	9	8	18		Reproduction	EC ₅₀	2.00×10^{-4}	10	2.00×10^{-5}	35		
60-57-1	Dieldrin	P	98 + C	0	7	43	45 + 1	0	7	2	1 + 1	(Bc)	Population growth	LOEC	1.00×10^{-3}	10	1.00×10^{-4}	35		
72-20-8	Endrin	P	89	0	1	22	66	0	1	0	1		Mortality	LC ₅₀	6.10×10^{-7}	50	1.22×10^{-8}	35		
2682-20-4	Methyl-sothiazolinone	P	3	0	0	1	2	0	0	0	0		Mortality	LC ₅₀	7.00×10^1	—	—	35		
51218-45-2	Metolachlor	P	63	0	37	12	14	0	35	6	5		Photosynthesis disturbance	LOEC	5.00×10^0	10	5.00×10^{-1}	35		
134-62-3	N,N-Diethyl-3-methylbenzamide	P	6	0	0	3	3	0	0	1	0		Growth	NOEC	6.00×10^{-1}	100	6.00×10^{-3}	35		
134-62-3	N,N-Diethyl- <i>meta</i> -toluamide	P	6	0	0	3	3	0	0	0	0		Growth	NOEC	6.00×10^{-1}	1000	6.00×10^{-4}	35		
26530-20-1	N-Octyl-sothiazolinone	P	6	0	0	1	5	0	0	0	1		Growth	NOEC	8.50×10^0	100	8.50×10^{-2}	35		

Table 2 (Contd.)

		Experimental ecotoxicity											Data used for PNEC calculation													
No. CAS	Compound	Family	Test species	Trophic level						Chronic test					Parameter	Endpoint	Value (µg L ⁻¹)	EF	PNEC (µg L ⁻¹)	Ref.						
				D	P	1C	2C	D	P	1C	2C	1C	2C	2C												
52645-53-1	Permethrine	P	4	0	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	LC ₅₀	3.00 × 10 ⁻²	1000	3.00 × 10 ⁻⁵	38
1610-18-0	Prometon	P	15	0	5	1	9	0	5	1	1	1	1	1	1	1	1	1	1	1	1	EC ₀₁	5.00 × 10 ⁰	10	5.00 × 10 ⁻¹	35
886-50-0	Terbutryn	P	21	0	9	2	10	0	6	0	0	0	0	0	0	0	0	0	0	0	0	EC ₁₀	1.90 × 10 ⁻²	1000	1.90 × 10 ⁻⁵	35
101-20-2	Triclocarban	P	11	0	1	5	5	0	1	2	1	1	1	1	1	1	1	1	1	1	1	NOEC	2.50 × 10 ⁻¹	10	2.50 × 10 ⁻²	35
319-84-6	α-HCH	P	21	0	1	8	12	0	1	2	4	4	4	4	4	4	4	4	4	4	4	EC ₅₀	6.50 × 10 ¹	10	6.50 × 10 ⁰	35
33213-65-9	β-Endosulfan	P	15	1	0	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	LC ₅₀	3.30 × 10 ⁰	1000	3.30 × 10 ⁻³	35
319-85-7	β-HCH	P	4	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NOEC	3.20 × 10 ¹	—	—	35
90-12-0	1-Methyl-naphthalene	PAH	6	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	EC ₅₀	1.42 × 10 ³	1000	1.42 × 10 ⁰	35
91-57-6	2-Methyl-naphthalene	PAH	5	0	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	EC ₅₀	1.46 × 10 ³	1000	1.46 × 10 ⁰	35
84-65-1	Anthraquinone	PAH	6	0	2	2	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	IC ₃₀	1.00 × 10 ⁻²	1000	1.00 × 10 ⁻⁵	35
132-65-0	Dibenzothiophene	PAH	3	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	LC ₅₀	4.20 × 10 ²	—	—	35
119-61-9	Benzophenone	PC	5	0	0	4	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	EC ₅₀	2.80 × 10 ²	100	2.80 × 10 ⁰	35
486-56-6	Cotinine	PC	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NOEC	1.00 × 10 ³	1000	1.00 × 10 ⁰	35
42399-41-7	Diltiazem	PC	3	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	LOEC	4.16 × 10 ³	1000	4.16 × 10 ⁰	35
58-73-1	Diphenhydramine	PC	2	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	LC ₅₀	3.94 × 10 ³	100	3.94 × 10 ¹	35
119-65-3	Isoquinoline	PC	5	0	1	2	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	LC ₅₀	1.40 × 10 ⁴	1000	1.40 × 10 ¹	35
94-13-3	Propylparaben	PC	2	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NOEC	1.00 × 10 ³	100	1.00 × 10 ¹	35
37680-73-2	PCB101	PCB	4	0	0	3	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	Growth	2.50 × 10 ⁰	100	2.50 × 10 ⁻²	35
35065-28-2	PCB138	PCB	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Growth	2.50 × 10 ⁰	100	2.50 × 10 ⁻²	35
35065-27-1	PCB153	PCB	4	0	0	1	3	0	0	1	1	1	1	1	1	1	1	1	1	1	1	Growth	2.50 × 10 ⁰	50	5.00 × 10 ⁻²	35

Table 2 (Contd.)

No. CAS	Compound	Family species	Experimental ecotoxicity										Data used for PNEC calculation				PNEC ($\mu\text{g L}^{-1}$)	Ref.	
			Test					Chronic test					Parameter	Endpoint	Value ($\mu\text{g L}^{-1}$)	EF			
			Test species	Trophic level		D	P	1C	2C	D	P	1C							2C
				D	P														
35065-29-3	PCB180	PCB	1	0	0	0	1	0	0	0	0	1		Growth	LOEC	2.50×10^0	100	2.50×10^{-2}	35
35693-99-3	PCB52	PCB	2	0	0	1	1	0	0	0	1		Reproduction	NOEC	2.50×10^1	100	2.50×10^{-1}	35	
335-67-1	Pentadecafluorooctanoic acid	PFAA	12	0	2	3	6+1 (Bc)	0	2	2	2		Mortality	LOEC	1.00×10^2	10	1.00×10^1	35	
335-76-2	Perfluorodecanoic acid	PFAA	3	0	1	1	1	0	1	0	0		Mortality	LC ₅₀	8.40×10^3	1000	8.40×10^0	35	
375-85-9	Perfluoroheptanoic acid	PFAA	1	0	1	0	0	0	1	0	0		Growth	EC ₅₀	1.90×10^6	1000	1.90×10^3	35	
375-95-1	Perfluorononanoic acid	PFAA	4	0	2	1	1	0	2	0	0		Growth	LOEC	1.00×10^1	1000	1.00×10^{-2}	35	
1763-23-1	Perfluorooctanesulfonic acid	PFAA	4	0	0	2	2	0	0	1	0		Mortality	LOEC	1.00×10^1	100	1.00×10^{-1}	35	
2058-94-8	Perfluorodecylphthalate	PFAA	2	0	0	1	1	0	0	0	0		Immobilization	NOEC	5.64×10^4	—	—	35	
117-81-7	Diethylene glycol phthalate	PHT	29	0	3	5	21	0	3	2	3		Reproduction	NOEC	2.00×10^0	10	2.00×10^{-1}	35	
26761-40-0	Diisodecyl phthalate	PHT	7	0	1	1	5	0	1	1	0		Mortality	LOEC	6.00×10^1	50	1.20×10^0	35	
27554-26-3	Diisononyl phthalate	PHT	7	0	1	2	4	0	1	1	0		Reproduction	NOEC	6.20×10^1	50	1.24×10^0	35	
131-11-3	Dimethyl phthalate	PHT	9	0	1	2	6	0	1	0	0		Mortality	NOEC	3.20×10^3	1000	3.20×10^0	35	
98-86-2	Acetophenone	VOC	4	0	1	2	1	0	0	0	0		Population growth	EC ₅₀	4.28×10^4	1000	4.28×10^1	35	
119-61-9	Benzophenone	VOC	5	0	0	4	1	0	0	1	0		Immobilization	EC ₅₀	2.80×10^2	100	2.80×10^0	35	
75-25-2	Bromoform	VOC	5	0	1	2	2	0	1	0	0		Mortality	NOEC	2.90×10^3	1000	2.90×10^0	35	
138-86-3	p-Limonene	VOC	11	0	1	7	3	0	1	0	0		Mortality	LC ₅₀	1.13×10^3	1000	1.13×10^0	35	
107-21-1	Ethylene glycol	VOC	20	1	1	7	11	0	1	1	1		Reproduction	NOEC	4.20×10^3	10	4.20×10^2	35	
120-72-9	Indole	VOC	3	0	1	2	0	0	1	1	0		Mortality	LC ₅₀	1.00×10^3	100	1.00×10^1	35	
124-76-5	Isoborneol	VOC	1	0	0	0	1	0	0	0	0		Mortality	LC ₅₀	5.90×10^4	—	—	38	

3.2 Ecotoxicity of the pollutants sought in UWWD

Of the 207 compounds sought in the UWWD samples (total, dissolved and/or particle phases), 161 were obtained by calculation using data from the European databases and the international literature. In addition, we ourselves formulated 52 new PNEC values based on experimental data and 4 PNEC values were obtained using the ECOSAR model, as detailed in Table 2. Finally, we listed 165 PNEC values. Fig. 1 presents the distribution of available PNEC values for each pollutant family. Fig. 1 shows that five families had a large number of molecules for which PNEC data were not available: PAH, PC, alkylphenols (ALK), others and PCB. This lack of data poses a problem for estimating the hazards of compounds found in UWWD.

The 165 PNEC values obtained were separated into three categories according to the ranking of Orias and Perrodin (2014): a PNEC $<0.1 \mu\text{g L}^{-1}$ (the most hazardous and the most ecotoxic) (Fig. 2), a PNEC between 1 and $0.1 \mu\text{g L}^{-1}$ (intermediate ecotoxicity) (Fig. 3) and a PNEC $>1 \mu\text{g L}^{-1}$ (less ecotoxic) (Fig. 4). Due to the results obtained, 59 of the compounds were ranked as “very ecotoxic”, 30 as “ecotoxic” and 76 as “slightly ecotoxic”.

Among the 59 very ecotoxic compounds, pesticides featured predominantly in the most hazardous category of pollutants sought in UWWD. Indeed, 33 of the compounds with a PNEC $<0.1 \mu\text{g L}^{-1}$ were pesticides. Moreover, with regard to the PNEC values of a very large number of pesticides sought in UWWD (54 compounds), they represent a potentially considerable source of hazard for this type of matrix: 43 compounds had a PNEC value $<1.0 \mu\text{g L}^{-1}$ and 33 had a PNEC value $<0.1 \mu\text{g L}^{-1}$. Two other families represented a considerable share of the most ecotoxic

pollutants: PC (7 compounds) and PAH (7 compounds). The share of the other families was lower. The most ecotoxic compound was endrin, a pesticide with a PNEC of $1.22 \times 10^{-8} \mu\text{g L}^{-1}$. However, when examining slightly ecotoxic compounds (PNEC $>1 \mu\text{g L}^{-1}$), many volatile organic compounds (VOC) were represented, with 24 of such compounds listed in this category. The gap between the share of the other families was narrower: flame retardants (FR; 3 compounds), PHT (6), perfluoroalkyl acids (PFAA; 3), PAH (5), pesticides (11), others (5), metals (6), PC (10), and ALK (1). The least toxic compound sought in UWWD was iohexol, a PC with a PNEC of $45\,705 \mu\text{g L}^{-1}$.

The large number of very ecotoxic pollutants sought in UWWD can be because the many substances found in UWWD were developed to eliminate certain families of organisms. Thus, they have a considerable impact on many aquatic species belonging to the same family (the most relevant example being pesticides). Nevertheless, many pollutants of this family have low toxicity in terms of PNEC values. This observation may be explained by three possibilities: (i) the pollutant specifically affects the organism targeted without having any effect on the others (as in the case of similar pesticides); (ii) it is only slightly toxic for aquatic organisms; (iii) there are not enough data to lower the PNEC value.

No or insufficient ecotoxicity data were available for 13 of the compounds (3 PC, 6 PAH, 2 ALK, 1 ORG, 1 PFAA) detected in UWWD. These compounds included many PC and PAH. More ecotoxicity data are necessary for these families of compounds to avoid underestimation of the hazard represented by UWWD. Moreover, Table 2 shows that the PNEC values of certain pollutants, such as PCB, propylparaben and cotinine, were

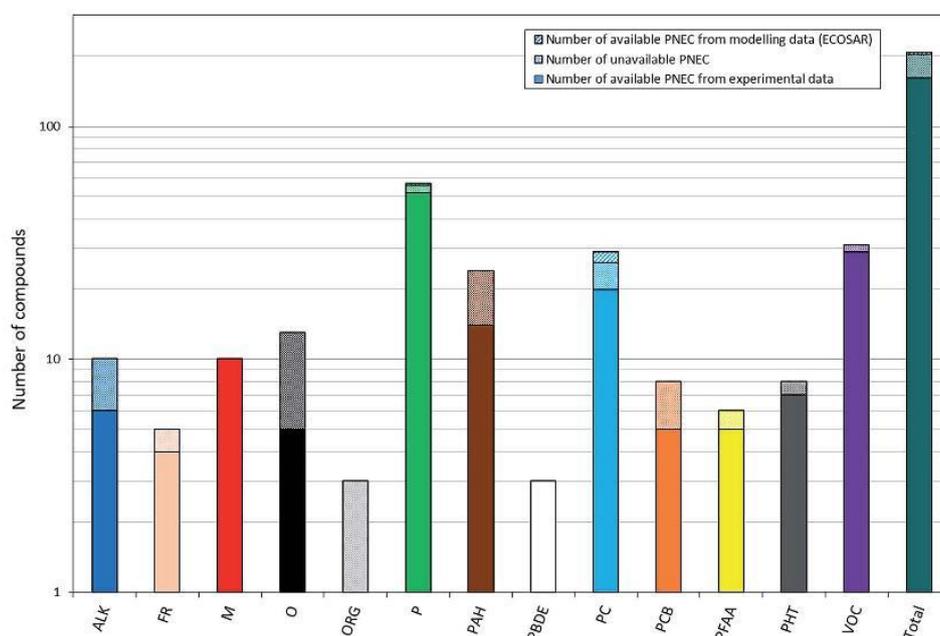


Fig. 1 Distribution of the number of molecules and their PNEC values for the different families of pollutants identified in UWWD.

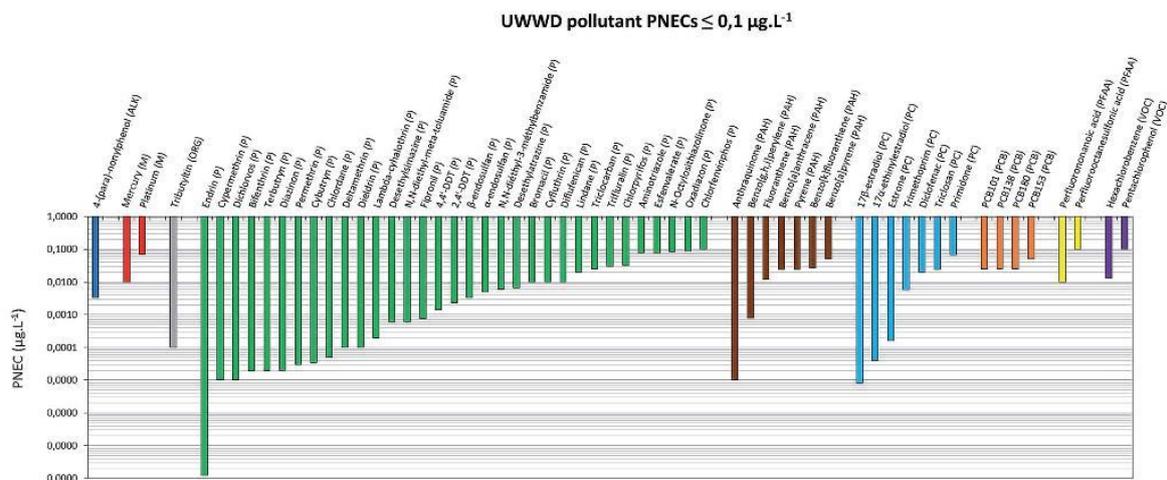


Fig. 2 PNEC values $\leq 0.1 \mu\text{g L}^{-1}$ for the different compounds sought in UWWD. Letters in brackets correspond to the classification in Table 1. PNEC values are ranked in ascending order into each separate pollutant family.

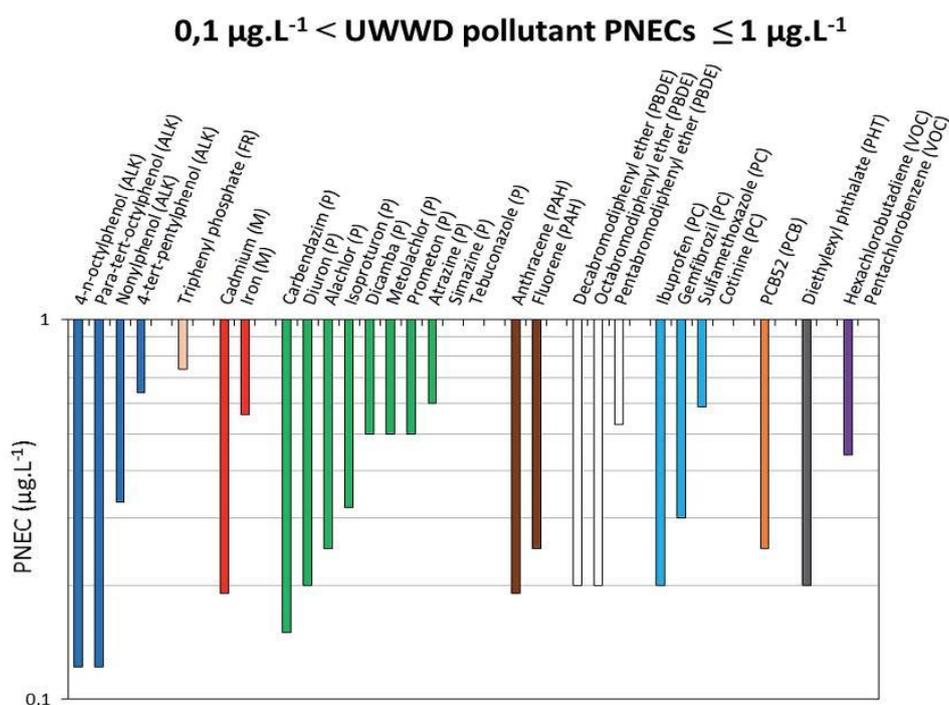


Fig. 3 PNEC values between $0.1 \mu\text{g L}^{-1}$ and $1 \mu\text{g L}^{-1}$ for the different compounds sought in UWWD. Letters in brackets correspond to the classification in Table 1. PNEC values are ranked in ascending order into each separate pollutant family.

calculated using a small number of ecotoxicological data, and consequently their toxicity could have been underestimated. However, these PNEC values were created with high safety factors (1000).

3.3 Concentration of pollutants in separate and combined systems

Maximum pollutant concentrations found in UWWD were assessed using available literature and are presented in Tables 3 and 4. MEC_{max} values were very variable, with some pollutants

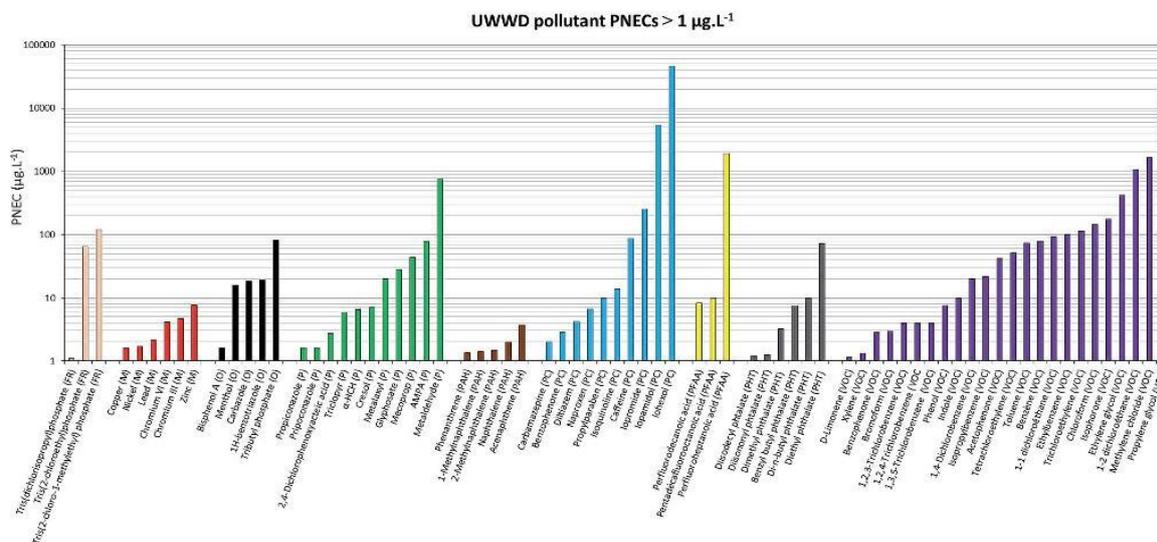


Fig. 4 PNEC >1 µg L⁻¹ for the different compounds sought in UWWD. Letters in brackets correspond to the classification in Table 1. PNEC values are ranked in ascending order into each separate pollutant family.

(e.g., hexachlorobenzene) found at several ng L⁻¹, whereas others (e.g., copper) were found at a few mg L⁻¹. However, the compound concentrations generally ranged between ng L⁻¹ and several hundred µg L⁻¹.

As observed in Table 3, 61 compounds were detected in stormwater samples, and 50 were sought but not detected. We found the highest concentrations for zinc (2.12 mg L⁻¹). High concentrations of pollutants, such as other heavy metals (copper, nickel) and pesticides (e.g. glyphosate and Amino-methylphosphonic acid (AMPA)) and PAH (e.g. pyrene), were also detected. The main families of pollutants detected at high concentrations were PAH, VOC, ALK, PHT, pesticides and metals.

Out of the 81 chemicals of interest sought in the dissolved part of the CSO samples, 49 were reported (see Table 4). The highest concentration was obtained for zinc (236 µg L⁻¹). The main families found at high concentrations were metals, pesticides and PC. The occurrence of high concentrations of heavy metals in separate systems and CSO is not surprising due to the source of wastewater and rainfall runoff. More precisely, tire wear, deposition of vehicle brakes, conveyance-system infrastructure (e.g., downspouts and culverts), and atmospheric deposition can lead to the presence of heavy metals.⁴ Moreover, the remobilization of wastewater and in-sewer deposits (due to pollutant water/particle partition) can represent a large share of the pollutant load in CSO, such as copper and PAH.⁵⁸ Launay *et al.*⁶⁴ observed the same phenomenon for PC and industrial chemicals. Contrary to stormwater, the presence of PC compounds sought and detected in the dissolved phase (18 compounds) was due to the contribution of urban wastewater to the pollution of CSO.

When we compared the maximum concentrations of UWWD pollutants with other urban effluents (e.g. urban wastewater and

hospital wastewater), such as PHT, heavy metals, PC and pesticides, MEC_{max} were close to those detected in these other matrices.^{60–63} For some of them, such as bisphenol A, the concentrations in UWWD (maximum 130 µg L⁻¹) can be even higher than that in wastewater (maximum, 2.14 µg L⁻¹). This phenomenon was also observed with heavy metals such as copper and zinc. Consequently, because they undergo hardly, any or no treatment, and because most of few treatments employed do not reduce the concentration of the many identified pollutants, considerable amounts of these substances may enter surface waters.

Nevertheless, it is important to point out that the data collected and discussed above have two main limitations. First, few studies have analyzed the dissolved phase of discharges and, thus, many compounds identified and sought in UWWD were not taken into account here because they were found in only the particle or total phase (due to their low solubility and high affinity for the particle phase). Consequently, the number of compounds detected here was limited (see Tables 3 and 4). Second, concentrations found in UWWD must be considered with caution because, as stated in the study of Huber *et al.*,⁵² concentrations measured in stormwater samples can be influenced by different parameters, such as the collection, preparation and analysis of samples, which can also affect the concentrations observed in discharges.

3.4 Hazard of pollutants in separate and combined systems

Using ecotoxicological (PNEC) and chemical (MEC_{max}) results enunciated previously, we were able to calculate a very wide range of HQ values in discharges (see Fig. 5 and 6). The wide dispersion of HQ values calculated could be explained by the heterogeneity of PNEC values observed in Fig. 2–4 and the heterogeneity of pollutant concentrations due to the high

Table 3 Maximum measured effluent concentrations (MEC_{max}) of micropollutants in the dissolved part sought in stormwater samples (ND: not detected, *: unspecified)

Pollutant family	Pollutant	MEC_{max} ($\mu\text{g L}^{-1}$)	Country	Ref.	Pollutant family	Pollutant	MEC_{max} ($\mu\text{g L}^{-1}$)	Country	Ref.
ALK	4- <i>n</i> -Octylphenol (ALK)	ND	France	4	PAH	Dibenzothiophene (PAH)	0.0417	USA (Washington D.C.)	2
ALK	4-Nonylphenol diethoxylate (ALK)	ND	France	4	PAH	Acenaphthylene (PAH)	0.070	Australia; France; Sweden; USA (Alabama); USA (Washington D.C.)	2, 4, 5, 47 and 48
ALK	4- <i>tert</i> -Pentylphenol (ALK)	0.7	Sweden	5	PAH	Perylene (PAH)	0.12	USA (Washington D.C.)	2
ALK	<i>para-tert</i> -Octylphenol (ALK)	0.99	France, Sweden	4 and 5	PAH	Dibenzo[<i>a,h</i>]anthracene (PAH)	0.13	France; Sweden; USA (Alabama); USA (Washington D.C.)	2, 4, 5 and 47
ALK	4-Octylphenol monoethoxylate (ALK)	4.39	Sweden	5	PAH	Benzo[<i>a</i>]anthracene (PAH)	0.34	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
ALK	4-(<i>para</i>)-Nonylphenol (ALK)	7.7	France, Sweden	4 and 5	PAH	Benzo[<i>b</i>]fluoranthene (PAH)	0.40	France; Sweden; USA (Alabama); USA (Washington D.C.)	2, 4, 5 and 47
ALK	4- <i>tert</i> -Butylphenol (ALK)	7.82	France, Sweden	4 and 5	PAH	Benzo[<i>e</i>]pyrene (PAH)	0.42	USA (South Carolina); USA (Washington D.C.)	2 and 48
ALK	4-Nonylphenol monoethoxylate (ALK)	8.36	Sweden	5	PAH	Indeno[1,2,3- <i>cd</i>]pyrene (PAH)	505	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
M	Platinum (M)	ND	France	4	PAH	Benzo[<i>a</i>]pyrene (PAH)	0.54	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
M	Mercury (M)	0.01	Canada; France	4 and 41	PAH	Benzo[<i>g,h,i</i>]perylene (PAH)	0.55	Australia; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 5, 47–49
M	Lead (M)	15.9	Australia; China; France; USA (Alabama); USA (California); *	4, 5, 47, 50–52	PAH	Anthracene (PAH)	0.56	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
M	Cadmium (M)	31.1	Australia; France; USA (Alabama); USA (California); *	4, 47, 50, 52 and 55	PAH	Chrysene (PAH)	0.62	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
M	Chromium (M)	32	Australia; France; USA (Alabama); USA (California); *	4, 47, 50, 52 and 55	PAH	Acenaphthene (PAH)	0.72	Australia; France; Sweden; USA (Alabama) USA (Washington D.C.)	4, 5, 47 and 49
M	Nickel (M)	49	France; USA (Alabama); USA (California); *	4, 5, 47 and 52	PAH	Benzo[<i>b</i>]fluoranthene (PAH)	0.98	Australia; France; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 47–49

Table 3 (Contd.)

Pollutant family	Pollutant	MEC _{max} (µg L ⁻¹)	Country	Ref.	Pollutant family	Pollutant	MEC _{max} (µg L ⁻¹)	Country	Ref.
M	Copper (M)	1520	Australia; China; France; USA (Alabama); USA (California); *	4, 5, 47, 50–52	PAH	Phenanthrene (PAH)	1.32	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
M	Zinc (M)	2118	Australia; China; France; USA (Alabama); USA (California); *	4, 5, 47, 50–52	PAH	Fluorene (PAH)	1.37	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
O	Bisphenol A (O)	130	Sweden	5	PAH	Fluoranthene (PAH)	14	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
ORG	Dibutyltin (ORG)	ND	France	4	PAH	Naphthalene (PAH)	17	Australia; France; Sweden; USA (Alabama); USA (Washington D.C.)	2, 4, 5, 47 and 49
ORG	Tributyltin (ORG)	ND	France	4	PAH	Pyrene (PAH)	20	Australia; France; Sweden; USA (Alabama); USA (South Carolina); USA (Washington D.C.)	2, 4, 5, 47–49
ORG	Monobutyltin (ORG)	0.025	France	4	PBDE	Decabromodiphenyl ether (PBDE)	ND	France	4
P	2,4'-DDT (P)	ND	France	4	PBDE	Octabromodiphenyl ether (PBDE)	ND	France	4
P	4,4'-DDT (P)	ND	France	4	PBDE	Pentabromodiphenyl ether (PBDE)	ND	France	4
P	Alachlor (P)	ND	France; USA (Alabama)	4 and 47	PCB	PCB101 (PCB)	ND	France	4
P	Aldrin (P)	ND	France; USA (Alabama)	4 and 47	PCB	PCB118 (PCB)	ND	France	4
P	Atrazine (P)	ND	France; USA (Alabama)	4 and 47	PCB	PCB138 (PCB)	ND	France	4
P	Chlordane (P)	ND	France; USA (Alabama)	4 and 47	PCB	PCB153 (PCB)	ND	France	4
P	Chlorfenvinphos (P)	ND	France	4	PCB	PCB180 (PCB)	ND	France	4
P	Chlorpyrifos (P)	ND	France	4	PCB	PCB194 (PCB)	ND	France	4
P	Desethylsimazine (P)	ND	France	4	PCB	PCB28 (PCB)	ND	France	4
P	Dieldrin (P)	ND	France	4	PCB	PCB52 (PCB)	ND	France	4
P	Endrin (P)	ND	France; USA (Alabama)	4 and 47	PFAA	Perfluoroheptanoic acid (PFAA)	0.011	Japan; Singapore; Switzerland; USA (Minnesota)	42–45
P	Esfenvalerate (P)	ND	France; USA (Alabama)	4 and 47	PFAA	Perfluoroundecanoic acid (PFAA)	0.045	Japan; Singapore; Switzerland; USA (Minnesota)	42–46
P	Lindane (P)	ND	France	4	PFAA	Perfluorodecanoic acid (PFAA)	0.077	Japan; Singapore; Switzerland; USA (Minnesota)	42–46
P	Simazine (P)	ND	France	4	PFAA	Perfluorononanoic acid (PFAA)	0.093	Japan; Singapore; Switzerland; USA (Minnesota)	42–46
P	Trifluralin (P)	ND	France	4	PFAA	Perfluorooctanesulfonic acid (PFAA)	0.16	Japan; Singapore; Switzerland; USA (Minnesota)	42–46

Table 3 (Contd.)

Pollutant family	Pollutant	MEC _{max} (µg L ⁻¹)	Country	Ref.	Pollutant family	Pollutant	MEC _{max} (µg L ⁻¹)	Country	Ref.
P	α-Endosulfan (P)	ND	France	4	PFAA	Pentadecafluorooctanoic acid (PFAA)	0.17	Japan; Singapore; Switzerland; USA (Minnesota)	42–46
P	β-Endosulfan (P)	ND	France	4	PHT	Dimethyl phthalate (PHT)	ND	USA (Alabama)	47
P	Lambda-cyhalothrin (P)	0.007	USA (California)	40	PHT	Di-n-octyl phthalate (PHT)	ND	France	4
P	Cyfluthrin (P)	0.023	USA (California)	40	PHT	Di-n-butyl phthalate (PHT)	1	Sweden; USA (Alabama)	5 and 47
P	Cypermethrin (P)	0.026	USA (California)	40	PHT	Diisodecyl phthalate (PHT)	1.2	Sweden	5
P	Bifenthrin (P)	0.034	USA (California)	40	PHT	Diisononyl phthalate (PHT)	2	Sweden	5
P	Metaldhyde (P)	0.05	France	4	PHT	Diethyl phthalate (PHT)	15.9	Sweden; USA (Alabama)	5 and 47
P	Permethrin (P)	0.13	USA (California)	40	PHT	Benzyl butyl phthalate (PHT)	16	Sweden; USA (Alabama)	5 and 47
P	Deltamethrin (P)	0.25	USA (California)	40	PHT	Diethylhexyl phthalate (PHT)	45	France; Sweden; USA (Alabama)	4, 5 and 47
P	Aminotriazole (P)	0.5	France	4, 47 and 48	VOC	1,2,3-Trichlorobenzene (VOC)	ND	France	4
P	Isoproturon (P)	0.6	France	4	VOC	1,2,4-Trichlorobenzene (VOC)	ND	France	4
P	Diuron (P)	0.64	France	4	VOC	1,3,5-Trichlorobenzene (VOC)	ND	France	4
P	AMPA (P)	19	France; Switzerland	4, 53 and 54	VOC	1,2-Dichloroethane (VOC)	ND	France	4
P	Glyphosate (P)	150	France; Switzerland	4, 53 and 54	VOC	Benzène (VOC)	ND	France	4
					VOC	Chloroform (VOC)	ND	France	4
					VOC	Ethylbenzene (VOC)	ND	France	4
					VOC	Hexachlorobenzene (VOC)	ND	France	4
					VOC	Hexachlorobutadiene (VOC)	ND	France	4
					VOC	Isopropylbenzene (VOC)	ND	France	4
					VOC	Pentachlorobenzene (VOC)	ND	France	4
					VOC	Tetrachloroethylene (VOC)	ND	France	4
					VOC	Toluene (VOC)	ND	France	4
					VOC	Trichloroethylene (VOC)	ND	France	4
					VOC	Xylene (VOC)	ND	France	4
					VOC	Pentachlorophenol (VOC)	0.2	France	4
					VOC	Dichloromethane (VOC)	13	France	4
					VOC	1,4-Dichlorobenzene (VOC)	47	USA (Alabama)	48

Table 4 Maximum measured effluent concentrations (MEC_{max}) of the micropollutants sought in the dissolved part of CSO samples (ND: not detected, *: unspecified)

Pollutant family	Pollutant	MEC_{max} ($\mu\text{g L}^{-1}$)	Country	Ref.	Pollutant family	Pollutant	MEC_{max} ($\mu\text{g L}^{-1}$)	Country	Ref.
ALK	4- <i>n</i> -Octylphenol (ALK)	ND	France	57	PC	Estrone (PC)	ND	South Korea	12
ALK	<i>para-tert</i> -Octylphenol (ALK)	ND	France	57	PC	Diltiazem (PC)	ND	South Korea	12
ALK	4- <i>tert</i> -Butylphenol (ALK)	0.080	France	57	PC	Trimethoprim (PC)	ND	South Korea	12
ALK	Nonylphenol (ALK)	0.390	France	57	PC	Meprobamate (PC)	ND	South Korea	12
FR	Tris(2-chloro-1-methylethyl) phosphate (FR)	1.140	South Korea	12	PC	Diclofenac (PC)	ND	South Korea	12
M	Nickel (M)	1.1	France	15, 57 and 58	PC	Gemfibrozil (PC)	0.006	South Korea	12
M	Chromium (M)	1.8	France	15, 57 and 58	PC	Diphenhydramine (PC)	0.023	South Korea	12
M	Cadmium (M)	9.9	France, United Kingdom	15, 57-59	PC	Primidone (PC)	0.040	South Korea	12
M	Copper (M)	65.4	France, United Kingdom	15, 57-59	PC	Triclosan (PC)	0.055	South Korea	12
M	Lead (M)	143.4	France, United Kingdom	15, 57-59	PC	Carbamazepine (PC)	0.060	South Korea	12
M	Zinc (M)	236.1	France, United Kingdom	15, 57-59	PC	Propylparaben (PC)	0.069	South Korea	12
O	1 <i>H</i> -Benzotriazole (O)	0.078	South Korea	12	PC	Sulfamethoxazole (PC)	0.070	South Korea	12
ORG	Dibutyltin (ORG)	ND	France	57	PC	Trimethoprim (PC)	0.073	South Korea	12
ORG	Tributyltin (ORG)	ND	France	57	PC	Benzophenone (PC)	0.136	South Korea	12
ORG	Monobutyltin (ORG)	0.013	France	57	PC	Atenolol (PC)	0.363	South Korea	12
P	Aldrin (P)	ND	France	57	PC	Acetulfame (PC)	0.727	South Korea	12
P	Dieldrin (P)	ND	France	57	PC	Naproxen (PC)	0.750	South Korea	12
P	Desethylatrazine (P)	0.017	France	57	PC	Ibuprofen (PC)	0.773	South Korea	12
P	Atrazine (P)	0.030	France	57	PC	Sucralose (PC)	0.818	South Korea	12
P	<i>N,N</i> -Diethyl-3-methylbenzamide (P)	0.035	South Korea	12	PC	Iopamidol (PC)	1.550	South Korea	12
P	Isoproturon (P)	0.040	France	57	PC	Iohexol (PC)	1.770	South Korea	12
P	Triclocarban (P)	0.061	South Korea	12	PC	Caffeine (PC)	2.54	South Korea	12
P	Aminotriazole (P)	0.460	France	57	PC	Iopromide (PC)	2.66	South Korea	12
P	Diuron (P)	0.470	France	57	PCB	PCB101 (PCB)	ND	France	57
P	AMPA (P)	1.600	France; Switzerland	53 and 57	PCB	PCB118 (PCB)	ND	France	57
P	Glyphosate (P)	3.4	France; Switzerland	53 and 57	PCB	PCB138 (PCB)	ND	France	57
PAH	Acenaphthene (PAH)	ND	France	15 and 57	PCB	PCB153 (PCB)	ND	France	57
PAH	Benzo[<i>a</i>]anthracene (PAH)	ND	France	15 and 57	PCB	PCB180 (PCB)	ND	France	57
PAH	Benzo[<i>b</i>]fluoranthene (PAH)	ND	France	15 and 57	PCB	PCB194 (PCB)	ND	France	57
PAH	Benzo[<i>k</i> , <i>l</i>]perylene (PAH)	ND	France	15 and 57	PCB	PCB28 (PCB)	ND	France	57
PAH	Benzo[<i>k</i>]fluoranthene (PAH)	ND	France	15 and 57	PCB	PCB52 (PCB)	ND	France	57
PAH	Chrysene (PAH)	ND	France	15 and 57	PHT	Diethylhexyl phthalate (PHT)	ND	France	57
PAH	Dibenzo[<i>a,h</i>]anthracene (PAH)	ND	France	15 and 57	VOC	Ethylbenzene (VOC)	ND	France	57
PAH	Benzo[<i>a</i>]pyrene (PAH)	0.005	France	15 and 57	VOC	Tetrachloroethylene (VOC)	ND	France	57
PAH	Benzo[<i>e</i>]pyrene (PAH)	0.006	France	15 and 57	VOC	Toluene (VOC)	ND	France	57
PAH	Indeno[1,2,3- <i>cd</i>]pyrene (PAH)	0.008	France	15 and 57	VOC	Trichloroethylene (VOC)	ND	France	57
PAH	Anthracene (PAH)	0.009	France	15 and 57	VOC	Xylene (VOC)	ND	France	57
PAH	Acenaphthylene (PAH)	0.020	France	15 and 57	VOC	Hexachlorobenzene (VOC)	0.002	USA (New York)	56
PAH	Pyrene (PAH)	0.021	France	15 and 57					
PAH	Fluoranthene (PAH)	0.025	France	15 and 57					
PAH	Fluorene (PAH)	0.027	France	15 and 57					
PAH	Phenanthrene (PAH)	0.085	France	15 and 57					
PAH	Naphthalene (PAH)	0.180	France	15 and 57					

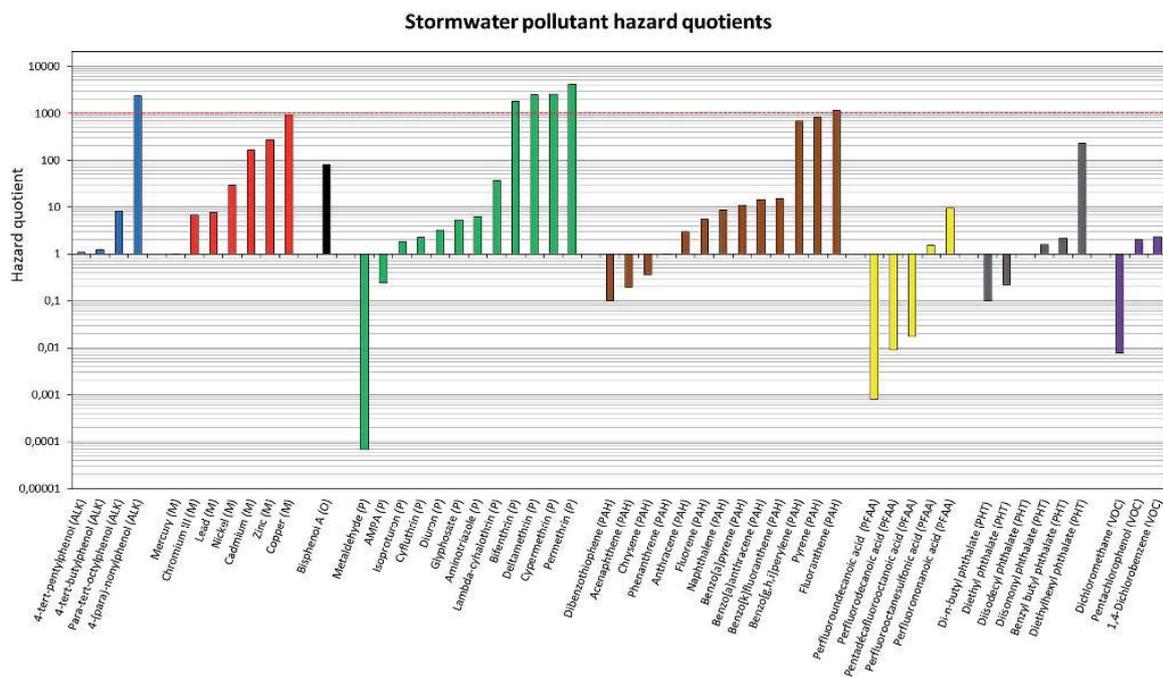


Fig. 5 Hazard quotient values calculated for micropollutants detected in stormwater samples (the red line delimits the compounds with an HQ up to 1000, which is considered very hazardous). Hazard quotient values are ranked in ascending order into each separate pollutant family.

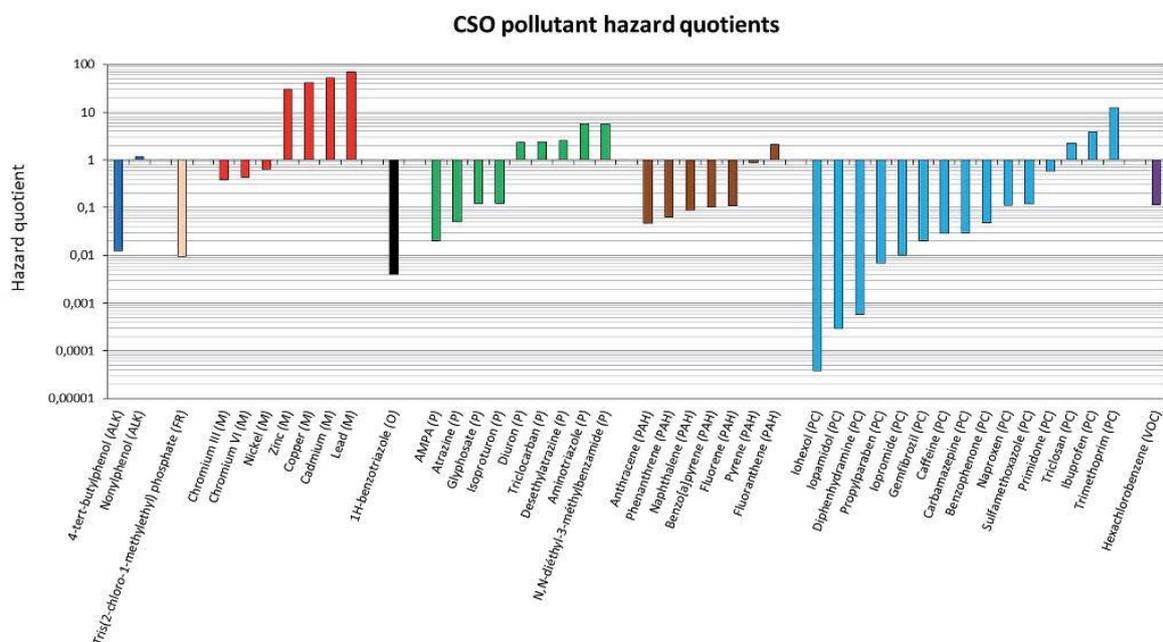


Fig. 6 Hazard quotient values calculated for micropollutants detected in CSO samples. Hazard quotient values are ranked in ascending order into each separate pollutant family.

variability of anthropic activities, such as the catchment leached by rain, and the variability of rainfall events (length, height, flow rate). Due to the relatively different nature and composition of stormwater and CSO, the pollutant hazards associated with these two matrices are discussed separately in the following sections.

3.4.1 HQ values in separate systems. With regard to stormwater samples, only 51 HQ values were calculated due to the small number of compounds found in the dissolved part (Fig. 5). We found 14 compounds with an HQ < 1. The lowest HQ was calculated for metaldehyde (pesticide) with an HQ of 6.7×10^{-5} . Most of the micropollutants detected in stormwater samples had an HQ ≤ 1 and were, therefore, potentially hazardous for aquatic ecosystems. Among these 37 compounds, 10 (27.1%) were pesticides, and 9 PAH (24.32%). Four of the six most hazardous compounds (HQ > 1000) were pesticides. Among these very hazardous compounds, we also observed the presence of one ALK (4-*p*-nonylphenol) and one PAH (fluoranthene). The most hazardous compound found was permethrin, with a HQ of 4166.6. The important hazard from pesticides (pyrethroids) linked to a high HQ could be explained by the high ecotoxicity of these pesticides rather than by the concentrations found in discharges ($\approx 100 \text{ ng L}^{-1}$). As noted above, most of these pesticides (*e.g.*, bifenthrin, cypermethrin) have a very low PNEC value ($< 0.1 \text{ ng L}^{-1}$).

3.4.2 HQ values in combined systems. With regard to CSO, only 43 HQ values were calculated due to the small number of molecules measured in the dissolved phase in CSO (Fig. 6). The HQ values obtained were lower than those for separate systems. Indeed, the highest HQ was found for lead (68.29). Contrary to separate systems, the highest HQ values were due to heavy metals, pesticides and PC. Only five families were found in this ranking (HQ > 1): PC (3 compounds), metals (4), PAH (1), pesticides (5) and ALK (1), and were potentially hazardous for aquatic ecosystems. Results highlighted for heavy metals could be because certain heavy metals in these effluents were at high concentrations (*e.g.* $2.1 \mu\text{g L}^{-1}$ for lead and $1.6 \mu\text{g L}^{-1}$ for copper), and not because of their low ecotoxicity, as shown by their relatively high PNEC values. The presence of hazardous PC in CSO could be explained by the discharge of large amounts of these often very ecotoxic molecules arising from urban wastewater in stormwater without having any treatment. Comparison of HQ values from CSO samples to those of separate systems revealed the calculated HQ values to be lower (*e.g.* 65.4 in CSO against 950 in stormwater for copper). These results were probably due to a dilution of stormwater pollutants by wastewater in CSO.

3.4.3 PC in CSO. With the hazardous PC highlighted above, 15 HQ values were calculated and are presented in Fig. 6. Urban wastewater is mixed with stormwater in combined sewers, so all the PC consumed in cities could be found in this type of discharge. However, few of these PC (28) have been taken into account in the scientific literature. Consequently, our study and all the other ecotoxicological studies dealing with CSO contain a bias because the potentially considerable hazard represented by these types of molecules was not taken into account. Indeed, as shown by Orias *et al.*,³⁶ many of these PC can have very low

PNEC values (*e.g.*, 17 β -estradiol, 8 pg L^{-1} ; clotrimazole, 0.5 pg L^{-1}), and/or are widely consumed (*e.g.*, ibuprofen, salicylic acid).

3.4.4 Molecules (*e.g.*, pesticides) forbidden by European legislation. Some of the pesticides involved in the hazard of UWWD (found in both stormwater and CSO, such as diuron) were forbidden in 2008 by European legislation. However, several European studies^{65–67} have detected the presence of these pesticides (with concentrations reaching $0.64 \mu\text{g L}^{-1}$) in discharges. This observation could be because farmers have stored pesticides purchased previously that they continue to use. Moreover, some of them linger in the environment for long periods (due to their high half-life) and their release can continue due to rainfall events. Consequently, the hazard represented by these pesticides continues to exist even if they are no longer on the market.

3.5 The method and data used

The method presented in the present study was shown to be relevant to detect pollutant hazards in UWWD. It could be applied for many other matrices, and included in many risk-assessment scenarios or chemical-prioritization programs. Nevertheless, our method and results are subject to limitations that need to be developed.

The first limitation is non-consideration of certain parameters, such as the bioconcentration, bioaccumulation³⁰ and biomagnification of pollutants in the aquatic food web, which could amplify considerably the hazard of pollutants in UWWD. Indeed, these phenomena have been demonstrated for many families of pollutants, such as the most hazardous found in UWWD: heavy metals,⁶⁸ pesticides^{69,70} and PC.^{71–74} Other parameters, such as pH, temperature, water chemistry (pH, hardness), can also modify pollutant toxicity. The “mixture” effect, which is linked to the antagonistic or the synergistic effects between sought pollutants, but also non-potentially thousand molecules not yet identified in UWWD, were not considered into our study and can modify the hazard they elicit. Second, the use of no observed effect concentration (NOEC) values for the construction of PNEC values should be discussed. We are aware that EC_x values are more accurate than NOEC values. EC_x values are calculated from modeling of the dose-response curve, which is itself based on the results of the whole range of concentration. NOEC values correspond only to identification of the last concentration of the range without effect. However, in the context of this work, we chose to use the largest set of values available in the literature and international databases for two reasons: (i) in the specific context of the PNEC calculation (*i.e.*, a no-effect value for all aquatic organisms), considering the scarcity of existing data in the literature, it seems more useful to consider values for one or several additional species than using accurate values for a limited number of species (*i.e.*, it is more important to consider inter-species variability than intra-species variability); (ii) this is the most common approach adopted by the international agencies in charge of the establishment of PNEC values for substances, such as INERIS in France, or the European Chemical Bureau in

Europe, through TGD guidelines. Third, our method considered only the maximum concentrations of the different pollutants in UWWD. Consequently, these values represent only the maximum hazard for each compound. This work could also have been done with median values if sufficient data had been available. This problem of limited data collection is the cause of another limitation: most of the chemical studies focusing on the concentrations of dissolved compound found in stormwater and CSO have dealt with relatively small areas of the world and many of them were published in Europe. Thus, data are lacking for some continents, a factor that limits our studies due to differences in, for example, PC consumption, pesticide use (*e.g.* glyphosate is used widely in South America) and sewer systems. Consequently, we can confidently assert that the HQ values calculated here are underestimated and would probably increase if more data were available. Fourth, only the dissolved parts of UWWD were considered in our study because the PNEC values were established for dissolved substances, and it was not possible to extend these values to pollutants whose bioavailability is not known but is present on particles in UWWD. Indeed, pollutant bioavailability is linked to particle desorption, which is dependent on multiple parameters such as pH, molar mass, solubility/lipophilicity, and the number of carboxyl groups (for organic pollutants).⁷⁵ Hence, our method could have

underestimated the hazard of these UWWD pollutants for aquatic environments. For example, as shown in a study by Zgheib *et al.*,⁴ if we had considered the concentration of fluoranthene in the dissolved phase ($0.018 \mu\text{g L}^{-1}$) and the total effluent ($0.832 \mu\text{g L}^{-1}$), assuming that all the pollutants were available, the HQ of the pollutants would have increased from 1.5 to 69.33, making it one of the most hazardous compounds for aquatic organisms. This point is illustrated in Fig. 7. Similar findings have been recorded by Baun *et al.*,³⁰ who prioritized 56 organic pollutants in the water phase of stormwater against 121 in the solid phase. Fifth, many chemical studies have considered only mineral and organic priority pollutants from the EU Water Framework Directive and other international legislation. However, many emerging compounds used currently have proven ecotoxic effects but have not yet been analyzed. For example, pesticides used widely in Europe such as metam-sodium and epoxiconazole have relatively low PNEC values (0.79 and $0.18 \mu\text{g L}^{-1}$, respectively). We can, therefore, expect high HQ values to be associated with these types of molecule. Finally, the presence of high concentrations of bacteria in CSO has already been detected, for example, by Passerat *et al.* (with concentrations $\leq 10^7$ cell per L for *Escherichia coli*).⁷⁶ It is now known that bacteria can transform pollutants and create byproducts that are more or less toxic than their parent

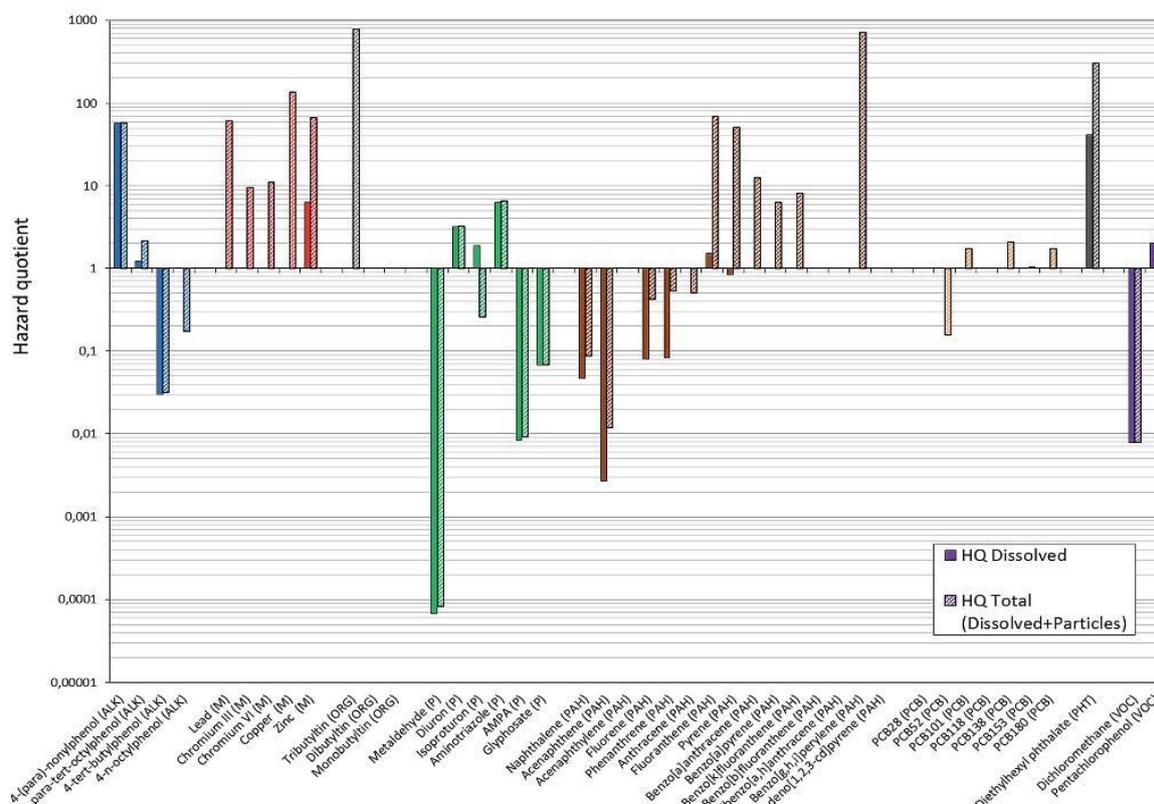


Fig. 7 Example of hazard quotient (HQ) values calculated with dissolved and total concentrations of pollutants in stormwater samples from Zgheib *et al.*⁴

compounds detected in CSO. These bacteria can, therefore, influence the composition of CSO and their toxicity with time.

Despite these limitations, our method enabled highlighting of new outlooks in terms of prioritization and management of UWWD pollutants. We can compare our results with previous works undertaken with the same purpose. When considering only the intrinsic characteristics (*e.g.*, particle solubility, sorption potential, ecotoxicity) of the pollutants selected, Baun *et al.*³⁰ classified 56 organic compounds as priority pollutants dissolved in water using their ranking and identification of chemical hazards method against 37 in our case (of which 31 were organic). The difference between the two studies is particularly notable for pesticides (10 against 33 pesticides for the method of Baun *et al.*³⁰). This difference can be explained by undetected or low environmental concentrations of many pesticides dissolved in water of our studied discharges. We revealed 9 hazardous PAH and 3 hazardous PHT instead of, respectively, 5 and 2 by Baun *et al.*³⁰ This discrepancy could have been due to the multiplication of decision filters (sorption/volatilization, technical problems, persistence, bioaccumulation, toxicity) applied by Baun *et al.* when selecting their compounds, and the lack of data for some pollutants that can lead to the rejection or selection of the priority list, as suggested by Baun *et al.* In the case of CSO-linked hazards, no studies have investigated a similar prioritization method. Consequently, no comparison can be made.

Searching the scientific literature enabled us to find new hazardous and priority pollutants. The same observation could be made for the European (Urban Wastewater Directive (UWD 91/271/EEC)) and American (USA EPA) priority pollutant lists. In fact, as stated in Table S2,[†] some pollutants identified in our study to be hazardous, such as diuron or fluoranthene, are already under the regulation of international agencies. On the contrary, some pollutants are absent from these lists. For example, some heavy metals (*e.g.*, copper and zinc in the UWD list), pesticides (*e.g.*, glyphosate, pyrethrinoids) in stormwater, PTH (*e.g.*, diisononyl phthalate) and PC (*e.g.*, ibuprofen, trimethoprim) in CSO are absent in UWD and EPA priority lists, and revealed to be at the origin of an important hazard for aquatic receiving ecosystems. Thus, these compounds should be the subject of reinforced controls and could be proposed to be added to these lists.

4. Conclusions and perspectives

The aim of our study was to develop and apply a methodology to determine and calculate the HQ values associated with the different pollutants sought and found in UWWD. A wide range of pollutants was sought in the latter, with highly variable ecotoxicity and concentrations. Although several families of pollutants (PC, PAH, VOC, ALK, metals, pesticides, others, PFAA and PHT) play a part in the UWWD hazards identified, we must emphasize that they comprise many pesticides and heavy metals (*e.g.*, pyrethrinoids, copper and cadmium). The hazards of the pollutants identified in our study have different origins, *i.e.*, the presence of: (i) high concentrations of certain highly ecotoxic compounds (*e.g.*, pyrene in stormwater); (ii) high

concentrations of slightly ecotoxic compounds (*e.g.*, heavy metals in CSO); (iii) low concentrations of certain highly ecotoxic compounds (*e.g.*, pesticides such as bifenthrin and permethrin in stormwater samples); (iv) intermediate concentrations of compounds with intermediate ecotoxicity (*e.g.*, ALK in UWWD). We showed that the hazard linked to CSO was lower than that linked to stormwater from separate systems. However, these results should be considered with caution because only a few PC (28 compounds) were sought in CSO compared with the huge range of PC consumed by humans. Moreover, ecotoxicity data were not available for 13 of the compounds (3 PC, 6 PAH, 2 ALK, 1 ORG, 1 PFAA) detected in UWWD and, therefore, no PNEC or HQ values could be calculated. Consequently, it will be necessary in further studies to obtain more ecotoxicity data on these compounds. Moreover, current hazard and risk-assessment methods must be improved to take into account pollutant bioconcentration/accumulation, which can represent a serious risk for higher trophic levels.

This work enabled identification of the potential hazard of UWWD and the pollutants involved. We now know which pollutants require an in-depth monitoring. We have also identified different parameters that require in-depth studies. In particular, it is now necessary to better assess the hazard of certain pollutants bound to the particulate phase. Lastly, this work should help managers to identify the largest and most hazardous pollution sources so that their treatment is prioritized.

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Supplementary data

Table S1: Extrapolation Factor assessment criteria derivate from Orias and Perrodin³⁶ and TDG³⁷.

Available data	EF (Extrapolation Factor)
At least one short-term L(E)C50 from each of three trophic levels of the base-set (e.g. algae, daphnids and fishes)	1000
One long term chronic data (e.g. invertebrate or fish)	100
Two long term chronic data from species representing two trophic levels (e.g. algae and/or daphnids and/or fishes)	50
Two long term chronic data from species representing three trophic levels (e.g. algae, daphnids and fishes)	10
Statistics method (e.g. species sensitivity distribution (SSD)), field data or model ecosystem	5-1 (Reviewed case by case)

Table S2: Comparison of hazardous compounds list identified in UWWDs in this study with the classification of these compounds as priority pollutants or not by European Water Framework Directive and in Environmental Protection Agency.

Pollutant	Identified as hazardous		Identification as a priority substance	
	In stormwater	In combined sewer overflows	In European Water Framework Directive (WFD, 2000/60/EC) pollutant priority list	In Environmental Protection Agency pollutant priority list
4-(para)-nonylphenol (ALK)	Yes	No	Yes	No
4-tert-pentylphenol (ALK)	Yes	No	Yes	No
4-tert-butylphenol (ALK)	Yes	No	Yes	No
Nonylphenol (ALK)	No	Yes	Yes	No
Para-tert-octylphenol (ALK)	Yes	No	Yes	No
Cadmium (M)	Yes	Yes	Yes	Yes
Chromium III (M)	Yes	No	No	Yes
Copper (M)	Yes	Yes	No	Yes
Lead (M)	Yes	Yes	Yes	Yes
Nickel (M)	Yes	No	Yes	Yes
Zinc (M)	Yes	Yes	No	Yes
Bisphenol A (O)	Yes	No	No	No
Aminotriazole (P)	Yes	Yes	No	No
Bifenthrin (P)	Yes	No	No	No
Cyfluthrin (P)	Yes	No	No	No
Cypermethrin (P)	Yes	No	Yes	No
Deltamethrin (P)	Yes	No	No	No
Desethylatrazine (P)	Yes	Yes	No	No
Diuron (P)	Yes	Yes	Yes	No
Glyphosate (P)	Yes	No	No	No
Isoproturon (P)	Yes	No	Yes	No
Lambda-cyhalothrin (P)	Yes	No	No	No
N,N-diéthyl-3-méthylbenzamide (P)	No	Yes	No	No
Permethrin (P)	Yes	No	No	No
Triclocarban (P)	No	Yes	No	No
Anthracene (PAH)	Yes	No	Yes	Yes
Benzo[a]anthracene (PAH)	Yes	No	Yes	Yes
Benzo[k]fluoranthene (PAH)	Yes	No	Yes	Yes
Benzo[g,h,i]perylene (PAH)	Yes	No	Yes	Yes
Benzo[a]pyrene (PAH)	Yes	No	Yes	Yes
Fluoranthene (PAH)	Yes	Yes	Yes	Yes
Fluorene (PAH)	Yes	No	Yes	Yes
Naphthalene (PAH)	Yes	No	Yes	Yes
Pyrene (PAH)	Yes	No	Yes	Yes
Ibuprofen (PC)	No	Yes	No	No
Triclosan (PC)	No	Yes	No	No
Trimethoprim (PC)	No	Yes	No	No
Perfluorooctanesulfonic acid (PFAA)	Yes	No	Yes	No
Perfluorononanoic acid (PFAA)	Yes	No	Yes	No
Benzyl butyl phthalate (PHT)	Yes	No	No	Yes
Diethylhexyl phthalate (PHT)	Yes	No	Yes	Yes
Diisononyl phthalate (PHT)	Yes	No	No	No
1,4-Dichlorobenzene (VOC)	Yes	No	No	Yes
Pentachlorophenol (VOC)	Yes	No	Yes	Yes



1.1.3 Synthèse et discussion

Cette première étude nous a permis de mettre en évidence un danger important, tant dans les eaux pluviales strictes que dans les rejets issus de déversoirs d'orage. Pour ces deux catégories de rejets, plusieurs familles de polluants sont à l'origine du danger écotoxique de ces rejets. Dans le cas des eaux pluviales strictes, trois familles se distinguent très nettement : les pesticides, les métaux lourds et les hydrocarbures aromatiques polycycliques (avec des HQ supérieurs à 1000). Dans le cas des rejets de surverses de déversoirs d'orages, les produits pharmaceutiques et métaux lourds sont fortement représentés parmi les polluants les plus dangereux.

Ce travail présente cependant quelques limites avec notamment le fait de ne considérer que les fractions dissoutes des RUTP. Les mécanismes d'adsorption/désorption sur les particules minérales et organiques des rejets étant compliqués à analyser et prédire, il est difficile de quantifier la part de la pollution particulaire disponible pour les organismes aquatiques. La seconde limite de l'étude est l'utilisation de données écotoxicologiques sans la prise en compte de l'influence de la bioconcentration/bioaccumulation/biomagnification des polluants au sein des chaînes trophiques.

Une perspective intéressante serait de mener un travail dans une même optique que celui présenté ci-avant, en tenant compte cette fois directement de la concentration finale des polluants dans les milieux recevant les pollutions (MEC : Measured Environmental Concentration). Cette prise en compte des MEC, du fait de la dilution des RUTP, permettrait de définir des valeurs de quotient de risque (RQ), plus faibles que celles des quotients de dangers (HQ) calculées précédemment. Ainsi, Angerville et al. (2013) ont par exemple observé un facteur de dilution de rejets issus d'un déversoir d'orage (situés à Grezieux-la-Varenne, dans l'Ouest Lyonnais) égal à 17 dans le ruisseau récepteur (Chaudanne). Toutefois, de nombreuses études ont démontré une pollution (issue des RUTP diluée) avérée des écosystèmes aquatiques récepteurs malgré le phénomène de dilution. Basé sur l'analyse chimique de ces milieux aquatiques, le calcul des quotients de risque devrait conduire à mettre en évidence un risque significatif pour les écosystèmes receveurs.



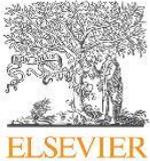
1.2 Méthodes d'étude de l'impact des Rejets Urbains par Temps de Pluie sur les écosystèmes aquatiques

1.2.1 Contexte et enjeux

Si l'objectif de la première sous-partie bibliographique était de contextualiser et de rationaliser le danger écotoxique représenté par les RUTP sur la base de données chiffrées de concentration en polluants, cette deuxième sous-partie détaille les méthodes d'études de l'impact des RUTP sur les écosystèmes aquatiques récepteurs et les organismes qui s'y trouvent. La majorité des travaux de recherche s'est attachée à identifier et quantifier la pollution chimique des RUTP *via* des techniques analytiques poussées. La détermination de l'impact de ces molécules sur les milieux aquatiques est peu abordée. Cette seconde sous-partie se découpe en cinq volets : (1) l'étude des indices biologiques basée sur l'étude des communautés d'organismes déjà présents sur sites et contaminés par les événements pluvieux, (2) les bioessais écotoxicologiques réalisés en laboratoire et *in situ*, (3) les méthodes d'évaluation des risques à priori, (4) les modélisations et (5) les biocapteurs. Pour chacun des volets, l'objectif a été d'établir une vue synthétique et critique des connaissances disponibles, de définir les avantages et les inconvénients liés aux méthodes employées. Finalement, cette étude a permis de formuler des recommandations pour les recherches à venir.

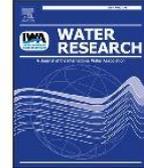
1.2.2 Article 2 - *Methods for evaluating the pollution impact of urban wet weather discharges on biocenosis: a review.*

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Review

Methods for evaluating the pollution impact of urban wet weather discharges on biocenosis: A review



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ABSTRACT

Rainwater becomes loaded with a large number of pollutants when in contact with the atmosphere and urban surfaces. These pollutants (such as metals, pesticides, PAHs, PCBs) reduce the quality of water bodies. As it is now acknowledged that physico-chemical analyses alone are insufficient for identifying an ecological impact, these analyses are frequently completed or replaced by impact studies communities living in freshwater ecosystems (requiring biological indices), ecotoxicological studies, etc. Thus, different monitoring strategies have been developed over recent decades aimed at evaluating the impact of the pollution brought by urban wet weather discharges on the biocenosis of receiving aquatic ecosystems. The purpose of this review is to establish a synthetic and critical view of these different methods used, to define their advantages and disadvantages, and to provide recommendations for futures researches. Although studies on aquatic communities are used efficiently, notably on benthic macroinvertebrates, they are difficult to interpret. In addition, despite the fact that certain bioassays lack representativeness, the literature at present appears meagre regarding ecotoxicological studies conducted *in situ*. However, new tools for studying urban wet weather discharges have emerged, namely biosensors. The advantages of biosensors are that they allow monitoring the impact of discharges *in situ* and continuously. However, only one study on this subject has been identified so far, making it necessary to perform further research in this direction.

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1. Introduction

It is now acknowledged that growing urbanisation has a major environmental impact (Paul and Meyer, 2001), whether on hydrology, physico-chemistry or the biology of aquatic ecosystems (Stearman and Lynch, 2013). One major type of urban pollution which remains relatively ignored by the public authorities is urban wet weather discharges. These stem in particular from the atmosphere (Gunawardena et al., 2013) and water running off impervious surfaces in urban areas (roads, roofs, etc.) (Göbel et al., 2007) which are undergoing constant expansion (Foster, 2001). Thus rainwater is returned to the natural environment with or without still limited treatments (retention ponds, infiltration basins, bio-filtration systems, reed-bed systems, etc.), because of the important volume to treat. Furthermore, rainwater is subject to other forms of urban contamination. Chocat et al. (2007) described urban wet weather discharges (UWWD) as all the water discharged by sewerage installations (mixture of treated wastewater and rainwater), by combined sewer overflows (CSO) (mixture of non treated wastewater and rainwater), and stormwater outfalls (generally non treated rainwater), during a rain event and the dry period following it.

Pollution by urban wet weather discharges is therefore complex due to the diversity of catchments and human activities, and significant concentrations of macro and micropollutants. Weibel et al. (1964) and Burm et al. (1968) were the first to focus on the physico-chemical nature of urban wet weather discharges. Many studies have since been devoted to their characterisation and they permitted highlighting the diversity of pollutants they contain (House et al., 1993; Pitt et al., 1995; Mulliss et al., 1997; van Buren et al., 1997; Admiraal et al., 2000; Lee and Bang, 2000; Lee et al., 2002a,b; Chebbo and Gromaire, 2004; Gasperi et al., 2008 or Curren et al., 2011). They can, in many cases affect adversely water utilisation (drinking fishing, bathing, etc.). The presence of molecules featuring in the list of priority organic pollutants established by the European directives was detected in urban wet weather discharges. The presence of Polycyclic Aromatic Hydrocarbons (PAHs) was detected by Hwang and Foster (2006), Terzakis et al. (2008), Zgheib et al. (2011), Kalmykova et al. (2013), diethylhexylphthalate by Bjorklund et al. (2009), Zgheib et al. (2011), 4 nonylphenol by Bjorklund et al. (2009), Zgheib et al. (2011), and bisphenol A and alkylphenolethoxylate by Cladière et al. (2012). Deffontis et al. (2013) also detected traces of hormones, the presence of which had already been revealed in river water by Pailler et al. (2009) and Peng et al. (2008). Other families of micropollutants like heavy metals (copper, lead and zinc, etc.), polychlorobiphenols (PCBs), pesticides, and phthalates have been detected too. The inventory of all these micropollutants has led to characterising urban wet weather discharges as highly complex chemical cocktails. This pollution is also subject to considerable variability (Kafi et al., 2008) due in particular to surfaces leached by rain, considerable variations of runoff flows through time and climatic conditions prior to rain events. Thus characterising urban wet weather discharges is complicated due to the intermittent release of micropollutants during rainfall events (Karlavičienė et al., 2009). The pollution transported by urban wet weather discharges is also

biological, with the inflow of many bacteria (such as *Escherichia coli*, the enterococcus, faecal coliforms, etc.) (Jeng et al., 2005; Parker et al., 2010). The same problem of variability has been highlighted for bacterial contamination as for chemical contamination (McCarthy et al., 2012). Urban wet weather discharges are therefore the source of substantial sanitary and environmental risks.

In order to monitor the quality of the discharges, different parameters have to be studied. It is important to observe these parameters as they are liable to have a major impact on the living conditions of the organisms living in aquatic receiving ecosystems. Many studies have focused on erosion phenomena in rivers, on the basis of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), pH, and total concentrations of phosphates, nitrates and ammonia (Mulliss et al., 1997; Chebbo and Gromaire, 2004; Kafi et al., 2008; Lee and Bang, 2000; Mallin et al., 2009). The biological component (especially the inflow of faecal bacteria) has also been studied (McLellan, 2004). Massive inflows of ammonia were observed at contaminated sites (Mulliss et al., 1997), as well reductions of the concentration of dissolved oxygen in certain environments. Stormwater is the main vector of underground contamination by nitrogen and phosphorous (Lee et al., 2003; Wakida and Lerner, 2005). Monitoring these different parameters is important as they play an essential role in the survival of aquatic species. Indeed, concentrations of ammonia and dissolved oxygen in water are the leading causes of mortality in fish in environments contaminated by urban wet weather discharges (Magaud et al., 1997). In addition, a reduction of dissolved oxygen leads to an increase in the sensitivity of certain fish to micropollutants such as heavy metals (Lloyd, 1961). It has also been shown that suspended particles play a fundamental role in the toxic impact of urban discharges (Tsui and Chu, 2003; Angerville, 2009). This can be explained by the fact that some of the micropollutants contained in urban wet weather discharges are adsorbed on the surfaces of particles, and predominantly found in this phase. A large amount of studies described this phenomenon, as Gasperi et al. (2008), Zgheib et al. (2011) or Li and Zuo (2013). If this one is particularly true for pollutants such as heavy metals, PAHs, PCBs (100% particle-bound in the study of Zgheib et al. (2011)), or phthalates, it has also been observed pesticide, heavy metals, alkylphenols, organotins or PAHs residues, in the dissolved phase of urban wet weather discharges (Zgheib et al., 2011), generally with weaker concentrations.

However, taken alone, these parameters are insufficient to evaluate the ecological impact of urban wet weather discharges. It is therefore necessary to carry out studies on the biological communities through bioassays *in situ* and in the laboratory in order to evaluate the stress on populations exposed to wet weather discharges (Burton et al., 2000).

In Europe, the chemical analyses performed are often compared to environmental quality standards (EQS) that are threshold values used to determine the good quality of rivers receiving rainwater, as it was done, for example, by Zgheib et al. (2011). However, the ecological impact of urban wet weather discharges cannot be evaluated only in comparison to these standards and a more inclusive view of the impact is needed (Novotny and Witte, 1997).

Due to global urban expansion, urban wet weather discharges

could have a deleterious impact on numerous aquatic areas and different water bodies. For example, effects have been observed in lotic aquatic environments (Custer et al., 2006; Scholes et al., 2007; Casadio et al., 2010; Gillis, 2012), underground environments (Foulquier et al., 2009, 2011), lacustrine environments (Hatch and Burton, 1999) and marine environments (Schiff and Bay, 2003; Roberts et al., 2007). Furthermore, all the trophic levels in these aquatic ecosystems (producers, decomposers, primary and secondary consumers) are potentially affected (Miltner et al., 2004; McQueen et al., 2010; Tang et al., 2013). The organisms living in waste water treatment plants (especially in infiltration basins) are also affected (Bartlett et al., 2012a, 2012b). Animal and plant species in the water column are not alone in being contaminated, since the deposits of particles contained in urban runoff, in rivers or in discharge treatment infrastructures leads to severe contamination of benthic species (such as annelids) (Schiff and Bay, 2003). Urban wet weather discharges therefore constitute a complex matrix for study due to their variable composition and the diversity of organisms and sites affected.

In Europe, the Urban Wastewater Directive (91/271/EEC) requires the management of urban wet weather discharges, though the means are relatively limited (mainly retention and/or infiltration basins), notably due to the large volumes that have to be treated. Furthermore, the European Water Framework Directive (WFD, 2000/60/EC) requires maintaining the good ecological status of water bodies exposed to such large volumes of discharges. To achieve this, it is important to implement effective monitoring methods to ensure compliance of the quality of aquatic environments. As expressed before, chemical analyses alone are not sufficient to evaluate water quality (Mankiewicz-Boczek et al., 2008), and thus the impact of urban wet weather discharges on the environment. Laboratory bioassays (algae and daphnia tests, etc.) are often chosen as they require few resources, they are relatively fast and simple to implement (Blaise, 1991), and they are sensitive and sufficiently repeatable (Marsalek et al., 1999b). Methods combining both approaches, the “Toxicity Identification Evaluation” (TIE), have been developed to help understand the action mechanisms of the most prevalent families of pollutants responsible for the toxicity of urban wet weather discharges (Gersberg et al., 2004; Custer et al., 2006). However, analyses that better integrate the variability of discharges, such as *in situ* studies, provide more realistic views of the consequences of exposure to urban wet weather discharges on an aquatic ecosystem (Burton et al., 2000); however, they remain to be developed (caging, biosensors, etc.).

The purpose of this review is therefore to take stock of all the techniques currently used to study the impact of pollution brought by urban wet weather discharges on the biocenosis of receiving aquatic ecosystems. To this end, the following are described and

discussed: (i) the different biological indices, ecotoxicological bioassays and *a priori* approaches used to evaluate the impact of discharges on contaminated aquatic organisms; (ii) a critical appraisal of these often used methods; and lastly, (iii) the need to turn to new methods that take better account of the variation of the quality of receiving environments and which permit the rapid acquisition of information on the impacts of discharges, such as prediction/prevention tools that can be used before the damage caused does not jeopardise the survival of ecosystems.

2. Evaluation of the pollution impact of urban wet weather discharges on biocenosis

This study considers contamination by combined sewer networks and by separate sewer systems, that in particular drain urban and road flows in urban and outer-urban areas, during wet weather events only. Moreover, all the potential receiving environments are considered (whether freshwater, seawater or artificial environments such as infiltration basins with their own biocenoses).

The evaluation of the ecological impact on the biocenose requires studying the effect of pollutant matrixes on the organisms present in contaminated aquatic environments. Evaluating the impact of urban wet weather discharges, and more particularly ecotoxicological impacts, can be approached in two different ways:

- The first is known as the “substance approach” (Fig. 1). It is often associated with a chemical analysis of a sample studied to determine the most prevalent pollutants, and as a function of the ecological pertinence, their risk, bioavailability, and whether or not they can be chosen as risk tracers. It then entails studying the impact of these risk tracers on key organisms. The impact of these pollutants is often assessed by laboratory tests that expose these organisms to a range of pollutant concentrations, or by acquiring data from international databases (BDI). There are several sub-types of substance approach: simple substance approaches, where only one pollutant is taken into account, and combined effect substance approaches where several pollutants are tested in mixtures (Perrodin et al., 2011). Although this approach is often used only for relatively well-known matrixes in which only a few toxic substances predominate, urban wet weather discharges have already been the subject of such studies. Mention can be made of the approach used by Oulton et al. (2014) who studied the impact of zinc present in urban wet weather discharges on the foraging behaviour of *Paratya australiensis*. One of the main criticisms levelled at the substance approach is the lack of representativeness of the tests performed in comparison to reality in the field. Indeed, since the matrixes discharged represent complex mixtures of all families of molecules, synergetic, antagonistic or additive effects may occur between them. This was shown in particular by Angerville (2009).
- The second is the “matrix approach” (Fig. 1), which is a much more realistic approach in relation to the matrix and the scenarios studied. The tests are then carried out on the whole matrix to be studied and not on one or several selected molecules, by exposing organisms to a range of effluent concentrations (Perrodin et al., 2011; Orias and Perrodin, 2013). In this case, the impact observed takes into account the synergetic, antagonistic and additive phenomena of the pollutants present. However, it is then much more difficult to determine which pollutants are responsible for the impact observed and their share of responsibility in the effects observed (Orias and Perrodin, 2013). It is nonetheless recommended to perform chemical analyses to determine the pollutants most present in order to determine the causes of the toxicity observed. As with

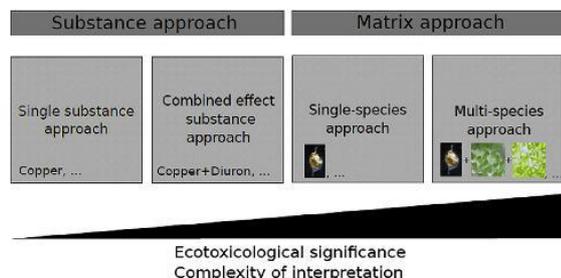


Fig. 1. Substance and matrix approaches for studying the biocenotic impact of urban wet weather discharges (modified, according to Angerville (2009)).

Table 1
Identification of the studies using biological indices for urban wet weather discharges ecological impact assessment.

Approach	Toxic matrix	Contaminated water	Studied community	Indices used	Author
Matrix	Combined sewer overflows	Coastal marine water	Macro-invertebrates (subtidal fauna)	Species relative abundance; Polychaeta, Mollusca and Arthropoda richness, abundance, and biomass; Dissimilarity; Polychaete feeding type proportion	Armstrong et al., 1980
Matrix	Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Freshwater (river)	Macro-invertebrates (benthic fauna)	Species diversity (Brillouin index); Hierarchical diversity; Major taxa composition; Dissimilarity	Pratt et al., 1981
Matrix	Urban stormwater runoff	Freshwater (river)	Macro-invertebrates (benthic fauna)	Species diversity (Brillouin index); Population density	Medeiros et al., 1983
Matrix	Urban stormwater runoff	Coastal marine water	Macro-invertebrates (intertidal mudflat fauna)	Species abundance	Roper et al., 1988
Matrix	Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Receiving water (river, ponds, etc.)	(zoo- and phyto-) Plankton	Biomass (chlorophyll-a concentration); Species diversity; Saprobic level	Gast et al., 1990
Matrix	Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Receiving water (river, ponds, etc.)	Macro-invertebrates (benthic fauna)	Taxa richness; Relative abundance of taxa	Willemsen et al., 1990
Matrix	Urban stormwater runoff	Freshwater (creeks and rivers)	Macro-invertebrates (benthic fauna)	Relative abundance of taxa; Trophic level; Saprobic level Hilsenhoff family-level biotic index; EPT (Ephemeroptera, Plecoptera, Trichoptera) index; EPT/Chironomidae abundance; Taxa richness	Masterson and Bannerman, 1994
Matrix	Urban motorway runoff	Freshwater (streams)	Macro-invertebrates (benthic fauna)	Relative abundance; Diversity (log series index and modified Sorenson index); Biological Monitoring Working Party (BMWP) score; Average score per taxon (ASPT); Family richness	Maltby et al., 1995a
			Fungi (aquatic hyphomycetes)	Species Relative importance values (RIV); Diversity (log series index and modified Sorenson index); Similarity index	
			Algae	Sampled genera; Diversity (log series index and modified Sorenson index); Biomass (Chlorophyll-a concentration)	
Matrix	Combined sewer overflows	Coastal marine water and freshwater (river)	Macro-invertebrates (benthic fauna)	Organisms abundance; Species richness	Hall et al., 1998
Matrix	Urban land use (associated to urban impervious surfaces and urban stormwater runoff)	Freshwater (little streams)	Algae (Epilithic and epiphytic benthic diatom)	Genus and species richness; Relative abundance	Sonneman et al., 2001
Matrix	Urban land use (associated to urban impervious surfaces and urban stormwater runoff)	Freshwater (little streams)	Macro-invertebrates (benthic fauna)	Ephemeroptera, Plecoptera and Trichoptera (EPT) richness	Walsh et al., 2001
Matrix	Urban land use (associated to urban stormwater runoff)	Freshwater (streams)	Fishes	Fish abundance; Species richness; Diversity (Shannon index); Index of biotic integrity (IBI)	Wang et al., 2001
Matrix	Urban land use (associated to urban stormwater runoff)	Freshwater (streams)	Macro-invertebrates (benthic fauna)	Generic and species richness; Diversity (Shannon Wiener index); Biotic index (BI); Ephemeroptera, Plecoptera, and Trichoptera pollution intolerant individuals; Feeding groups proportion (Filterers, Scrapers, Shredders, Collectors, Gatherers); Pollution tolerance (Hilsenhoff biotic index)	Stepenuck et al., 2002
Matrix	Urban stormwater runoff	Coastal marine water (estuaries)	Macro-invertebrates (benthic fauna)	Number of taxa; Total and relative abundance of taxa	Morrisey et al., 2003
Matrix	Urban stormwater runoff	Coastal marine water	Macro-invertebrates (benthic fauna)	Total and mean taxa abundance and biomass; species richness; Diversity (Shannon–Wiener index); Evenness (Pielou's index); Benthic Response Index (BRI)	Schiff and Bay, 2003

(continued on next page)

Table 1 (continued)

Approach	Toxic matrix	Contaminated water	Studied community	Indices used	Author
Matrix	Urban wet weather discharges (combined sewer overflows and urban untreated or treated stormwater)	Freshwater (creeks and river)	Macro-invertebrates (benthic fauna)	Benthic communities abundance and richness; Evenness; Taxonomic composition	Grapentine et al., 2004
Matrix	Urban stormflows	Freshwater (river)	Macro-invertebrates (benthic fauna)	Total abundance and biomass (dry mass); Ephemeroptera, Plecoptera, and Trichoptera (EPT) contribution to the community (EPT taxa, EPT density and EPT biomass); Similarity (Jaccard index); Community tolerance index	Gray, 2004
Matrix	Urban land use (associated to sites impacted directly by discharges from combined or sanitary sewer overflows, sites receiving wastewater treatment plant (WWTP) discharges, sites impacted by instream sewer line placement and construction, etc.)	Freshwater (creeks and run)	Fishes	Index of Biotic Integrity (IBI); Relative abundance of sensitive and high sensitive species	Miltner et al., 2004
Matrix	Combined sewer overflows	Neither (batches and flow chambers)	Periphyton (algae and bacteria)	Total biomass (Dry mass, Ash free dry mass and Organic mass); Algal biomass (chlorophyll-a and phaeopigment concentrations); Bacteria abundance	Parent-Raoult, 2004; Parent-Raoult et al., 2005
Matrix	Urban stormwater runoff	Freshwater (run and branch)	Macro-invertebrates (benthic fauna)	Taxa abundance (Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Chironomidae, Tipulidae, Simuliidae, Isopoda, Platyhelminthes)	Gresens et al., 2007
Matrix	Urban stormwater runoff	Coastal marine water	Epifauna (invertebrates inhabiting the alga <i>Sargassum linearifolium</i>)	Total and Taxa (Anemones, Copepods, Gammariids, Gastropods, Isopods, Ostracods and Polychaetes) abundance	Roberts et al., 2007, 2008
Substance	Zn, Cu and Pb solutions	Neither (flow chambers)	Bacteria	Bacterial communities composition based on 16S rRNA gene sequences analysis	Ancion et al., 2010
Matrix	Combined sewer overflows	Freshwater (creeks)	Macro-Invertebrates (benthic fauna)	Global Normalised Biological Index (GNBI); Biological Monitoring Working Party (BMWP) score; Family and genus diversity	Rollin et al., 2010
Matrix	Urban road runoff	Neither (mesocosms)	Algae	Biological Diatom Index (BDI); Specific Pollutant sensitivity Index (SPI)	Johnson et al., 2011
Matrix	Urban Stormwater Runoff	Freshwater (streams)	Sediment denitrifying Bacteria	Algal taxa cell numbers and biovolumes; Biomass (chlorophyll-a concentration and ash-free dry mass (AFDM))	Perryman et al., 2011
Matrix	Urban freeway and residential stormwater runoff	Stormwater Treatment facility ponds	Macro-invertebrates (benthic fauna)	Communities structure (Based on terminal restriction fragment length polymorphisms of nosZ genes)	Tixier et al., 2011, 2012
Matrix	Urban stormwater impoundments	Freshwater (creek)	Fishes	Taxonomic richness; Abundance; Diversity (Shannon Wiener index); Evenness (Simpson's index); Oligochaete Index of Sediment Bioindication (IOBS); Index of sediment biological quality (IOBL) (based on Oligochaete taxonomic richness and abundance)	Stearman and Lynch, 2013

the substance approach, there are two main types of matrix approach: the single species bioassay approach where a single species is exposed to a matrix (generally in the laboratory), and the multi species approach where a community of species (generally species of different levels of biological organisation) is exposed all together and at the same time to the matrix studied (in microcosms or mesocosms). It is possible to perform these tests *in situ* by exposing the organisms directly in the contaminated environment. One of the drawbacks of this approach is the high variability when setting up the tests (inter-test variability) (Chapman, 2000).

There are two types of assessment to study the impact of urban wet weather discharges on ecosystems: ecotoxicological studies and studies requiring biological indices. Contrary to ecotoxicological studies, assessing the disturbance of aquatic communities using biological indices is mainly done through the matrix approach, since the aim is to study how the structures of the communities present in the receiving aquatic environments are modified. However, studies using the substance approach have been performed, as in the case of Ancion (2010), who studied the modifications occurring in bacterial communities due to exposure to heavy metals such as zinc, copper and lead present in urban wet weather discharges.

Whatever the case, focussing on the total effluent is far more representative of the phenomena induced as it is based on studies that underline the reactivity of the ecosystem, taking into account physico-chemical descriptors, environmental quality criteria, the habitat, the hydraulic regime, autochthonous biological communities and toxicological data.

2.1. Biological indices of ecosystem disturbance

Biological indices have been used in view to assessing the impact of urban wet weather discharges on the biodiversity of the communities in place. Most studies are performed on urban and sub-urban rivers and streams as they receive the water discharged from combined sewer overflows. Very few (6 studies identified) have concerned lacustrine environments or coastal areas and only one study has focused on groundwater. The studies performed most usually involve evaluations of abundance, species richness, community diversity (for example, using the Shannon index), and standardised indices for indicator species specific to the environment (invertebrates, fish, diatoms, oligochaetes, macrophytes). Three of them, the Global Normalised Biological Index (GNBI), the Biological Diatom Index (BDI) and the River Fish Index (RFI), have a good status reference framework. This is determined by the statistical exploitation of a large number of data obtained from field studies. However, these indicators are often designed with a single reference that does not take into account the type of watercourse. The different studies using indices to characterise urban wet weather discharges are summarised in Table 1.

2.1.1. Indices on decomposers (bacteria and fungi)

Modifications of bacterial communities in receiving environments mainly stem from the inflow of faecal bacteria from animal faeces (carried by runoff, etc.) and human faeces (combined sewer overflows, etc.). Thus the study by Dwight et al. (2011) on 78 stations on the south coast of California highlighted an imbalance in bacterial composition with the presence of pathogenic species which can therefore have an impact on human health. Bacterial community composition disturbance have relative unclear long-term effects for aquatic ecosystem, but probably leads to a modification of processes as decomposition, nutrient cycling, etc. (Wakelin et al., 2008). Moreover, fecal bacteria would be

responsible of the oxygen depletion (linked to biodegradable dissolved organic carbon) often observed with combined sewer overflows, and impacting survival of many organisms, as the fishes (Even et al., 2004).

Modifications can also occur within river communities as has been pointed out by several studies. Ancion et al. (2010) studied the modification of the composition of bacterial communities present in biofilms after exposure to metals mostly found in urban runoff (zinc, copper and lead), by using the polymorphism of DNA sequences located between genes coding for RNA 16S and RNA 23S.

Other authors (Maltby et al., 1995a) studied the impact of highway runoff (located on the outskirts of cities) on other communities of decomposers in rivers and streams, in this case fungi, more specifically hyphomycetes, by comparing their assemblages (with the determination of their species richness and the use of Relative Importance Values (RIV)) upstream and downstream of outfalls of contaminated water. It is important to monitor the composition of fungal communities, and more particularly aquatic hyphomycetes, as they are responsible for decomposing and modifying the structure of leaves (notably via the transformation of indigestible compounds into consumable ones) for their consumption by macro-invertebrates, and thus the distribution of both particulate organic matter and aquatic macro-invertebrates in rivers (Bärlocher, 1985; Maltby et al., 1995a).

2.1.2. Indices for monitoring primary producers

Since algae are specific species to the environment in which they exist, they are an excellent indicator of the impact of urban discharges on receiving environments. Contrary to certain benthic macro-invertebrates and fish, most of them are unable to migrate (drifting, burrowing, etc.) to habitats with more favourable conditions and less subject to the impact of discharges (Parent-Raoult and Boisson, 2007). Algae, along with detritus, are also the main source of energy in small watercourses (Maltby et al., 1995a), and the largest (macro-algae) shelter macro-fauna. Thus they play an essential role in watercourses.

Regarding algae, diatoms have been relatively well used for studying urban wet weather discharges and have been the subject of several studies (Gast et al., 1990; Willemssen et al., 1990; Sonneman et al., 2001; Rollin et al., 2010). In particular, diatoms are subject to a standardised index often used in the works cited previously, namely the Biological Diatom Index (BDI) based on the presence of species, their abundance and their sensitivity to pollutants, among other things. The determination of species and their abundance is often done by microscope on the basis of a minimum of 300–400 algae per sample taken randomly.

Assessing the impact of urban wet weather discharges on algae is not necessarily expressed by a reduction of abundance, biomass or the number of species (Maltby et al., 1995a; Rollin et al., 2010). For example, Maltby et al. (1995a) noted that neither abundance in epilithic algae, nor their diversity was affected by runoff discharges from roads. However, several authors have observed changes in the composition of algal communities. Sonneman et al. (2001) studied communities of epilithic and epiphytic algae in watercourses located around Melbourne and observed changes in the diatom communities exposed. However, contrary to the macro-invertebrates also affected, the impact on algae in this case was mainly explained by the high inflow of nutrients in the discharges, making these algae good indicators of the enrichment of the environment by urban activities. Rollin et al. (2010) studied colonisation by diatoms of media placed directly downstream of stormwater outflows using the BDI and a second index, the SPI (Specific Pollutant sensitivity Index) also based on the abundance of the species observed (400 individuals observed per sample recovered) and the sensitivity of species. They nonetheless

observed that the impact on diatoms was less marked than for other compartments (in this case macro-invertebrates). This could be due to a greater capacity for renewal in algae. Algae communities therefore present several drawbacks for monitoring the impact of urban wet weather discharges.

Although the study of communities has often been performed *in situ*, several authors have carried out experiments in the laboratory, namely on periphytons (algae and bacteria) subjected to simulated stormwater outflow discharges in artificial canals (Parent-Raoult, 2004; Parent-Raoult et al., 2005) and on communities studied in 1300 L mesocosms (Johnson et al., 2011).

2.1.3. Indices for monitoring benthic macro-invertebrates

The aqueous phase of urban wet weather discharges is not the only phase containing pollutants. Studies have shown that the particulate phase is the most pollutant discharge since most of the priority substances are found adsorbed to the surfaces of the particles (Parent-Raoult and Boisson, 2007; Zgheib et al., 2011). What is more, during the discharge of urban effluents, pollutants and particles fall progressively in the water column and deposit on sediments on the beds of watercourses (in the benthic zone). It has also been shown that the pollutants from discharges are also liable to accumulate in estuary sediments at concentrations likely to cause adverse biological effects (Morrisey et al., 2003). Therefore it is important to monitor the composition of macro-invertebrate communities since the compartment in which they live is highly exposed to dissolved and particulate fractions of urban wet weather discharges. Furthermore, since they colonise these sediments, they are heavily exposed to cumulative effects which makes them excellent bio-indicators (Rochfort et al., 2000; Komínková et al., 2005). This is why benthic macro-organisms are the indicators most often used to monitor the impact of urban wet weather discharges on aquatic ecosystems (see Table 3). The studies performed involved organisms of both the benthic and hyporheic zones.

Several authors have emphasised that the type of catchment cannot be the only parameter to be taken into account when evaluating the impact of urban wet weather discharges. Rainfall intensity and the sampling site play a major role. This is why, regarding the choice of sector to be prospected, Angerville (2009) hypothesised on the existence of a preferential zone governed by suitable conditions (gentle longitudinal slope, slow flow rate, laminar flow, etc.) favouring the settling of particles contained in urban wet weather discharges. Therefore interstitial organisms are exposed to a larger share of the particulate pollution contributed by urban wet weather discharges. This choice seems consistent to observe a strong impact on communities. Indeed, as shown in Borchardt et al. (1990), studying the loss of Gammarus populations, low flow rates cause a stronger effect. This may be due to of the sedimented particles containing adsorbed pollutants but also to a preferential particles deposition due to low flow (Brunke, 1999). This can lead to a siltation of interstitial zones hindering macro-invertebrates to protect themselves inside, and therefore leads to greater exposure to discharges (Borchardt et al., 1990).

The first studies of this type applied to urban wet weather discharges emerged as early as the beginning of the 1980s, notably those of Armstrong et al. (1980), Pratt et al. (1981) and Medeiros et al. (1983), with observations of a marked disturbance of communities by discharges. Contrary to other types of community (algae, decomposer and fish), benthic macro-invertebrates have been studied in most aquatic environments: marine (Schiff and Bay, 2003), estuarine (place of transition) (Morrisey et al., 2003), freshwater (Gray, 2004; Gresens et al., 2007) and artificial (treatment basins) (Tixier et al., 2011, 2012). Three orders of macro-invertebrates (Ephemeroptera, Plecoptera and Trichoptera (EPT))

were studied in particular by Walsh et al. (2001), Stepenuck et al. (2002), and Gresens et al. (2007). One of the reasons for this is that organisms of these three orders are relatively sensitive to pollution (Walsh et al., 2001) and their density and relative abundance declines rapidly as pollution increases.

There are two types of study dedicated to benthic macro-invertebrates for assessing the impact of urban wet weather discharges: direct sampling of the communities present on site, with the constraints of the sampling site mentioned above (meaning most studies), and studies based on the installation of an artificial substrate colonised by the organisms. But, in the two cases, an important limit is to find a reference site with no pollution, which is now very difficult.

Although most authors have sought to assess the direct impact of urban wet weather discharges (from separate or combined sewer networks) on benthic communities, others, on the contrary have sought to find a link between impervious urban area (in direct connection with quality and quantity of rainwater runoff) and the impacts on these communities. However, the use of these proxies is not always relevant. As explained by Walsh et al. (2001) in their study, alterations of the structure of macroinvertebrate communities between low and highly urbanised areas are not always explained by the imperviousness rate only, because some natural parameters can interact, as climate, habitat and catchment geology. Moreover, as they wrote, sites having the same rate of imperviousness don't always show the same impact on communities. It can be explained by: on one hand, the presence of systems of drainage infrastructure, and on another hand, the difficulty to make a direct link between imperviousness and pollutants concentrations in discharges, which depends on the watershed studied.

Measures of the species richness of benthic communities have often made it possible to assess the impact of pollution on the benthic communities. Generally, biological indices provide information in accordance with the decrease of richness. However, several studies have shown that if no change of species richness is observed, it is not possible to use biotic indices to draw conclusions as they often depend on this parameter (Cao et al., 1996). In addition, changes to some benthic macroinvertebrate communities can occur over different timescales (from a few months to a few years) following contamination (Damasio et al., 2008). These indices are used to show the effects of pollution already present for a long time. So, it is not advisable to use them to assess the impact of a one-time event, but rather a more diffuse pollution in time. Bioassays remain preferable in the other case.

2.1.4. Indices for monitoring fishes

In general, the impact of urbanisation on fishes has been given far less attention than to invertebrates (Paul and Meyer, 2001); likewise for urban wet weather discharges. Indeed, it is much more difficult to monitor their impact on fish communities as they are not confined to local receiving environments. Besides, due to their mobility, it is difficult to assess the time in which fish are really in contact with urban wet weather discharges (La Point and Waller, 2000). However, it is interesting to observe modifications of fish communities, as they have relatively long lifetimes and thus good indicators of long term disturbances. Indeed, several studies have sought to demonstrate the correlation between the imperviousness of soils due to the urbanisation of catchments (responsible for rainwater runoff which picks up all the molecules described previously from these surfaces) and the disturbance of fish communities in urban and sub-urban watercourses (Wang et al., 2001; Miltner et al., 2004). But, this method has the same limits than those for benthic communities. In these two studies, a common good health index of fish communities and watercourses was used: the IBI (Index of Biotic Integrity), calculated following the capture of

Table 2
Identification of the studies using a substance approach for the ecotoxicity assessment of urban wet weather discharges.

Pollutant	Approach	Organism type	Organism	Bioassay type	Exposure time	Endpoint	Author
Diazinon, Chlorpyrifos	Single and combined substance	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	24 h; 48 h; 72 h; 96 h	Mortality	Bailey et al., 1997
Zn, Cu, Pb	Single substance	Phytoplankton	x	Biomarker	6 h	Respiration/ Photosynthesis	Seidl et al., 1998
Zn	Single substance	Vertebrate	<i>Oncorhynchus mykiss</i>	Monospecific bioassay	96 h	Mortality	Bailey et al., 1999
Paraquat, Norflurazon, Atrazine, Flazasulfuron	Single substance	Plant	<i>Lemna minor</i>	Monospecific bioassay	48 h	Growth	Frankart et al., 2003
Zn	Single substance	Invertebrate	<i>Ceriodaphnia dubia</i>	Biomarker	48 h	Photosynthesis	Kszos et al., 2004
Atrazine, Diazinon	Single and combined substance	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	48 h	Mortality	Banks et al., 2005
Cu	Single substance	Vertebrate	<i>Oncorhynchus kisutch</i>	Biomarker	3 h	Neurotoxicity (olfactory system olfactory sensitivity) and predator avoidance behaviours	Sandahl et al., 2007
Cd, Cu, Pb, Zn, NH4	Single and combined substance	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	24 h	Mortality	Angerville, 2009
		Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	72 h	Growth	
NaCl	Single substance	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	4 weeks	Growth	Bartlett et al., 2012a
				Monospecific bioassay	1 week; 2 weeks; 3 weeks; 4 weeks	Mortality	
Galaxolide, Cd	Single and combined substance	Vertebrate	<i>Carassius auratus</i>	Biomarker	7 days; 14 days; 21 days	Oxidative stress	Chen et al., 2012
Zn	Single substance	Invertebrate	<i>Paratya australiensis</i>	Biomarker	72 h	Foraging behaviour	Oulton et al., 2014

fish by electrofishing in sampling zones. This index is especially easy to assess as fish are easy to collect and identify. Stearman and Lynch (2013) sought to establish a link between urbanisation, urban fragmentation, and rainwater management practices (especially those of reservoirs and basins), rather than the rainwater itself, and the disturbance of stream fish communities. Their study relied on the analysis of two indices: total species richness and a tolerance index based on the proportions of fish classed into categories of tolerance to habitat degradation and environment quality.

2.2. Ecotoxicological bioassays

The main studies and methods found for ecotoxicological bioassays carried out on urban wet weather discharges using substance and matrix approaches are summarised in Tables 2 and 3.

There are two possibilities for classing ecotoxicological tests: (1) classification into three categories: genotoxicity tests, acute tests and chronic tests; (2) classification with respect to the scale of the study. We have chosen the second solution for the sake of clarity. The methods used are classed into four groups: biomarker tests (genotoxicity, enzymatic activity, etc.), single species tests (organism scale), multi species bioassays (several species studied simultaneously) and *in situ* tests.

2.2.1. Biomarker tests

Several authors, including Tang et al. (2013), have observed that in the case of contamination by urban wet weather discharges, parameters such as photosynthesis (a toxicity biomarker) responded with greater sensitivity than more global parameters such as algae population growth (in particular due to the presence of herbicides). This is why biomarkers are especially important when studying the impact of urban wet weather discharges. The biomarkers are early pollution detectors. They enable both to identify the presence of toxic effects of pollutants faster than global parameters, but also to detect effects at concentrations below those

that product irreversible effects (van Gestel and van Brummelen, 1996; Burton et al., 2000). However, Marsalek et al. (1999a) observed that some biomarkers could be less sensitive than single-species assays (e.g. SOS-Chromotest less sensitive than testing *Daphnia magna* mortality to 48 h). One explanation mentioned is the difference in sensitivity to particles of discharges.

Several families of biomarker have been studied such as exposure and effect biomarkers, genotoxicity, cytotoxicity and biomarkers of enzymatic activity or more global metabolic activity.

Regarding genotoxicity and cytotoxicity biomarkers, tests such as the SOS-chromotest, which uses strains of the bacteria *E. coli* and measures beta-galactosidase activity to determine the level of induction of the DNA repair mechanism, and the umuC test, which is similar but uses a strain of *Salmonella typhimurium*, have already been the subject of several studies (Marsalek et al., 1999a, 1999b; Tang et al., 2013). Cytotoxicity tests based on the inhibition of mitochondrial electron transfer (Sub-mitochondrial Particle bioassays) have also been used to assess the impact of urban wet weather discharges (Tang et al., 2013).

Recent works on the primary producer compartment have studied chlorophyll fluorescence, which indicates photosynthetic efficiency, and enzymatic activity inhibition (esterase and alkaline phosphatase) underlying the metabolism of nutrients. These tests are used to assess the impact of diffuse pollution in runoff in urbanised coastal areas (Durrieu et al., 2011), as well as the quality of combined sewer overflow discharges and water collected at the inlets of infiltration basins (Ferro, 2013). Substance approach type tests focussing on the inhibition of several fluorescence parameters linked to photosynthesis due to pesticides found in urban wet weather discharges were also developed by Frankart et al. (2003). In addition, measures of metabolic activity (net primary productivity and respiration) by Seidl et al. (1998) and Boisson and Perrodin (2006) on the behaviour of primary producers have also been carried out to study discharges from CSO and road runoff.

What is more, Tang et al. (2013) also used measures of

Table 3

Identification of the studies using in matrix approach for the ecotoxicity assessment of urban wet weather discharges.

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
Urban road stormwater runoff	Downstream contaminated sediments (fractioned extracts)	Bacteria	<i>Photobacterium phosphoreum</i>	Monospecific bioassay	15 min	Luminescence inhibition	Boxall and Maltby, 1995
		Invertebrate	<i>Gammarus pulex</i>	Monospecific bioassay	14 days	Mortality	
Airport stormwater runoff	Discharges	Vertebrate	<i>Pimephales promelas</i>	Monospecific bioassay	48 h	Mortality	Fisher et al., 1995
		Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	48 h	Mortality	
Urban stormwater runoff	Downstream contaminated freshwaters and treatment pond discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	7 days	Mortality	Katznelson et al., 1995
Urban motorway stormwater runoff	Artificial pond waters and contaminated waters/sediments/sediments extracts	Invertebrate	<i>Gammarus pulex</i>	Monospecific bioassay	14/15/17 days	Mortality	Maltby et al., 1995b
Urban stormwater runoff	Urban stormwater pond sediment porewaters	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	48 h	Mortality	Wenholz and Crunkilton, 1995
Combined sewer outfalls and larger stormwater outfalls	Downstream contaminated freshwaters	Invertebrate	<i>Gammarus pulex</i>	<i>In situ</i> assay (caging)	36 days (daily assessment)	Mortality	Mulliss et al., 1997
Urban stormwater runoff	Urban stormwater pond sediments and discharges	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	10 days	Mortality	Karouna-Renier and Sparling, 1997
				Monospecific bioassay	10 days	Growth	
Sawmills stormwater runoff	Discharges	Vertebrate	<i>Oncorhynchus mykiss</i>	Monospecific bioassay	96 h	Mortality	Bailey et al., 1999
Urban stormwater runoff	Discharges and marine contaminated waters	Invertebrate	<i>Shongyocennofus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	Jirik et al., 1998
Combined sewer overflows	Downstream contaminated sediments	Invertebrate	<i>Rhepoxymius abronius</i>	Monospecific bioassay	10 days	Mortality	Hall et al., 1998
		Invertebrate	<i>Mytilus edulis</i>	Monospecific bioassay	48 h	Mortality	
Combined sewer overflows	Discharges (filtered and centrifuged)	Phytoplankton	x	Biomarker	6 h	Respiration and Photosynthesis (Oxygen production)	Seidl et al., 1998
Urban stormwater events	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	Exposition: 15 min – 6 h (very toxic samples)/12 h – 48 h (low toxic samples) Post exposition: 48 h	Mortality	Brent and Herricks, 1999
					48 h; 10 days/48 h; 7 days; 10 days	Mortality	
Urban stormwater runoff	Downstream contaminated freshwaters and sediments	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay/ <i>In situ</i> assay (caging)	10 days/7 days; 10 days	Feeding behaviour	Hatch and Burton, 1999
				Monospecific bioassay/ <i>In situ</i> assay (caging) Biomarker	20 min	Enzymatic activities (IQ toxicity assay)	
		Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay/ <i>In situ</i> assay (caging) Biomarker	48 h/48 h	Mortality	
		Vertebrate	<i>Pimephales promelas</i>	Monospecific bioassay/ <i>In situ</i> assay (caging)	48 h/48 h	Enzymatic activities (IQ toxicity assay) Mortality	
Urban stormwater runoff	Inflows and outflows from ponds	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	48 h	Mortality	Marsalek et al., 1999a

Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
	Inflows and outflows from ponds (concentrated 10 times)	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	15 min	Luminescence inhibition	
		Bacteria	<i>Escherichia coli</i>	Biomarker	?	Genotoxicity (SOS chromotest)	
		x	x	Biomarker	x	Mitochondrial toxicity (Sub-mitochondrial Particle bioassay)	
Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Inflows and outflows from ponds (concentrated 10 times)	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	48 h	Mortality	Marsalek et al., 1999b
		Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	15 min	Luminescence inhibition	
		Bacteria	<i>Escherichia coli</i>	Biomarker	x	Genotoxicity (SOS chromotest)	
		Inflows and outflows from ponds and downstream contaminated sediments	x	x	Biomarker	x	Mitochondrial toxicity (Sub-mitochondrial Particle bioassay)
	Bacteria		<i>Vibrio fischeri</i>	Monospecific bioassay	20 min	Luminescence	
	Bacteria		<i>Escherichia coli</i>	Biomarker	4 h	Genotoxicity (SOS chromotest assay)	
		Invertebrate	<i>Panagrellus redivivus</i>	Monospecific bioassay	96 h	Mortality	
				Monospecific bioassay	96 h	Growth	
Urban stormwater runoff	Discharges	Vertebrate	<i>Oryzias latipes</i>	Monospecific bioassay	9–12 days	Embryotoxicity (teratogenesis, hatching and mortality of eggs)	Skinner et al., 1999
		Vertebrate	<i>Menidia beryllina</i>	Monospecific bioassay	7–9 days	Embryotoxicity (teratogenesis, hatching and mortality of eggs)	
Urban stormwater runoff	Downstream contaminated freshwaters (after outlet of a detention pond)	Invertebrate	<i>Gammarus minus</i>	<i>In situ</i> assay (caging)	42 days	Mortality	Lieb and Carline, 2000
Urban stormwater runoff	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	96 h	Mortality	Schiff et al., 2002
		Invertebrate	<i>Mysidopsis bahia</i>	Monospecific bioassay	96 h	Mortality	
		Invertebrate	<i>Strongylocentrotus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	
Urban stormwater runoff	Discharges, downstream contaminated freshwater, marine contaminated sediments and interstitial waters from sediments	Invertebrate	<i>Strongylocentrotus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	Bay et al., 2003
		Invertebrate	<i>Rhepoxymius abronius</i>	Monospecific bioassay	10 days	Mortality	
Urban stormwater runoff	Downstream contaminated freshwaters	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	48 h; 96 h	Mortality	Gersberg et al., 2004
Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Stream sediments contaminated by untreated or treated runoffs/Contaminated downstream waters column by untreated runoffs	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay/ <i>In situ</i> assay (caging)	28 days/7 weeks	Mortality	Grapentine et al., 2004

(continued on next page)

Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
				Monospecific bioassay/ <i>In situ</i> assay (caging)	28 days/7 weeks	Growth	
	Stream sediments contaminated by untreated or treated runoffs	Invertebrate	<i>Chironomus riparius</i>	Monospecific bioassay	10 days	Mortality	
		Invertebrate	<i>Hexagenia spp.</i>	Monospecific bioassay	10 days	Growth	
				Monospecific bioassay	21 days	Mortality	
		Invertebrate	<i>Tubifex tubifex</i>	Monospecific bioassay	21 days	Growth	
				Monospecific bioassay	28 days	Mortality	
				Monospecific bioassay	28 days	Reproduction	
Simulated urban parking lot runoffs	Discharges	Invertebrate	<i>Shongyocennofus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	Greenstein et al., 2004
Urban stormwater runoff	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	48 h	Mortality	Kszos et al., 2004
Urban motorway stormwater runoff	Discharges	Periphyton	x	Plurispecific bioassay (artificial river)/Biomarker	1 h or 4 h, three time a week over 30 days	Biomass	Boisson and Perrodin, 2006
				Plurispecific bioassay (artificial river)/Biomarker	1 h or 4 h, three time a week over 30 days	Metabolic activity (net primary production and respiration)	
Urban and motorway stormwater runoff	Downstream contaminated freshwaters (whole and SPE concentrated), sediment porewaters and suspensions	Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	48 h	Growth	Christensen et al., 2006
	Downstream contaminated freshwaters	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	48 h	Mortality	
Urban and agricultural stormwater runoff and treated wastewaters	Downstream contaminated freshwaters and substrates (cobble)	Invertebrate	<i>Isonychia spp.</i>	<i>In situ</i> assay (exposure chamber)	96 h	Mortality	Custer et al., 2006
		Invertebrate	<i>Hydropsyche spp.</i>	<i>In situ</i> assay (exposure chamber)	96 h	Mortality	
		Invertebrate	<i>Psephenus herricki</i>	<i>In situ</i> assay (exposure chamber)	96 h	Mortality	
Urban and motorway stormwater runoff	Discharges	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	15 min	Luminescence inhibition	Scholes et al., 2007
		Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	48 h	Growth	
		Invertebrate	<i>Brachionus calyciflorus</i>	Monospecific bioassay	48 h	Reproduction	
Urban stormwater highway runoff	Discharges	Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	96 h	Growth	Kayhanian et al., 2008
		Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	7 days	Mortality	
				Monospecific bioassay	7 days	Reproduction	
		Vertebrate	<i>Pimephales promelas</i>	Monospecific bioassay	7 days	Mortality	
				Monospecific bioassay	7 days	Growth	
		Bacteria	<i>Photobacterium phosphoreum</i>	Monospecific bioassay	22 h	Luminescence inhibition	
		Invertebrate	<i>Strongylocentrotus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	
Urban stormwater runoff	Sediment porewaters from stormwater pond	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	7 days	Mortality	Mayer et al., 2008

Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
Urban stormwater runoff	Downstream contaminated freshwaters	Vertebrate	<i>Oncorhynchus kisutch</i>	<i>In situ</i> assay	5 year study	Mortality	McCarthy et al., 2008
Urban stormwater runoff	Stormwater ponds discharges and sediments Stormwater ponds discharges	Invertebrate	<i>Hydra hexactinella</i>	Monospecific bioassay Monospecific bioassay	96 h (daily assessment) Exposition: 1 h; 3 h; 5 h or 7 h; Post-exposition: 144 h (measurement each day)	Mortality Growth (Pulse toxicity test)	Rosenkrantz et al., 2008
Urban roadway stormwater runoff	Stormwater ponds discharges and sediments Stormwater ponds sediments Stormwater pond sediments	Vertebrate	<i>Bufo americanus</i>	Monospecific bioassay Monospecific bioassay	144 h (daily assessment) 40–48 days	Growth Avoidance behaviour (sediment avoidance) Embryotoxicity (delay of metamorphosis and mortality of embryos)	Snodgrass et al., 2008
Urban highway runoff	Discharges	Vertebrate	<i>Rana sylvatica</i>	Monospecific bioassay	13 days	Mortality (embryos)	
		Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	5 min; 15 min; 30 min	Luminescence inhibition	Waara and Färm, 2008
		Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	24 h; 48 h	Mortality	
		Invertebrate	<i>Thamnocephalus platyurus</i>	Monospecific bioassay	24 h	Mortality	
		Bacteria	<i>Lemna minor</i>	Monospecific bioassay	7 days	Growth	
Urban industrial and road stormwater runoff	Treated or not discharges	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	30 min	Luminescence inhibition	Wium-Andersen et al., 2009
		Algae	<i>Scenedesmus subspicatus</i>	Monospecific bioassay	72 h	Growth	
		Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	24 h; 48 h	Mortality	
Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Discharges, downstream contaminated surface freshwater, benthic and hyporheic zone samples	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	20 min; 30 min	Luminescence inhibition	Angerville (2009)
	Discharges	Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	72 h	Growth	
	Discharges and downstream contaminated freshwater	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	24 h; 48 h	Mortality	
	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	21 days	Reproduction	
	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	7 days	Reproduction	
	Discharges and downstream contaminated freshwater	Invertebrate	<i>Brachionus calyciflorus</i>	Monospecific bioassay	48 h	Reproduction	
	Discharges, downstream contaminated benthic and hyporheic zone samples	Invertebrate	<i>Heterocypris incongruens</i>	Monospecific bioassay	6 days	Growth	
Urban stormwater runoff	Downstream contaminated sediments	Plant	<i>Lepidium sativum</i>	Monospecific bioassay Monospecific bioassay	6 days 48 h	Mortality Germination	Karlavicienė et al., 2009

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Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
				Monospecific bioassay	48 h	Roots growth	
Urban stormwater runoff	Contaminated coastal marine waters and freshwaters	Invertebrate	<i>Strongylocentrotus purpuratus</i>	Monospecific bioassay	20 min	Egg fertilisation by spermatozoa	Reifel et al., 2013
Urban highly travelled roadway stormwater runoff	Stormwater pond sediments	Vertebrate	<i>Hyla versicolor</i>	Monospecific bioassay	x	Embryotoxicity (delay to metamorphosis and mortality of embryos)	Brand et al., 2010
Urban combined sewer overflows	Downstream contaminated freshwaters	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	15 min	Luminescence inhibition	Casadio et al., 2010
Highway and urban stormwater runoff	Discharges	Vertebrate	<i>Bufo viridis</i>	Monospecific bioassay	65 days	Embryotoxicity (teratogenesis, delay to metamorphosis and mortality)	Dorchin and Shanas, 2010
Urban stormwater runoff	Urban stormwater ponds and tanks discharges and sediments (inlet and outlet)	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	30 min	Luminescence inhibition	Karlsson et al., 2010
Campus parking lot stormwater runoff	Discharges	Invertebrate	<i>Ceriodaphnia dubia</i>	Monospecific bioassay	7 days	Mortality	McQueen et al., 2010
				Monospecific bioassay	7 days	Reproduction	
				Monospecific bioassay	7 days	Mortality	
Urban Stormwater runoff	Discharges	Vertebrate	<i>Oncorhynchus mykiss</i>	Monospecific bioassay	21 days	Growth	Milukaite et al., 2010
Urban Stormwater runoff	Urban contaminated streams	Vertebrate	<i>Oncorhynchus kisutch</i>	<i>In situ</i> assay	x (survey between 2000 and 2009)	Mortality	Feist et al., 2011
Urban highway runoff	Discharges	Algae	Algal community	Plurispecific bioassay (mesocosm (1300 L))	28 days	Biomass	Johnson et al., 2011
				Plurispecific bioassay (mesocosm (1300 L))/ Biomarker	28 days	Metabolic activity	
		Invertebrate	<i>Physa</i> sp.	Plurispecific bioassay (mesocosm (1300 L))	28 days	Growth	
				Plurispecific bioassay (mesocosm (1300 L))	28 days	Mortality	
		Vertebrate	<i>Lepomis gibbosus</i>	Plurispecific bioassay (mesocosm (1300 L))	28 days	Growth	
				Plurispecific bioassay (mesocosm (1300 L))	28 days	Mortality	
Urban Industrial stormwater runoff	Discharges	Algae	<i>Scenedesmus subspicatus</i>	Monospecific bioassay	96 h (daily assessment)	Growth	Kaczala et al., 2011
Urban highway and residential stormwater runoff	Stormwater pond sediments	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	4 weeks	Growth	Bartlett et al., 2012a, 2012b
				Monospecific bioassay	4 weeks	Mortality	
Urban Combined Sewer Overflows	Downstream contaminated surface freshwater, benthic and hyporheic zone samples (water phase) Downstream contaminated surface freshwater, benthic and hyporheic zone samples (particle and water phase separately)	Invertebrate	<i>Daphnia magna</i>	Monospecific bioassay	24 h	Mortality	Becouze-Lareure, 2012
				Monospecific bioassay	24 h	Mortality	
		Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	20 min (particles)/ 30 min (water)	Luminescence inhibition	
		Invertebrate	<i>Brachionus calyciflorus</i>	Monospecific bioassay	48 h	Reproduction	
		Invertebrate	<i>Heterocypris incongruens</i>	Monospecific bioassay	6 days	Growth	
				Monospecific bioassay	6 days	Mortality	

Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
Urban wet weather discharges (Urban road stormwater runoff and municipal wastewater)	Downstream contaminated freshwaters	Invertebrate	<i>Lasmsgona costata</i>	Biomarker/ <i>In situ</i> assay (sampling)	x	Immune function	Gillis, 2012
				<i>In situ</i> assay (sampling)	x	Age	
				<i>In situ</i> assay (sampling)	x	Condition (physical)	
				<i>In situ</i> assay (sampling)	x	Reproductive status	
Urban highway and residential stormwater runoff	Stormwater pond sediments	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	28 days	Mortality	Tixier et al., 2012
				Monospecific bioassay	28 days	Growth	
		Invertebrate	<i>Hexagenia spp.</i>	Monospecific bioassay	21 days	Mortality	
				Monospecific bioassay	21 days	Growth	
Urban stormwater runoff	Downstream contaminated freshwaters	Invertebrate	<i>Hyalella azteca</i>	Monospecific bioassay	96 h	Mortality	Weston and Lydy, 2012
Urban stormwater (runoff)	Discharges	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	30 min	Luminescence inhibition	Chong et al., 2013
Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Discharges	Algae	<i>Pseudokirchneriella subcapitata</i> ; <i>Chlorella vulgaris</i>	Monospecific bioassay	72 h	Growth	Ferro, 2013
		Algae	<i>Chlorella vulgaris</i> ; <i>Chlamydomonas reinhardtii</i>	Biomarker	2 h; 24 h; 48 h	Photosynthesis	
				Biomarker	2 h; 24 h; 48 h	Enzymatic activities (esterase, alkaline phosphatase, catalase)	
Urban wet weather discharges (combined sewer overflows and urban stormwater runoff)	Discharges (SPE extracts)	Bacteria	<i>Vibrio fischeri</i>	Monospecific bioassay	30 min	Luminescence inhibition	Tang et al., 2013
		Algae	<i>Pseudokirchneriella subcapitata</i>	Monospecific bioassay	24 h	Growth	
				Biomarker	2 h	Photosynthesis	
		x	x	Biomarker	x	Endocrine perturbation (E-SCREEN assay)	
		x	x	Biomarker	x	dioxin-like activity (AhR-CAFLUX assay)	
Bacteria	<i>Salmonella typhimurium</i>	Biomarker	x	Genotoxicity (umuC assay)			
		Biomarker	x	Oxidative stress biomarker (AREc32 assay)			
Urban parking lot runoff	Stormwater ponds discharges	Vertebrate	<i>Oryzias latipes</i>	Monospecific bioassay	14 days	Embryotoxicity (teratogenesis, hatching and mortality of eggs)	Colton et al., 2014
				Biomarker	20 h	Genotoxicity (LA-QPCR assay on embryos)	
Urban highway stormwater runoff	Treated and untreated discharges by stormwater pond	Vertebrate	<i>Danio rerio</i>	Monospecific bioassay	48 h; 96 h	Embryotoxicity	McIntyre et al., 2014

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Table 3 (continued)

Toxic matrix	Samples used for assays	Organism type	Studied organism	Bioassay type	Exposure time	Endpoint	Author
Urban highway stormwater runoff	Discharges	Bacteria	<i>Vibrio qinghaiensis.Q67</i>	Biomarker Monospecific bioassay Monospecific bioassay	3 h 96 h (daily assessment) 15 min	Neurotoxicity Mortality Luminescence inhibition	Wu et al., 2014
		Vertebrate	<i>Danio rerio</i>	Monospecific bioassay	96 h (daily assessment)	Embryotoxicity (FET-test) (teratogenesis, hatching and mortality of eggs)	

"Urban stormwater runoff" refers to discharges which may originate from several sources as residential, industrial, road or commercial area runoff.

enzymatic activity, though on recombinant DNA and reporter genes to detect specific classes of pollutants (notably PAHs) (E-screen test, Ahr CAFLUX, AREc32; see Table 3).

Lastly, oxidative stress biomarkers were observed in *Carassius auratus* by Chen et al. (2012) following exposure to reconstituted urban runoffs (samples consisting of distilled water and minerals regularly found in urban runoffs, added with the targeted pollutants).

2.2.2. Single species bioassays

These bioassays have undergone the most development and are those most often used in the laboratory. Some of them are now the subject of standards that allow comparing results between tests performed in different countries. The main organisms and single species bioassays associated with the study of urban and sub-urban runoff discharges are presented below.

2.2.2.1. Bacteria bioassays. It is important to study the decomposer compartment as the organisms of this group permit the transformation and degradation of many types of organic matter from animals and dead plants that decompose, for example, on the beds of watercourses. Generally, two main types of marine bacteria are used for bioassays to assess the toxicity of urban runoff discharges: *Vibrio fischeri* (Marsalek et al., 1999a, 1999b; Scholes et al., 2007; Waara and Färm, 2008; Wium-Andersen et al., 2009; Angerville, 2009; Casadio et al., 2010; Karlsson et al., 2010; Becouze-Lareure, 2012; Chong et al., 2013; Tang et al., 2013) and *Photobacterium phosphoreum* (Boxall and Maltby, 1995; Kayhanian et al., 2008). The assays identified for this purpose (Microtox® bioassays) are exclusively acute toxicity tests based on the inhibition of bioluminescence (caused by the oxidation of luciferin for *V. fischeri* and *P. phosphoreum*), expressing a dysfunction of the energetic pathway. The effects are generally visible after 15–30 min exposure. Microtox® bioassays are known to be relatively sensitive (Parvez et al., 2006) for samples of both the aqueous and sediment phases. In addition, they are fast, easy to perform and inexpensive (Parvez et al., 2006; Karlsson et al., 2010). This was also observed by Scholes et al. (2007), when setting up a battery of bioassays applied to urban and road runoff. However, some studies have shown the contrary, such as that by Wium-Andersen et al. (2009) for whom the Microtox® test presented the least sensitive response to road and industrial runoff in comparison to the other bioassays performed. These discrepancies show that the sensitivity of Microtox® for urban stormwaters toxicity assessment can vary widely depending on their origins, and thus their composition. Indeed, Boxall and Maltby (1995) reported that the Microtox® have a limited sensitivity for substances with a log Kow (coefficient of octanol/water partition) upper to 3 (for example, PAHs with a number of ring between 2 and 5). Moreover, Marsalek et al. (1999a) call into question the use of these marine bacteria to assess the

toxicity of chlorine-laden discharges.

2.2.2.2. Bioassays on primary producers: algae and plants. Bioassays on single cell algae are highly pertinent for studying urban wet weather discharges, as they represent the primary producer compartment, the first link in the food chain. Disturbances of the functioning of primary producers can precede damage to higher links in the trophic pyramid. Thus, bioassays on primary producers can lead to the early detection of pollution. Single cell planktonic algae (1–20 µm) have been the subject of many studies to assess both the impact of urban runoff from separate and combined networks. Two species have been studied for this purpose: *Pseudokirchneriella subcapitata* (Christensen et al., 2006; Scholes et al., 2007; Kayhanian et al., 2008; Angerville, 2009; Ferro, 2013; Tang et al., 2013) and *Scenedesmus subspicatus* (= *Chlamydomonas reinhardtii*) (Wium-Andersen et al., 2009; Kaczala et al., 2011). They are known to be relatively sensitive to pesticides (Rojčková and Marsálek, 1999) which are often found in urban wet weather discharges. The main bioassays on algae applied to urban wet weather discharges are based on the exposure of a population for a given time (generally 72 h, though exposures can vary from 48 to 96 h according to the study). Counting the number of cells before and after exposure shows the impact of the discharges studied on algal growth. The rapid growth of algae also facilitates performing chronic toxicity tests. Chronic toxicity is generally expressed by the concentration for which 10, 20 or 50% effect (growth inhibition) is observed (EC10, EC20, EC50), or more simply by the percentage of growth inhibition. The advantage of algae tests is that they require few technical and financial resources and the algae are easy to cultivate in the laboratory (Angerville, 2009). However, they suffer from a lack of reproducibility owing to considerable intra and inter-test variations.

Bioassays using macrophytes are also important though less used. Bioassays on aquatic plants such as *Lemna minor* are used to measure the impact of highway runoff (matrix approach) (Waara and Färm, 2008) and micropollutants such as pesticides often present in stormwater (substance approach) (Frankart et al., 2003). The tests applied on the plant are based on an assessment of frond growth inhibition after 7 days' exposure to discharges (generally expressed by percentage inhibition or by EC50). On the contrary, other plants such as *Lepidium sativum* are used to assess the toxicity of sediments (notably river sediments) contaminated by runoff from industrial and residential areas (Karlavicienié et al., 2009). In this case the test is based on the assessment of germination or root development after 48 h exposure to sediments.

As specified before, algal and macrophyte assays with a "matrix approach" are more realistic than "substance approach" assays, because they take into account all the pollutants and molecules in these complex discharges. But, one of the criticisms levelled at these tests is that they are quite sensitive to the presence of

nutrients (such as phosphorus, nitrates, etc.) and some trace elements (as metals) in the water (Kaczala et al., 2011), as these can mask the toxicity induced by the pollution of urban wet weather discharges and stimulate their growth in comparison to controls. This problematic phenomenon has already been observed by Kayhanian et al. (2008), Kaczala et al. (2011) or Ferro (2013). But as Kaczala et al. (2011) noted, if these nutrients lead to a stimulation of growth, another important problem can appear and need to be taken into account: a potential eutrophication created by the rejection of urban wet weather discharges in aquatic environments. So, primary producer's growth assays remain important to perform.

2.2.2.3. Bioassays on invertebrates

- Bioassays on daphnia and ceriodaphnia:

Tests on daphnia (*Daphnia magna*), organisms belonging to the family *Daphniidae*, small freshwater zooplanktonic crustaceans (<6 mm), are those most often used in ecotoxicology since they are easy to breed in the laboratory, they are ubiquitous, and because of their trophic level (Adema, 1978). The main tests performed to assess the impact of urban wet weather discharges on these organisms concern acute toxicity, for which the mortality of a population is observed for 24–48 h exposure to urban wet weather discharges or to water (river, sea, oceanic, etc.) receiving stormwater (Fisher et al., 1995; Hatch and Burton, 1999; Marsalek et al., 1999a, 1999b; Christensen et al., 2006; Waara and Färm, 2008; Wium-Andersen et al., 2009; Angerville, 2009; Becouze-Lareure, 2012). However, Schiff et al. (2002) extended the exposure time to 96 h. These tests are those used most often as they are very easy to implement and require few material or financial resources for tests using either a substance or matrix approach to assess the impact of urban wet weather discharges. For the most part they involve the calculation of LC50 values expressing the toxicity of the discharges. Chronic toxicity tests have also been used to study the reproduction inhibition of daphnia after 21 days exposure (Angerville, 2009).

Other organisms of the same family, though smaller, namely ceriodaphnia (*Ceriodaphnia dubia*), are also often used for the substance approach (Bailey et al., 1997; Kszos et al., 2004; Banks et al., 2005) and the matrix approach (Katznelson et al., 1995; Wenholz and Crunkilton, 1995; Brent and Herricks, 1999; Gersberg et al., 2004; Kayhanian et al., 2008; Angerville, 2009; McQueen et al., 2010). The principle of using ceriodaphnia for tests to study urban wet weather discharges is similar to that of tests using daphnia except that the exposure times are different. Regarding mortality tests, exposure times vary from 48 h to 7 days according to study, while 7 days is the duration usually used for reproduction inhibition tests.

- Bioassays on rotifers:

Rotifers (*Brachionus calyciflorus*) are freshwater pseudocoelomate metazoans. They play an important role in planktonic communities as they consume algae and are hunted by other invertebrates and fish. Rotifer tests applied to urban wet weather discharges consist in assessing the reproduction inhibition of organisms by counting them after 48 h exposure (Scholes et al., 2007; Angerville, 2009; Becouze-Lareure, 2012). These tests are of great interest for urban wet weather discharges as the effects of the toxicity of PAHs and PCBs on these organisms present sensitive and reproducible results (Dahms et al., 2011).

- Bioassays on ostracods:

Like many environmental matrixes, urban wet weather discharges contain a substantial particulate phase. Zgheib et al. (2011) showed that a large share of the pollution of urban wet weather discharges was found in the particulate phase of these discharges (pollutants such as heavy metals, PCBs and PAHs). Ostracods are bivalve arthropods and consume particles in suspension, on the surface and in sediments. Consequently, bioassays using ostracods are of interest in the presence of a matrix containing a sufficiently large proportion of particles. In addition, these organisms are quite sensitive to pollutants such as hydrocarbons, herbicides, pesticides and heavy metals (Ruiz et al., 2013) which deposit on the bottom of rainwater basins and on the beds of contaminated watercourses. Thus they are useful for assessing the impact of urban wet weather discharges. Tests using these organisms are based on 6 days' exposure to discharges, contaminated water and sediments, after which the survival of the organisms is observed and their size is measured to compare them with the measures taken at the beginning of the assay. The inhibition of their growth is determined and compared to that of controls (Angerville, 2009; Becouze-Lareure, 2012).

- Bioassays on sea urchins:

Urban wet weather discharges from urban areas located on the coast affect marine environments which has led to the use of single species bioassays on marine organisms of which one is used in particular, the sea urchin *Strongylocentrotus purpuratus* (Jirik et al., 1998; Schiff et al., 2002; Bay et al., 2003; Greenstein et al., 2004; Kayhanian et al., 2008; Reifel et al., 2013). The bioassays performed on this organism are based on assessing the inhibition of their reproduction. Thus, they are carried out on eggs and sperm. The sperm is exposed for 20 min to urban wet weather discharges or contaminated media, then brought into contact with the eggs for 20 more minutes to determine the inhibition of egg fertilisation in comparison to controls. Several studies performed to assess the toxicity of urban and road runoff in California liable to affect the coast showed that these organisms are highly sensitive to discharges, and represent the most sensitive of the batteries of bioassays performed (Schiff et al., 2002; Kayhanian et al., 2008). Greenstein et al. (2004) suggested that the toxicity observed on sea urchins caused by road runoff in particular (obtained from a university carpark) was due to the presence of metals (especially zinc) whose presence in these discharges is considerable. Kayhanian et al. (2008), proposed the same hypothesis.

- Bioassays on amphipods and crustaceans:

Crustaceans are organisms found in abundance in both marine environments and in streams and rivers. Therefore ecotoxicological bioassays have been carried out on freshwater organisms such as amphipods: *Gammarus pulex* (Boxall and Maltby, 1995; Maltby et al., 1995b) and *Hyalella azteca* (Karouna-Renier and Sparling, 1997; Hatch and Burton, 1999; Mayer et al., 2008; Bartlett et al., 2012a, 2012b; Tixier et al., 2012; Weston and Lydy, 2012) as well as on marine crustaceans: *Rhepoxynius abronius* (Hall et al., 1998; Bay et al., 2003). These organisms are often used to both assess the toxicity of contaminated discharges, water and sediments as they are detritivores. They are used to represent benthic organisms (notably in stormwater basins and in lentic systems) affected by urban wet weather discharges (Mayer et al., 2008; Bartlett et al., 2012a, 2012b). Generally, the tests performed on these organisms and applied to urban wet weather discharges are lethality tests. The main factor differentiating studies is exposure time, which can range from 48 h to 28 days. Certain authors have studied other sublethal parameters: growth and feeding behaviour (by studying

the consumption of leaf discs) (see Tables 2 and 3). Certain studies, such as those by Mayer et al. (2008), Bartlett et al. (2012a, 2012b), appear to show that amphipods are sensitive to salts and metals found in road discharges and stormwater basins and the sources of toxicity found in these species.

- Other organisms:

Many other freshwater and marine invertebrate species have been the subject of laboratory studies of urban wet weather discharges (all the organisms are listed in Tables 2 and 3). Mention can be made of the chironomid (*Chironomus riparius*) and worms (such as *Tubifex tubifex* and *Panagrellus redivivus*) which have been used to assess the toxicity of stormwater basins and that of the sediments of contaminated rivers. Also, other marine crustaceans have been used like *Mysidopsis bahia* to assess the toxicity of urban wet weather discharges on aquatic environments.

2.2.2.4. Bioassays on vertebrates. Regarding vertebrates, fish are frequently used for ecotoxicological tests as they are common in aquatic environments and also because they are of great economic value (Milukaite et al., 2010). Many species have been used in studies and the species represented in particular are: *Pimephales promelas* (Hatch and Burton, 1999; Kayhanian et al., 2008; McQueen et al., 2010) and *Oncorhynchus mykiss* (Bailey et al., 1999; Milukaite et al., 2010). The tests performed on fish are generally lethality tests (acute toxicity) with exposure times ranging from 48 h to 12 days according to study. The results of these tests are often expressed in survival percentage or LC50. Other tests have also been developed to assess the impact of urban wet weather discharges: embryotoxicity tests (Skinner et al., 1999) assess the lowest concentrations for which undesirable effects are observed (dysfunctions of eye size, pericardial effusion, etc.) are observed (LOAECs) and growth inhibition after 21 days exposure (Milukaite et al., 2010). Several studies have shown that tests on fish were more sensitive to urban wet weather discharges than classical tests on micro-crustaceans (especially *C. dubia*) (Kayhanian et al., 2008; Milukaite et al., 2010), although they are more cumbersome to set up. This sensitivity is due to the organic contamination of the discharges.

Other vertebrate organisms less known in ecotoxicology have been the subject of single species bioassays. Mention can be made of frogs (like *Bufo americanus*, *Rana sylvatica*, *Hyla versicolor* and *Bufo viridis*), for which the survival of embryos/larvae and delayed metamorphosis have been studied in particular (Snodgrass et al., 2008; Brand et al., 2010; Dorchin and Shanias, 2010) after exposure to discharges (especially from roads), and the sediments of contaminated stormwater basins, since frogs are often found in these basins where they lay their eggs and reproduce (Brand et al., 2010) due to the colonisation of the basins by plants.

2.2.3. Large scale multi-species bioassays: micro- and mesocosms

It is possible to change scales to obtain a more representative vision of the impact of urban wet weather discharges on the environment, by studying several trophic levels simultaneously. It is well-known that if a trophic link is affected, the links above it will also be affected. Consequently, multi-species bioassays and micro and mesocosm tests have been developed over the past few decades. However, these tests are more difficult to set up and are more expensive in terms of time, space and equipment. Also, the interpretation of the results is more complex. Few studies of this type have been performed for urban wet weather discharges. We only found one, performed by Johnson et al. (2011). They studied the impact of urban road runoff by using a 1300 L mesocosm composed biologically of an algal community (primary producer),

snails of the genus *Physa* (*Physa* spp.) (primary consumers) and the fish *Lepomis gibbosus* (secondary consumers) exposed for 28 days. Parameters such as biomass, metabolic activities, mortality and growth were measured. Only effects on algal populations were observed. They concluded that the quality of the water itself could not explain the impact on algae but supposed that the flow of runoff during renewal of events would be responsible. But, it is only assumptions without evidences. However, other authors such as Villeneuve et al. (2011) already showed the effects of diet flow (exposing periphyton to laminar or turbulent flow) of two pesticides (diuron and azoxystrobin) mixed on the composition of bacterial and algal communities exposed in mesocosms. They concluded that the flow regime had a real impact on the effects of the pesticides on the periphyton, with a higher sensitivity of communities to pesticides.

2.2.4. In situ bioassays

Contrary to laboratory bioassays, few studies have focused on the impact of urban wet weather discharges on the environment *in situ*. Indeed, we only identified 8 studies. Some authors nonetheless assert that the quality of receiving environments should be performed by *in situ* tests with an evaluation of communities (Marsalek et al., 1999b). These studies permit obtaining much better representativeness of the exposure of organisms to the matrices tested, as they take into account the temporal variations of discharge toxicity. Many of these studies (Mulliss et al., 1997; Hatch and Burton, 1999; Lieb and Carline, 2000; Grapentine et al., 2004; Custer et al., 2006) are based on the “caging” principle, which consists in caging the organisms studied and placing them in the contaminated matrix or in the environment receiving the discharges; the duration of exposure is fixed beforehand. Organisms such as gammarids, *H. azteca* and fish are often used for caging experiments. When setting up *in situ* tests, the exposure time for organisms is often longer than in laboratory tests. Mention can be made of Grapentine et al. (2004), who studied the toxicity of the sediments of basins and streams contaminated by urban wet weather discharges on *Hyalella* in the laboratory and *in situ* (caging) with exposure times of 28 days versus 7 weeks. This can be explained by the fact that organisms were exposed periodically to urban discharges during rainfall events, in renewed environments, and that the impacts were visible at the end of a longer period.

There are other types of *in situ* tests for evaluating urban wet weather discharges. Some consist in sampling organisms taken directly from contaminated environments and monitoring their physical condition through a reduction of immune system efficiency or monitoring the mean age of the organisms observed (Gillis, 2012). Others consist in monitoring, for example, the mortality over several years of fish species that swim upstream in water contaminated by discharges (McCarthy et al., 2008; Feist et al., 2011).

Several authors (Marsalek et al., 1999b; Bay et al., 2003; Tang et al., 2013) insist that in every case it is vital to multiply the number of bioassays (notion of battery) based on various organisms and toxicity parameters, as the discharges contain many micro-pollutants with very different modes of action, in view of refining the characterisation of urban wet weather discharges samples. As described above, the different organisms used for bioassays do not all have the same sensitivity to the different families of pollutants present (e.g., sea urchins are sensitive to metals whereas gammarids are more sensitive to PAHs; crustaceans (like ceriodaphnia) are more sensitive to pesticides than are sea urchins).

In addition, although batteries of bioassays permit observing the toxicity of urban wet weather discharges on different families of organisms, it remains vital to undertake chemical and ecotoxicological analyses in parallel (Karlsson et al., 2010).

2.3. An a priori method: the assessment of risks linked to urban wet weather discharges

Several risk assessment methods for urban wet weather discharges have already been used including for the assessment of ecological risks through the calculation of risk factors and the study of discharge pollution by methods like GIS (geographical information system).

The assessment of ecotoxicological risks by calculating risk factors is based on four major standard steps described by Perrodin et al. (2011). These are: 1 – The definition/formulation of the problem in which the study scenario is determined and the different parameters (such as discharge flows, flow rates, etc.) are presented and fixed. 2 – The characterisation of the exposure which permits calculating the Predicted Environmental Concentration (PEC) resulting from the concentrations of substances selected in the substance approach, and the percentage of the pollutant matrix in the environment in the matrix approach. 3 – The characterisation of the effects, which assesses the sensitivity of organisms to pollutants, especially through the utilisation of ecotoxicological databases (substance approach) and the organisation of ecotoxicological bioassays (matrix approach) which permits calculating the Predicted No Effect Concentration (PNEC), by assigning an extrapolation factor to this value. 4 – The characterisation of the risk, involving the calculation of a risk factor ($RF = PEC/PNEC$). This method has already been used by several authors such as Angerville (2009), Angerville et al. (2013) and Gooré Bi et al. (2015) for urban wet weather discharges. For example, the latter applied this method to combined sewer overflows in a river in a sub-urban district of Lyon, in France. A disadvantage of these methods is the number of organisms used to characterise the effects. Indeed, the smaller the number of organisms, the higher the extrapolation factor and thus also the risk (Angerville et al., 2013). It is therefore necessary to perform a large number of tests (in the case of a matrix approach) to obtain a risk as close as possible to that of the real risk. Eriksson et al. (2007) used a procedure (known as CHIAT: Chemical Hazard Identification and Assessment Tool) derived from the previous one, to calculate the risk due to xenobiotic organic compounds (XOCs) present in urban wet weather discharges into an urban stream in Denmark. Other authors like Hall et al. (1998) and Brix et al. (2010) used the same approach described by Perrodin et al. (2011), by following the principle of the four steps but by using a probabilistic approach considering, according to the authors, the variability of exposures between individuals of a population to determine the risk. Weinstein et al. (2010), used two risk assessment approaches, including one similar to that of Perrodin et al. (2011), but based on calculating two hazard coefficients representing the ecological risk linked to PAHs present in the retention basins studied and based on concentrations below which it is unlikely to observe harmful effects on (TEC: Threshold Effect Concentration), concentrations above which such harmful effects are probable (PEC: Probable Effect Concentration), and on PAHs concentrations observed in sediments.

One of the first objectives of risk assessment is to identify hazard. Thus Baun et al. (2006) created a method (RICH: Ranking and Identification of Chemical Hazards), which, on the basis of identifying different pollutants in urban stormwater, aims at establishing a hazard priority ranking, by taking into account physico-chemical, persistence, bioaccumulation and ecotoxicity data. This method permits, among other things, choosing the molecules to be studied more precisely in procedures like risk assessments based on calculating PEC/PNEC, the CHIAT method, etc.

In the study of ecological risks, the probability of occurrence of ecological impacts is synonymous with risk (Ellis, 2000). Therefore

Novotny and Witte (1997) formulated a risk assessment method related to the discharge of urban wet weather discharges in watercourses. It was based only on probabilistic statistical models that took into account parameters such as the probability of a rain event permits obtaining a certain concentration of pollutants in rainwater, and the probability that this contamination will affect species present in the environment (based on substance type bioassays for 96 h on priority pollutants).

Ellis (2000) described the advantages and disadvantages of these two types of risk assessment methods (approach by direct toxicity assessment (DTA) and the probabilistic approach). The latter only concerns acute risks due to urban wet weather discharges, and does not permit taking account of pollutant bioaccumulation phenomena or chronic effects due, for example, to contaminated sediments. Furthermore, this type of method is based only on bioassays most often performed for 48 or 96 h in the laboratory, and not *in situ*. Also it uses inert substrates (sediments) and does not take into account suspended particles. It is possible for risk to be underestimated as the models built fail to take into account the “cocktail” effect of urban wet weather discharges, thus the synergetic effect of pollutants, and the multiple exposures of aquatic organisms to pollutants. However, this was done in the methods employed in particular by Perrodin et al. (2011) and Angerville et al. (2013) and which are widespread. Nonetheless, another limitation of these methods is that they do not take into account the duration of exposure to discharges, since toxicity tests (often performed in the laboratory) do not take into account the transient effects of discharge pollution or seasonal or even diurnal variations (Ellis, 2000). What is more, the bioaccumulation phenomena occurring in trophic networks, and those of eutrophication in aquatic ecosystems, are rarely considered in this type of method (Perrodin et al., 2011). The same problem of ignoring synergy/antagonism also appears in substance type assessments.

Lastly, other authors use a GIS approach to predict the risk of urban wet weather discharges spreading contamination to water bodies. This was, for example, the case of Zandbergen (1998) who used a set of indicators (impervious areas, river habitat, pollutant loads, water quality, sediment quality, fish and human health) to which he assigned a score expressing good or bad status in order to draw a map showing the evolution of these indicators for the entire catchment of the River Brunette in the area of Vancouver. Ellis et al. (2012) also implemented a semi-quantitative method to assess the risk of discharges passing through a sustainable urban drainage system (SUDS) (such as retention basins, porous roads, etc.) and their capacity to reduce the risk of discharges. To do this they used a pollution index and a pollution reduction index based on a GIS that took into account different indicators like sealed surface, runoff concentration/load and the potential treatment performance of individual SUDS. However, these tools still need developing as they consider only the worst cases of pollution by runoff. Nonetheless, they encourage the creation of more refined methods for assessing the risks linked to them (Ellis et al., 2012).

More qualitative methods also exist for characterising the risk linked to pollutants in urban wet weather discharges, like that of Lundy et al. (2012), based on the formulation of numerical values (1–5) for both the probability of occurrence of these pollutants in discharges, and of their level of impact on receiving aquatic environments.

2.4. Methods for modelling the pollution impact of urban wet weather discharges

Other less frequently used methods of studying the impact of urban wet weather discharges have emerged in recent years. Among these, methods of modelling the toxicity of urban wet

weather discharges have been developed. The main causes of mortality observed in fish during rainfall events are hypoxia and the presence of ammonia in large quantities. Thus Magaud et al. (1997) developed a model based on laboratory tests performed on *O. mykiss*. They built a model expressing the probability that these organisms would die as a function of two key parameters for their survival: concentrations of ammonia and dissolved oxygen in water. The limitation of their model was that it failed to take into account other parameters crucial for the survival of the fish studied: exposure time and the concentration of the toxic pollutants in the discharges. Indeed, Zhang et al. (2011) developed a time-dependent model of the toxicity of PAHs present in urban road discharges based on a model built by Lee et al. (2002a, 2002b). To do this, they took into account spatial–temporal variations in the composition of discharges, with a pulse-exposure of organisms, to make it more realistic. They coupled the known ecotoxicological data of these compounds with field data (concentrations of PAHs in the discharges and the duration of rainfall events) to calculate the toxicity that these events would cause for different aquatic species. The aim of the model was to take into account parameters that classical laboratory tests cannot, such as the considerable variability of rainfall events. Indeed, it is acknowledged that it is difficult to characterise the impact of these events using aquatic organisms after short and intermittent exposures. However, if some authors tend to say that the flow regime (laminar or turbulent) during rainfall events could also have an effect on the impact of urban discharges during rainy weather on the ecosystem, no study considers the parameter in their models.

In addition, as shown by Magaud et al. (1997), it is important to couple the different parameters studied in models (in this case oxygen and ammonia) rather than simply add the effects of each parameter separately. Indeed, the authors obtained lower fish survival values when the fish were exposed to two combined stresses, rather than the effects obtained for each stress were added. These observations reflect the problem of the synergies and antagonisms between different pollutants and parameters.

3. The limits of current methods and the alternative techniques implemented

We saw previously that each of the techniques and organisms used has advantages and disadvantages for studying the impact of urban wet weather discharges. The aim here is to identify the different limits of the methods used classically, and the different solutions that may be proposed.

As explained above, physico-chemical analyses do not allow explaining the impact of discharges on organisms (Ellis, 2000). Furthermore, they often underestimate the severity of contamination in the water column, as this can vary very rapidly (Burton et al., 2000). To achieve this, it is therefore necessary to perform studies on living organisms. Although acute and chronic bioassays in the laboratory have allowed the observation of toxicity on different species, their use for assessing the impact of urban wet weather discharges is limited by several weaknesses:

- There is a lack of representativeness of the exposure of organisms to the discharges studied. Indeed, these studies often consider that organisms are exposed to pollutants continuously. However, it is acknowledged that in reality, due to short though intense rain events, these organisms are exposed episodically to discharges for several hours. This is why certain researchers have sought to develop alternative methods in which organisms are exposed to shorter periods (several hours) before observing the effects of this exposure over several days in a test medium for control. This is the pulse-exposure method implemented in

particular by Brent and Herricks (1999) and Rosenkrantz et al. (2008). These exposures often produce higher toxicity than continuous exposures to discharges (Burton et al., 2000). Laboratory tests therefore lead to underestimation of the toxicity of urban wet weather discharges.

- In addition, the samples (whether contaminated water or sediments) used in laboratory bioassays are often conserved by freezing. Thus the organic micropollutants (such as pesticides and PAHs) are liable to be degraded. Although this has been demonstrated with pharmaceutical compounds in wastewater (Fedorova et al., 2014; Negreira et al., 2014), no study has yet focused on this phenomenon in other families of toxic micropollutants. However, it is highly likely that this is the case with the different families of organic micropollutants found in urban wet weather discharges.
- Due to reasons that are often of a technical, human or material nature, the ecotoxicological studies performed on the urban wet weather discharges frequently use punctual samples taken from the sewer systems draining these discharges; temporal variability is not represented in this case regarding drainage installations.
- Lastly, often a single (or a relatively limited number) species is studied, whereas it is necessary to widen the scope (only one mesocosm study has been performed) of studies on the impact of urban wet weather discharges to obtain more results more representative of ecosystem impacts.

Although biological indices can be used to study modifications in the composition of communities exposed *in situ* to rain events, by conforming to the dilution of discharges in the environment and to exposure times, they nonetheless have major disadvantages:

- Species can also react to a stress other than that sought (contamination by pollutants) (Parent-Raoult and Boisson, 2007; Damasio et al., 2008) which makes the interpretation of the results much more difficult than with ecotoxicological tests.
- There is also a problem of natural variability (Roper et al., 1988) in space (notably in estuaries) and time (reproduction cycles during the year, etc.), which makes it difficult to assess the impacts that can be very different over very short distances and the times of the year when communities are analysed.

Risk assessments and impact modelling methods do not allow asserting that an impact will actually occur in an ecosystem, although they remain a good preventive tool for managers of urban wastewater treatment and drainage networks.

Consequently to offset the limits of different methods, most of them are in combination in order to complete and compensate the failings of each one, that is to say that ecotoxicological studies and biological indices are frequently coupled with chemical analyses to better understand the results obtained. The only disadvantage is that physico-chemical analyses do not allow to assess which group of pollutants is responsible in particular for the effects observed (except when only one group of pollutants is present). This is why studies are generalised in the assessment of urban wet weather discharges: the methods used in this case are known as Toxicity Identification Evaluations (TIE). These are initially based on establishing a general profile of the compounds likely to be the source of toxicity, after which the matrixes are studied. For example, Gersberg et al. (2004) used EDTA, a chelating agent, to remove all the divalent metals present in the samples in order to test toxicity once the target group of micropollutants had been eliminated.

Furthermore, other approaches have been developed, such as the triad approach for benthic organisms (in rivers, seas and basins), which consists in performing a physico-chemical analysis,

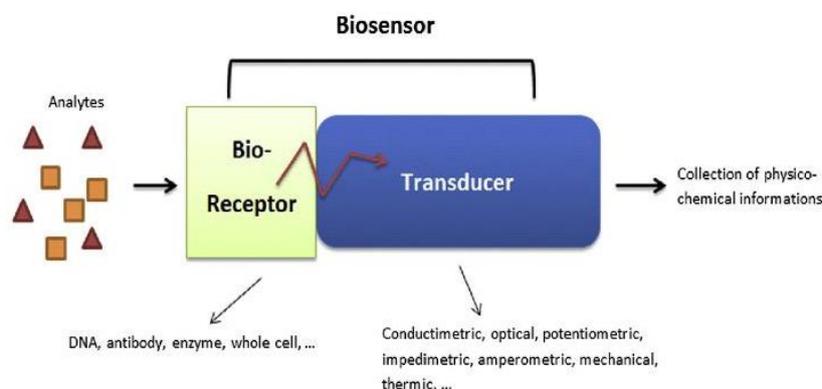


Fig. 2. Schematic diagram of how a biosensor functions. The red arrow shows the transmission of the biochemical information received by the bio-receptor to the transducer which changes it into a physico-chemical signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ecotoxicological bioassays and an *in situ* study at the same time on communities to obtain a global vision of the impact of urban wet weather discharges. This was done, for example, by Tixier et al. (2012). However, these studies are relatively complex to perform. *In situ* bioassays (like caging an organism on site before and after urban wet weather discharges) nonetheless represent a good compromise between laboratory studies and studies of communities.

4. Towards new procedures for studying the pollution impact of urban wet weather discharges: biosensors

As seen previously, “classical” impact studies do not appear to fully represent the harm really affecting the environments receiving urban wet weather discharges, and some of them are cumbersome to implement. It is therefore necessary to find new means for monitoring urban wet weather discharges. Certain studies have dealt with the utilisation of sensors capable of continuously monitoring physico-chemical parameters on receiving sites affected by urban wet weather discharges (Ruban, 1995; Zug et al., 2001). However, biological monitoring is required to evaluate the ecological impact of urban wet weather discharges. Biosensors have been developed over the last ten years to solve this shortcoming. They are tools comprising two components: a bio-receptor and a transducer capable of converting biochemical information (like a concentration into analytes) emitted by the receptor into physico-chemical signals (Thévenot et al., 2001) (see Fig. 2). The bio-receptors are generally in contact with a transducer or immobilised on it.

Biosensors have been applied more recently to studying the environment. Their sensitivity has been proven and they allow continuous monitoring of water pollution *in situ* (Rogers, 1995), contrary to laboratory tests. Thus, they are ecologically pertinent tools. They also have the advantage of being far less expensive than chemical analyses and classical ecotoxicological tests, while providing good sensitivity, selectiveness and reproducibility (Dennison and Turner, 1995; Rogers, 2006). Furthermore, certain biosensors are very useful tools as they do not only determine the presence and even the concentrations of chemical pollutants in an aquatic ecosystem, they can also be used to observe a biological response of the organisms or the aquatic environment analysed to the toxicity of a pollutant or discharge (Rodríguez-Mozaz et al., 2005).

The nature of bioreceptors used for monitoring aquatic environments is varied (see Fig. 2). DNA, antibodies or purified enzymes can for example constitute the bioreceptor, with high specificity

reaction with the analyte. However, their main drawback is that they are unrepresentative of the real impact of pollutants on the aquatic environment and only to detect the presence of their target compounds. They don't take into account the bioavailability of pollutant, and the fact that in cells some pollutant's sensitive enzymes can be conserved by native cellular machinery (Pancrazio et al., 1999). This weakness has been improved by development of whole-cell biosensors which often use unicellular microorganisms. They allow to take into account the complex relationships between different cellular compartments and enzymes. They also have the advantage to give informations on both the bioavailability and the presence of pollutants, and give various toxicity informations according to the studied metabolic reactions and transducers used (Hansen and Sorensen, 2001; Lagarde and Jaffrezic-Renault, 2011). Thus, unicellular organisms such as bacteria (Willardson et al., 1998; Kumar et al., 2006; Gavlaseva et al., 2008) algae (Chouteau et al., 2005; Tsopela et al., 2014) or yeast (Campanella et al., 1995; Hollis et al., 2000) have been used to measure the toxicity of pollutants. It is therefore possible to monitor the impact of toxic both in prokaryotes and eukaryotes (Lagarde and Jaffrezic-Renault, 2011). Furthermore, these whole-cell biosensors remain, despite the complexity of the organisms, relatively sensitive results in numerous contaminants, as shown Chouteau et al. (2005) who developed conductometric biosensor algal cell, allowing the observation of inhibition of the alkaline phosphatase enzyme activity (APA) to observe effects at concentrations of metals (Zinc, Cadmium) of 10 ppb. Thus, they should be preferred to allow a realistic observation (when they are not genetically modified) of the effects of urban wet weather discharges on the environment. However, whole-cell biosensors also have some drawbacks. For example, the bioreceptor immobilisation step on the transducer can lead to a decrease of the biosensor sensitivity (Eltzov and Marks, 2011). For example, immobilisation using matrix as alginate, albumin or sol-gel which can create a barrier against the diffusion of pollutant to the organisms. It has been shown by Awasthi and Rai (2005), with algae immobilised in alginate and exposed to nickel, zinc, and cadmium. In the case of physical adsorption of cells, as for conductometric biosensors, a release of cell can appear after few days. It has been demonstrated by Guedri and Durrieu (2008) with algae physically immobilised on self-assembled monolayers (SAMs) of alkanethiolate. Another important point is the conservation of the total integrity and activity of the cells, without any perturbation. Some procedure, as direct sol-gel matrix immobilisation of cells, can impact cell viability (Ferro et al., 2012). Finally, some problem of reproductivity can also appear (Gu et al., 2004). It's why further researches are still

necessary to improve these critical points.

A large number of biosensors have been developed according to the families of toxic pollutants to be studied. Thus biosensors have already been used to measure the presence and/or impact of pesticides (Védrine et al., 2003; Chouteau et al., 2005; Touloupakis et al., 2005; Mauriz et al., 2007; Durrieu et al., 2011), heavy metals (Bontidean et al., 2003; Chouteau et al., 2005), PAHs (Alarie et al., 1990; Willardson et al., 1998) and PCBs (Gavlasova et al., 2008), all pollutants found in urban wet weather discharges. However, biosensors are often based on a substance approach, where the pollutant studied is diluted in a solution of pure water. Thus they do not clearly establish the impact of pollutants in whole discharges.

Studies have already been performed to evaluate the hazard of certain urban discharges (*in situ* matrix monitoring). This is the case of wastewater treatment plant effluents, and bacterial biosensors have been developed to study PAHs, phenols, pharmaceutical compounds and pesticides (Farré and Barceló, 2003). However, there is a surprising lack of attention given to the continuous *in situ* monitoring of urban wet weather discharges as a study matrix. Only one study identified, by Ferro (2013), attempted to evaluate the impact of urban wet weather discharges using biosensors based on algal cells. He highlighted the sensitivity for studying discharges of certain enzymatic biomarkers such as Esterase Activity (EA) and Alkaline Phosphatase Activity (APA) in the implementation of conductimetric biosensors, Catalase Activity (CA) coupled with an oximetry transducer, and photosynthetic activity coupled with an optical transducer. Statistical treatments performed in comparison with the compositions of the discharges studied and the disturbances caused by these activities have shown that they are specifically inhibited by certain of the families of pollutants present (e.g., effects of PAHs on EA and effects of metals on APA). But, the specificity of enzymatic or metabolic response to pollutants remains challenging to bring out (Gu et al., 2004).

Although biosensors remain qualitative tools at present, they nonetheless represent an efficient resource for predicting and evaluating the pollution of urban aquatic environments.

5. Conclusion and outlook

In this study we attempted to summarise all the different techniques used at present to evaluate the impact of urban wet weather discharges on ecosystems. Despite the growing number of studies on evaluating the impact of urban wet weather discharges, their number still remains relatively low (as shown in Tables 1–3). The important spatial and temporal variation of urban wet weather discharges complicates the assessment of their impact (by substance and matrix approach) on ecosystems (marine and freshwater). It's why a large array of methods/tools has been developed, which generally include chemical and physicochemical analyses, batteries of bioassays and *in situ* tests (whether through the study of biological indices or ecotoxicological studies).

Nonetheless, chemical and physico-chemical analyses alone are not enough to predict the ecological impact of discharges on ecosystems. Ecotoxicological bioassays, most usually carried out in the laboratory (though sometimes *in situ*) only take into account a small number of selected organisms and lack realism (especially for the substance approach). It's why the utilisation of these bioassays is now questioned. They are performed under controlled conditions on samples taken and often stored for periods of varying length. However, in recent years, researchers have tended to show that the conservation of samples, even by freezing, leads to their modification (such a reduction in the concentrations of micropollutants present).

It is now well known that field studies (bioassays and indices) integrate the complexity of phenomena more completely. However,

in order to perform in-depth studies of the impact of urban wet weather discharges, many researchers combine ecotoxicological studies, biological indices and chemical analyses to determine which pollutants are responsible for the impact of discharges.

These methods are nonetheless expensive and time-consuming to implement, and they do not permit continuous monitoring of the pollution of urban wet weather discharges. Moreover, the complexity of the results obtained often leads to a limitation of their interpretation. This has led over the past few years to the development of new tools for monitoring them: biosensors. These are designed to evaluate the toxicity of matrixes for organisms exposed *in situ* and continuously. They are good tools that could be useful both for researchers and for managers of urban wet weather discharges. In an embryonic stage at present, they don't allow quantifying pollutants in environments, and chemical analyses remain essential. The aim of these tools is therefore to backup those already available. They are still subject to laboratory studies and only one study (using immobilised algae) has emerged. For all that, the potential of biosensors is strong, which makes it necessary to:

- Continue to develop the utilisation of existing biosensors (with algae) that appear promising for *in situ* and continuous monitoring and develop new biosensors using species from other compartments (primary and secondary consumers, decomposers) and adapted to the field. This would permit monitoring the toxicity of discharges continuously *in situ* at different trophic levels and lead to better understanding of their impact on ecosystems, since the effects observed by monitoring only one species cannot be extrapolated to other organisms.

In addition to developing biosensors:

- Favour more comprehensive *in situ* studies of the variation of pollution in watercourses during rainfall events. These would take into account the whole rainfall event, contrary to studies performed on samples taken punctually.
- Improve and facilitate the development of laboratory techniques to obtain more sensitive results, especially through longer exposures and chronic bioassays that often show toxic impacts not detected by acute toxicity tests, and tests that are more representative of the exposures actually incurred by organisms, something that has already been done, for example, with pulse exposures.

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1.2.3 Synthèse et discussion

Au cours de cette étude, nous avons pu observer qu'un nombre encore limité d'études s'intéressent à l'impact des RUTP sur le biotope (e.g. 57 études écotoxicologiques sur matrice entière recensées, un faible nombre comparé à celles menées sur les eaux usées de station d'épuration). Les études sur la perturbation des communautés aquatiques (utilisant bien souvent des indices biologiques) sont soumises à des facteurs externes rendant difficiles la discrimination des effets dus aux RUTP. Les études écotoxicologiques menées sur les RUTP ont été réalisées pour la plupart en laboratoire (8 seulement *in situ*), en conditions contrôlées, sur matrices entières (échantillons de RUTP prélevés sur le terrain, conservés (e.g. par congélation) ou utilisés directement). La mise en œuvre des essais *in situ* (plus représentatifs des conditions réelles de terrain) nécessite bien souvent beaucoup de matériel et de temps. De plus, les matrices pluviales polluantes apparaissent de manière épisodique, et non sous forme d'une pollution continue dans le temps ; il est donc difficile d'organiser la programmation des expérimentations. Il est intéressant de noter que des auteurs ont travaillé en laboratoire à la réalisation de bioessais en « pulse exposure » mimant des expositions transitoires dans le temps. En ce qui concerne les essais *in situ*, depuis plus d'une décennie des outils tels que les biocapteurs se développent. Ils permettent d'effectuer des mesures écotoxicologiques *in situ*, en continu, et constituent ainsi des outils d'alerte précoce en rendant possible la mesure rapide de l'impact des rejets. Cette orientation pourrait donc être une bonne alternative aux essais écotoxicologiques menés actuellement, et a fait l'objet d'un développement important durant ces travaux de thèse. Leur principe d'action, intérêt, points cruciaux de développement et des exemples sont présentés en préambule dans la sous-partie suivante.



1.3 Biocapteurs : application au monitoring environnemental

Cette partie présente un état de l'art sur les biocapteurs dédiés à la mesure de l'écotoxicité (notamment les biocapteurs à cellules entières microbiens) et les enjeux liés à leur conception. Les biocapteurs sont des outils d'intérêt pour le suivi *in situ* et en continu, de tout type de rejets polluants, et notamment des rejets urbains (comme les RUTP). Ils peuvent ainsi contribuer à la surveillance de la qualité des milieux aquatiques.

1.3.1 Biocapteurs : définitions et rappels

Les biocapteurs sont des outils qui permettent de mesurer et de transformer un phénomène biologique/biochimique en signal physique (e.g. optique, électrique). Comme vu dans l'article 2, ces outils sont généralement composés de deux éléments : un élément biologique, appelé bio-récepteur immobilisé sur un transducteur, qui émet le signal biologique/biochimique, converti en un signal électrique. L'ensemble est connecté à un appareil de mesure capable d'enregistrer les informations acquises (Figure 1). Initialement, ces outils ont été développés dans le domaine médical avec notamment la création du biocapteur de mesure du glucose sanguin, basé sur la réaction de l'enzyme glucose oxydase (Clark et Lyons, 1962). Depuis, leur principe a été appliqué à de nombreux domaines et notamment à l'environnement. Les principaux avantages des biocapteurs résident dans leur très grande sensibilité, leur bas coût, et leur rapidité de réponse.

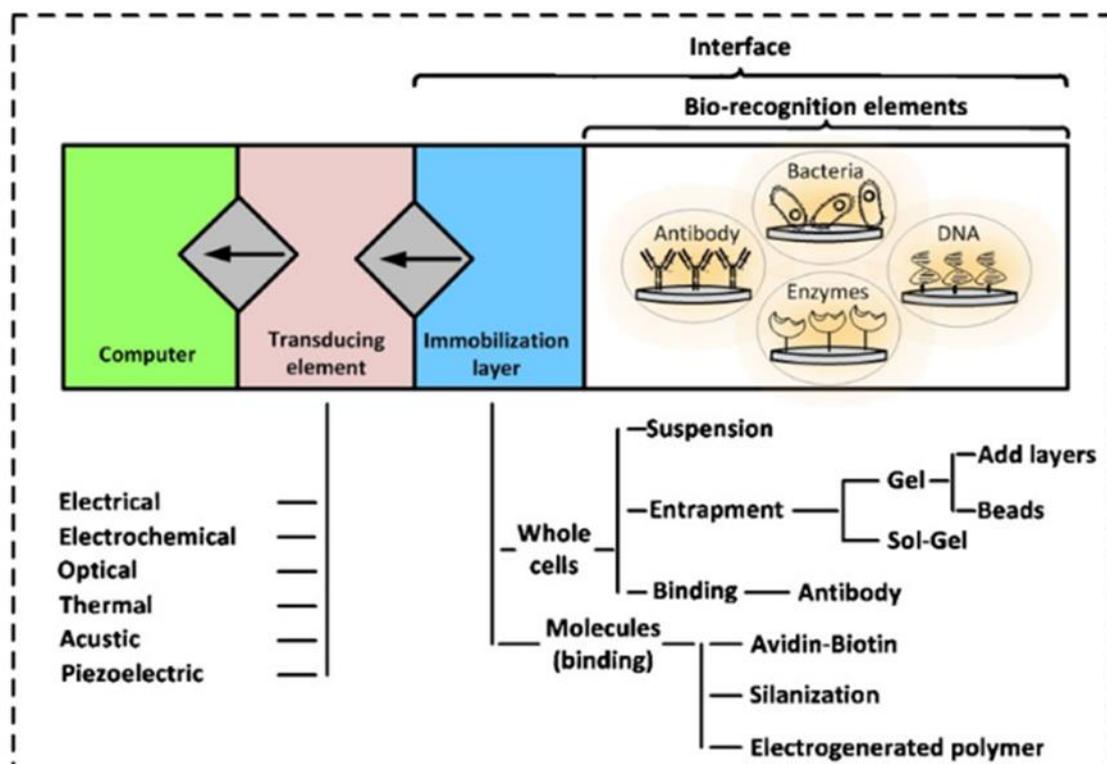


Figure 1 : Présentation du concept de biocapteurs et des différents bio-récepteurs, transducteurs et techniques de mise en contact des bio-récepteurs et transducteurs (d'après Eltzov et Marks, 2011).

1.3.2 Les différents types de bio-récepteurs

Le choix du bio-récepteur est très important et doit être adapté au type de transducteur utilisé. Le bio-récepteur doit répondre à des exigences telles que (i) la durée de vie, (ii) la facilité d'immobilisation, (iii) la non perturbation de ce dernier consécutive au processus d'immobilisation, (iv) la spécificité et (v) la sensibilité de réponse aux analytes étudiés. Parmi les bio-récepteurs existants pour la conception de biocapteurs, nous pouvons citer :

- Les acides nucléiques (ADN et ARN). Ces bio-récepteurs sont particulièrement utilisés pour le développement de puces à ADN/ARN permettant de détecter des toxiques, toxines ou des organismes pathogènes (e.g. bactéries alimentaires). L'utilisation de ces puces repose principalement sur une hybridation de morceaux d'ADN/ARN complémentaires aux séquences immobilisées (taille souvent inférieure à 60 bases), ou de molécules mimant cette hybridation.

- Les enzymes, historiquement les premiers bio-récepteurs étudiés, restent à ce jour les éléments biologiques les plus utilisés pour la création de biocapteurs. Les enzymes présentent plusieurs avantages dont la possibilité de modifier leurs propriétés catalytiques et leurs spécificités en fonction des substrats (en modifiant par exemple la structure de leurs sites actifs). Cependant, leur utilisation souffre de plusieurs limites telles qu'un coût élevé de purification et un manque de spécificité pour la différenciation des polluants d'une même famille (e.g. détection différenciée des métaux lourds dans les eaux polluées) (Rogers et al., 2006). Enfin, leur manque de stabilité les rend souvent inutilisables *in situ*. Si leurs domaines d'application sont très variés, leur utilisation la plus connue demeure à ce jour la création du biocapteur à glucose basée sur l'utilisation de la glucose oxydase (Clark et Lyons, 1962).

- Les protéines non enzymatiques (récepteurs membranaires). Les biocapteurs utilisant ces protéines s'appuient sur une perturbation de la protéine membranaire étudiée. La fixation de l'analyte sur la protéine membranaire entraîne une chaîne de transduction (cascade de signaux cellulaires) où des phénomènes tels que l'ouverture de canaux, la réaction avec d'autres protéines membranaires externes ou internes, etc., se produisent. La protéine la plus étudiée ces dernières années, est le récepteur aux œstrogènes α employé par exemple pour la détection impédimétrique de perturbateurs endocriniens tels que les œstrogènes, mais également le bisphénol A et la génistéine, un polyphénol (Granek et Rishpon, 2002).

- Les anticorps. Ils sont utilisés dans les immunocapteurs, plus polyvalents que les biocapteurs enzymatiques. Ils se fixent spécifiquement à des polluants individuels ou des familles de polluants avec une large gamme d'affinités. Cependant la complexité de leur conception et le nombre de réactifs spécialisés pour chaque capteur à développer freinent leur utilisation (Rogers et al., 2006). Le capteur

le plus connu actuellement utilisé avec ce bio-récepteur est le test ELISA (Enzyme-Linked Immuno Assay) qui vise à détecter la présence de protéines, anticorps ou antigènes.

- Les organites, tels que les mitochondries ou les thylakoïdes. L'utilisation d'organites peut s'avérer pertinent lorsque l'on souhaite détecter des familles de polluants et non une molécule particulière, contrairement aux biocapteurs enzymatiques, et ce malgré une durée de vie limitée. Suggérée par Wang et al. (2016), l'utilisation de biocapteurs à mitochondries (utilisant dans leur cas une détection électrochimique par voltamétrie cyclique de la perturbation de la respiration) permet de détecter de multiples familles de pesticides telles que les neurotoxiques, organophosphates ou des carbamates. Les mitochondries sont en effet des cibles primaires ou secondaires de ces différentes familles de composés.

- Les cellules entières. Ces dernières sont beaucoup utilisées en environnement car elles permettent à la fois de détecter la présence de polluants, mais également de mettre en évidence un effet toxique de ces molécules (Rodriguez-Mozaz et al., 2005). A l'inverse des précédents bio-récepteurs, ces derniers vont avoir tendance à capter la pollution globale des matrices étudiées (multiples familles de polluants). L'obtention d'une réponse spécifique à un composé précis est presque impossible sur cellules non génétiquement modifiées. Cela est dû aux nombreuses interactions intracellulaires entrant en jeu et à l'absorption multiple des contaminants aquatiques. L'utilisation de cellules entières pour la création de biocapteurs présente néanmoins de nombreux avantages tels que (i) la facilité de les cultiver et de les entretenir, (ii) la sensibilité de ces dernières aux polluants, (iii) la représentativité environnementale, ou encore (iv) leur faible coût. Parmi les cellules entières, les micro-organismes unicellulaires sont utilisés pour les biocapteurs environnementaux (dit « microbiens »). Parmi eux, trois types de micro-organismes sont fréquemment cités dans la littérature :

- Les bactéries : ce sont les micro-organismes les plus couramment cités pour la création de biocapteurs. Les bactéries sont utilisées par exemple pour la création de biocapteurs de mesure la DBO (Galindo et al., 1992 ; Jia et al., 2003) et de biocapteurs de détection de molécules toxiques (e.g. le phénol par Choi et Gu (2002)). Des bactéries génétiquement modifiées sont créées pour le développement de biocapteurs spécifiques à certaines molécules, tels que les métaux lourds (Charrier et al., 2011a,b). Le principe est d'associer dans leur génome un promoteur natif à un gène rapporteur qui sera responsable du signal capté par le transducteur (e.g. bioluminescence émise suite à l'activation du gène de la luciférase et captée par un système optique).
- Les microalgues : ces micro-organismes photosynthétiques présentent l'avantage d'être très simples à cultiver. Ils sont utilisés pour développer des biocapteurs de pollution car ils répondent à de nombreux polluants. Dans la majorité des cas de développement de biocapteurs à cellules algales, la mesure de la toxicité est basée soit sur la perturbation de la photosynthèse (*via* la mesure de la fluorescence chlorophyllienne, ou de la production d'O₂

ou la consommation de CO₂) (Tsopela et al, 2016), soit sur la perturbation de l'activité de certaines enzymes membranaires telles que les phosphatases alcalines ou les estérases (Chouteau et al., 2004, 2005).

- Les levures (e.g. *Saccharomyces cerevisiae*) sont notamment utilisées afin de créer des biocapteurs ampérométriques mesurant des perturbations de la respiration cellulaire (diffusion de l'O₂), ou des biocapteurs utilisant des cellules génétiquement modifiées (comme pour les bactéries) par l'implantation de gènes rapporteurs de la luciférase par exemple (Hollis et al., 2000). Cependant, les levures ont l'avantage d'être des cellules eucaryotes et d'être plus robustes et plus tolérantes aux variations de pH, d'osmolarité et de température que les bactéries (Baronian, 2004). Elles sont de plus très faciles à faire croître sur une large gamme de substrats.

Tout comme n'importe quel biocapteur, les biocapteurs à cellules entières présentent certaines limites d'usage. La présence de nutriments dans le milieu conduit à modifier le métabolisme cellulaire. C'est le cas par exemple des biocapteurs à cellules algales, très sensibles à la présence de phosphates ou nitrates qui influencent le fonctionnement et la production de protéines liées à la photosynthèse ou d'autres processus physiologiques (Geider et al., 1993), et peuvent cacher les effets toxiques de certaines matrices polluées. Les biocapteurs bactériens et fongiques (levures) présentent une contrainte qui peut être un réel frein pour une utilisation sur le terrain : la stérilité des cultures utilisées. De plus, très souvent le développement de ces biocapteurs est basé sur l'utilisation de souches génétiquement modifiées qui engendrent un problème éthique (Jarque et al., 2016). Cela interdit leur déploiement *in situ*. Ceci n'est cependant pas le cas pour la plupart des biocapteurs algaux qui ne sont pas modifiés génétiquement.

1.3.3 Les différents types de transducteurs

Le transducteur est un élément essentiel pour la création des biocapteurs car il permet de transformer l'information « biologique » émise par le bio-récepteur en un signal physique. Le signal physique est ensuite enregistré par un système d'acquisition. Par conséquent, le système de conversion doit être parfaitement maîtrisé afin de ne pas diminuer la sensibilité des biocapteurs. Différents types de transducteurs sont couramment utilisés pour la création de biocapteurs microbiens :

- Les transducteurs optiques permettant différents types de mesures :

- La bioluminescence : certaines bactéries (telle que *Vibrio Fischeri*, *Escherichia coli* ou *Phosphobacterium phosphoreum*) sont en effet capables de produire de la lumière. Le niveau de luminescence peut éventuellement être modifié en présence de composés toxiques. Ce dernier est généralement mesuré à l'aide de luminomètres dont la configuration est adaptée au biocapteur créé. Jouanneau et al. (2011) ont utilisé des souches d'*Escherichia coli* dont la perturbation de la bioluminescence par des métaux lourds a été mesurée à l'aide d'un

luminomètre apte à lire des microplaques au sein desquelles les bactéries étaient immobilisées.

- La fluorescence : méthode basée sur la mesure de lumière émise par un composé à une longueur d'onde donnée, après excitation à une longueur d'onde plus faible. Ce système de transduction est très largement utilisé pour la création de biocapteurs car il permet la conception de biocapteurs relativement simples à fabriquer. Deux types de biocapteurs à fluorescence sont développés : ceux utilisant la fluorescence de molécules intrinsèques aux organismes comme le biocapteur à fluorescence chlorophyllienne sur *Chlorella vulgaris* développé par Védrine et al. (2003) ; et ceux mesurant la transformation d'un substrat non fluorescent utilisé par le métabolisme du micro-organisme en un produit fluorescent (ou inversement).
- La colorimétrie : principalement utilisée dans les biocapteurs bactériens, est un signal basé sur la transformation d'un substrat chromogène en un produit coloré (ou inversement) sous l'action métabolique d'une enzyme ou d'un organisme. La concentration en produit coloré est alors déterminée optiquement (e.g. photographie et analyse d'intensité de couleur à l'aide de logiciels d'analyse d'image de type ImageJ) ou par spectrophotométrie.

- Les transducteurs électrochimiques permettant différents types de détection :

- L'ampérométrie : cette détection est basée sur le principe de l'application d'un potentiel fixé entre une électrode de travail et une électrode de référence. Un signal de courant est enregistré et corrélé à une concentration en analytes et, par conséquent, à une mesure de sa production ou consommation. Le signal de courant enregistré est le plus souvent dû à une réduction/oxydation d'un métabolite électro-actif ou d'un intermédiaire fixé sur une électrode (Ding et al., 2008).
- La voltamétrie cyclique, méthode également ampérométrique, mais qui permet de mesurer à la fois un potentiel et un courant. La détermination de la densité du courant de pointe mesurée est alors proportionnelle à la concentration en molécules étudiées.
- La conductimétrie : elle permet de mesurer des changements de conductivité de solutions dus à la disparition ou la création d'espèces ioniques. Chouteau et al. (2005) ont par exemple utilisé cette technique afin de créer un biocapteur algal (sur *Chlorella vulgaris*) utilisant un substrat non ionique se transformant au contact d'une enzyme membranaire (phosphatase alcaline) en un produit chargé entraînant une modification de conductivité du milieu. Ce changement de conductivité est alors réduit lors d'une inhibition de l'enzyme par les polluants étudiés.
- La potentiométrie basée sur une mesure différentielle de potentiel entre une électrode de travail et une électrode de référence.

-
- La « pile à combustible microbienne » qui permet de transformer une énergie chimique émise par une bactérie en énergie électrique (Rabaey et Verstraete, 2005). Une modification du métabolisme bactérien ou une consommation de substrat peut engendrer une modification de l'énergie électrique produite par la biopile à combustible.

1.3.4 Les différentes méthodes de mise en contact bio-récepteur/transducteur

Cette étape est probablement la plus cruciale dans la conception de biocapteurs car c'est bien souvent lors de la mise en contact du bio-récepteur avec le transducteur qu'une perte de sensibilité des biocapteurs apparaît. En effet, il est nécessaire de disposer d'une technique de mise en contact bio-récepteur/transducteur n'entraînant pas de stress biologique, ne créant pas de barrière physique entre les analytes et le bio-récepteur, assurant un contact entre les organismes et le transducteur, et enfin permettant une stabilité et durabilité du bio-récepteur fixé. Deux grands types de mise en contact ont généralement été développés et basés sur :

- Une immobilisation chimique : cette dernière a principalement lieu sous forme de :

- Couplages covalents directs ou indirects : citons l'utilisation de SAMs (Self-Assembled monolayers) largement employés pour l'immobilisation d'enzymes mais plus récemment pour des algues par Guedri et Durrieu (2008). Cette technique d'immobilisation est basée sur l'utilisation d'électrodes traitées afin de greffer en surface des groupes carboxyliques capables de se lier de manière covalente à des groupements amines présents en surface de cellules algales.
- Réticulations ou co-réticulations. Ces processus consistent à lier les bio-récepteurs (enzymes, organismes) entre eux à l'aide d'un agent réticulant. Dans le cas des co-réticulations, les éléments biologiques sont mélangés à une protéine inerte qui facilite la réticulation par l'agent réticulant. C'est le cas par exemple du biocapteur développé de Chouteau et al. (2005) où des microalgues sont immobilisées à l'aide de BSA (Bovine Serum Albumine) et de glutaraldéhyde comme agent réticulant. Cependant, ces techniques présentent plusieurs limites : le phénomène de barrière physique des protéines, ainsi que la toxicité potentielle du procédé pour tout type de bio-récepteur (p.ex. le glutaraldéhyde toxique pour les microalgues).

- Une immobilisation physique utilisant les principes :

- D'adsorption (e.g. sur nanofibres créées par électrospinning par Eroglu et al., 2012). Cette technique, comme les couplages covalents, permet d'immobiliser des cellules entières et a été largement utilisée. Toutefois, contrairement aux couplages covalents, des phénomènes de décrochages des cellules peuvent avoir lieu lors de variations de la température, du pH, avec la présence de solvants (Guedri et Durrieu, 2008) ou bien d'un débit de liquide trop intense à leur contact.

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- D'inclusion dans des gels/hydrogels, technique répandue pour la création de biocapteurs microbiens. Parmi les composés généralement utilisés pour leur création, on retrouve l'alginate (Ionescu et al., 2006), le chitosan ou l'agarose (Leth et al., 2002). Ce sont des polysaccharides (d'origine végétale ou animale) ayant un fort pouvoir gélifiant et capables d'emprisonner les cellules microbiennes, tout en permettant une diffusion des composés cibles et des composés des milieux de culture (et donc le développement et le maintien des cellules). D'autres composés comme la silice sont également utilisés pour la formation d'hydrogels à partir d'un processus sol-gel (Ferro et al., 2012). Ces gels/hydrogels sont généralement sélectionnés pour leur facilité de préparation, leur innocuité pour les micro-organismes et leurs propriétés optiques. Toutefois, ils peuvent présenter des limites en termes de diffusion des analytes (e.g. polluants) jusqu'aux cellules piégées.
 - De piégeage physique. Védrine et al. (2003) ont par exemple créé un biocapteur optique (fibre optique) en réalisant un piégeage de cellules algales (*Chlorella vulgaris*) par la filtration de ces dernières sur un filtre de microfibre en quartz. Les limites sont alors les mêmes que pour une adsorption (voir ci-avant).

Enfin, depuis une dizaine d'années, la microfluidique a été développée afin de créer des biocapteurs sans avoir recours à une immobilisation chimique ou physique des organismes à proprement parler. (e.g. Lefevre et al. 2012, Roda et al., 2016, Tahirbegi et al., 2017). La microfluidique est un domaine scientifique et technique qui s'intéresse aux écoulements de fluides, avec un régime d'écoulement des fluides laminaire (par opposition aux écoulements de type turbulents couramment rencontrés aux échelles de tailles supérieures), à l'échelle micrométrique, au sein de microcanaux dont l'une des dimensions, au moins, est comprise entre 1 et 100 μm (Yousefi et al., 2015). Ces microcanaux sont généralement créés au sein de systèmes appelés puces microfluidiques. Il est alors possible de faire s'écouler plusieurs liquides côte à côte au sein de ces microcanaux, sans que ceux-ci ne se mélangent. Cependant, des échanges des espèces peuvent avoir lieu entre ces fluides par simple diffusion moléculaire. A cette échelle, il est possible de faire pénétrer rapidement les fluides par capillarité dans la puce microfluidique grâce aux effets de surfaces au sein des canaux microfluidiques. De même, il est possible de les faire s'écouler à l'aide de systèmes de pompes seringues ou encore par électro-osmose. Les quelques études recensées (p. ex. Buffi et al., 2011 ; García-Alonso et al., 2009 ; Tsopela et al., 2016) de biocapteurs microfluidiques microbiens reposent principalement sur l'utilisation d'un transducteur électrochimique (e.g. électrode) ou d'un système optique mis en contact des microcanaux, ou des systèmes microfluidiques, afin de collecter le signal d'intérêt émis par les microorganismes insérés. Plusieurs méthodes ont été employées afin de réaliser ces puces microfluidiques, à partir de différents matériaux : du silicone, des polymères tels que le PDMS (polydiméthylsiloxane, le plus commun) ou encore du verre. La microfluidique présente de nombreux avantages pour la conception de biocapteurs : (i) la taille des microsystèmes qui autorise une faible consommation de réactifs, de bio-

récepteurs, d'échantillons à tester et qui permet le transport facile sur le terrain, (ii) le bas coût de cette technique, (iii) la flexibilité dans la conception des systèmes microfluidiques (Jokerst et al., 2012). Cependant, la limite importante des systèmes microfluidiques pour la création de biocapteurs environnementaux est la présence de colloïdes dans les rejets. Cette présence peut perturber le flux de cellules dans les microcanaux, ou entraîner un colmatage de ces canaux et donc une perturbation du système microfluidique (Yousefi et al., 2015).

Pour illustrer notre propos, un exemple de système microfluidique utilisé pour le développement de biocapteurs est présenté Figure 2. Lefevre et al. (2012) ont développé un biocapteur algal basé sur la perturbation de la fluorescence chlorophyllienne de *Chlamydomonas reinhardtii* afin de détecter la présence d'herbicides dans les eaux. Pour éviter toute barrière physique entre les algues et les herbicides, ces chercheurs ont exposé puis immobilisé les microalgues dans des canaux microfluidiques en série. Un système d'excitation/détection de la fluorescence basé sur la création d'OLED (Organic Light-Emitting Diode) et OPD (Organic PhotoDetector) ainsi qu'un système de filtres (servant à n'utiliser que les longueurs d'onde souhaitées) ont été placés de part et d'autre du système microfluidique créé. De très bonnes sensibilités ont alors été obtenues (avec une valeur d' EC_{50} de 11 nM).

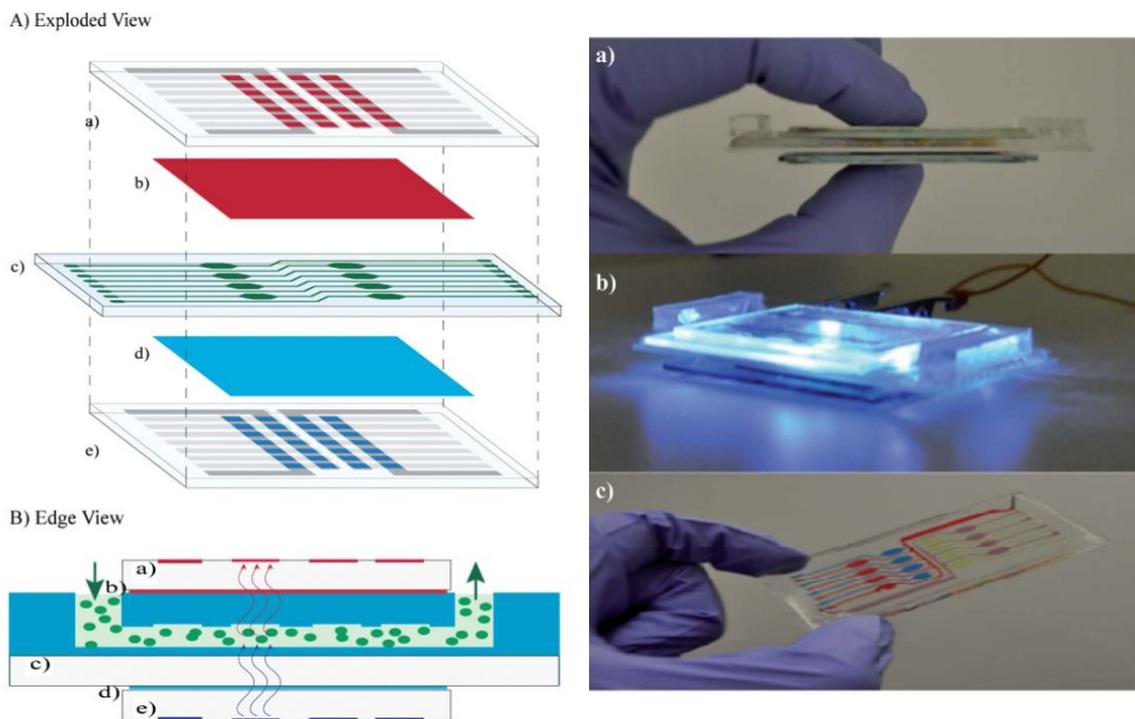


Figure 2 : Exemple de biocapteur algal utilisant la fluorescence chlorophyllienne, basée sur l'insertion de microalgues (*Chlamydomonas reinhardtii*) dans une puce microfluidique créée en PDMS, utilisant un système d'OLED (Organic Light-Emitting Diode) afin d'exciter les algues et d'OPD (Organic PhotoDetector) afin de collecter la fluorescence émise.: (A) Vue éclatée et (B) vue de face : la puce microfluidique (c) est prise en sandwich entre l'OLED (e) et l'OPD (a). Le filtre d'émission (d) est entre la puce microfluidique et l'OLED, tandis que le filtre d'excitation (B) est entre la puce microfluidique et l'OPD (d'après Lefevre et al., 2012).

1.3.5 Application des biocapteurs à cellules entières à la détection de micropolluants pour la surveillance des écosystèmes aquatiques récepteurs

Un schéma de présentation du large éventail des différentes utilisations des biocapteurs à cellules entières appliquées à l'environnement a été proposé par Eltzov et Marks en 2011 (Figure 3). L'application la plus courante de ces biocapteurs est la détection de micropolluants tels que les pesticides, les métaux lourds, les hydrocarbures aromatiques polycycliques, les surfactants ou encore les perturbateurs endocriniens. Toutefois, les biocapteurs algaux sont pour la plupart basés sur la perturbation de la fluorescence chlorophyllienne (liée à la photosynthèse) pour la détection d'herbicides (Peña-Vázquez et al., 2009), ou celle d'enzymes membranaires pour la détection de métaux lourds (Chouteau et al., 2004). Les biocapteurs bactériens et à levures ont quant à eux été essentiellement développés en laboratoire pour la mesure de DBO (Demande Biologique en Oxygène), permettant d'identifier la présence de métaux lourds, ainsi que de substances pathogènes ou biocides (Jusoh et Wong, 2014).

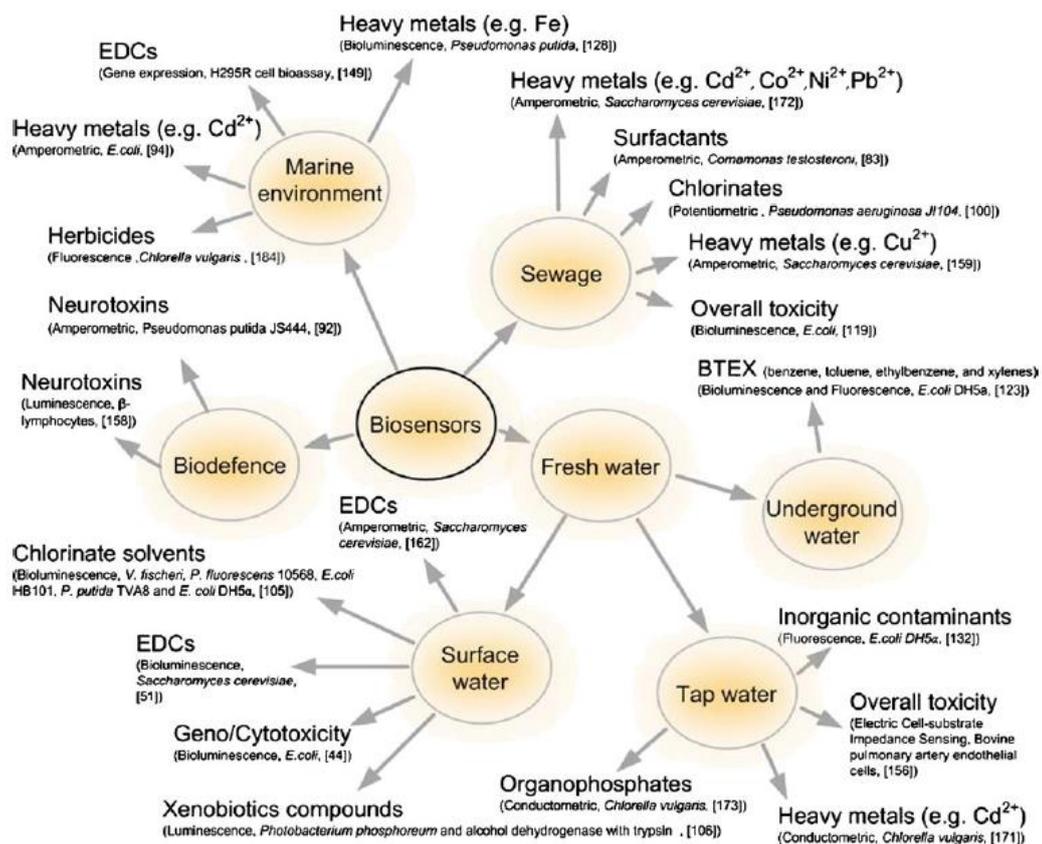


Figure 3 : Exemple de biocapteurs à cellule entière pour le monitoring environnemental (d'après Eltzov et Marks, 2011) (EDCs : Endocrine Disruptive Compounds).

A l'inverse, certains biocapteurs ont été développés dans l'objectif de détecter une toxicité globale des échantillons analysés, en optimisant la sensibilité, mais avec une faible spécificité. Ces biocapteurs sont généralement dédiés à l'étude de l'écotoxicité d'effluents de stations d'épuration. C'est

le cas du biocapteur bactérien mis au point par Choi et Gu (1999), basé sur la perturbation de la bioluminescence de souches recombinées d'*Escherichia coli* et de *Vibrio fischeri*. Farré et al. (2001) et Philp et al. (2003) ont réalisé des études similaires utilisant ces mêmes bactéries.

Enfin, notons qu'il existe encore peu de biocapteurs microbiens suffisamment développés pour un usage *in situ*. Cependant, depuis quelques années, de premières études commencent à apparaître (Michelini et al., 2013). Les capteurs à DBO sont pour l'instant les seuls à avoir réellement franchi l'étape de sortie du laboratoire pour une utilisation en routine par les collectivités publiques et les structures privées (Figure 4), et à être commercialisés par plusieurs entreprises sous forme de bioréacteurs (e.g. BioMonitor[®] par LAR, ou Ra-BOD[®] par AppliTek) (Jouanneau et al., 2014).

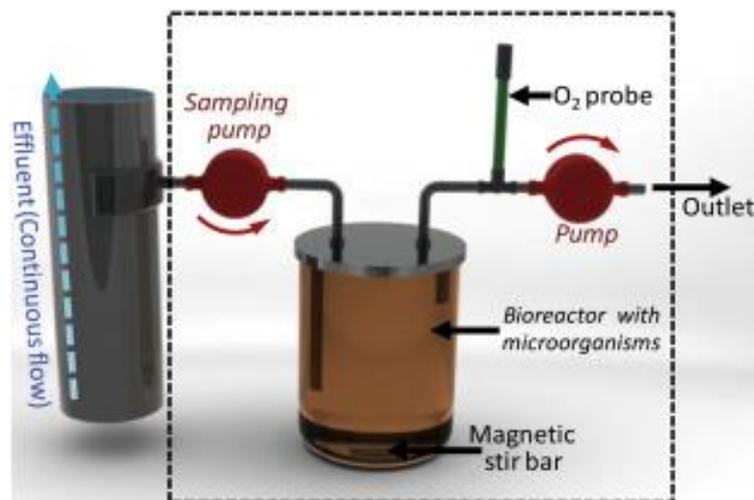


Figure 4 : Représentation schématique d'un biocapteur optique à DBO de terrain de type « bioréacteur », d'après Jouanneau et al. (2014).

Concernant les biocapteurs bactériens et à levures, dédiés à la détection de polluants et de matrices toxiques, aucune étude actuelle ne fait référence à de réelles campagnes de mesures *in situ*, mais à des campagnes de prélèvements de rejets analysés ensuite en laboratoire à l'aide du biocapteur développé, comme dans le cas de Philp et al. (2003). Quant aux biocapteurs algaux, une première étude a été menée par Ferro (2013) sur la réalisation d'essais *in situ* de biocapteurs conductimétriques, auparavant développés en laboratoire par Chouteau (2004), Guedri (2010). Ferro (2013) a développé un biocapteur algal conductimétrique basé sur une perturbation de la phosphatase alcaline et de l'estérase pour la détection de pesticides et métaux lourds dans les RUTP. Mais, le biocapteur a montré des limites et n'est pas encore opérationnel : un problème d'interférence entre le bruit de fond des rejets étudiés et le signal émis par les algues immobilisées sur les électrodes a empêché les mesures *in situ*.

1.3.6 Conclusion : défis et enjeux actuels et futurs liés au développement de biocapteurs

L'utilité et la sensibilité des biocapteurs microbiens pour le suivi et la détection de polluants sont clairement établies à l'échelle de tests en laboratoire. Leur usage *in situ* reste à démontrer (Su et al., 2011). Leur développement est en cours à l'échelle du laboratoire, avec encore peu de techniques commercialisées à l'heure actuelle (e.g GreenScreen®, un biocapteur de génotoxicité sur *Saccharomyces cerevisiae*) (Farré et al., 2005). Plusieurs raisons expliquent les difficultés de transfert de la technologie de laboratoire au terrain : la compacité des biocapteurs fait bien souvent défaut et empêche l'utilisation de ces outils sur le terrain (dû aux pompes, réserve de solvant, etc.). L'autonomie des biocapteurs peut aussi être une difficulté. Il est souvent nécessaire de relier les biocapteurs développés à une source électronique importante (prise), en raison des transducteurs utilisés (e.g. fibres optiques reliées à des fluorimètres, microélectrodes à des oscilloscopes). Enfin, le manque de sensibilité des biocapteurs dû aux méthodes d'immobilisation des micro-organismes (e.g. dans des hydrogels de silice), et la spécificité de réponse trop faible de ces derniers découragent les industriels à les utiliser. Il est donc nécessaire de travailler sur ces différents points afin de pouvoir créer des outils qui pourront être transférés vers des utilisateurs finaux (services publics, industriels). Pour remédier aux nombreuses difficultés rencontrées, Su et al. (2011) préconisent notamment l'utilisation de la technique de « lab-on-a-chip », en intégrant les micro-organismes dans des systèmes microfluidiques qui permettraient de diminuer considérablement le volume d'échantillons et permettraient d'améliorer la sensibilité et la rapidité des réponses observées. Une autre voie d'optimisation serait de poursuivre les investigations sur l'immobilisation des cellules *via* des immobilisations physiques (liaison covalente, réticulation) ou chimiques (adsorption et piégeage physique) tout en maîtrisant les problèmes de barrière physique entre les analytes (polluants) et les cellules immobilisées.



2 Démarche générale de la thèse

2.1 Présentation générale

L'objectif de ce travail a été de développer des outils de détections sensibles de la toxicité des RUTP en laboratoire et *in situ*, en utilisant les microalgues, premier compartiment trophique à la base des écosystèmes. La figure 5 présente la démarche générale que nous avons adoptée.

La première étape de notre étude a consisté à collecter des échantillons de RUTP au sein de sites de prélèvements situés sur la région Lyonnaise. Puis, à l'échelle du laboratoire, nous avons développé et appliqué sur des microalgues unicellulaires (*Chlorella vulgaris*) exposées à ces rejets sur de courtes périodes (2 et 24 heures), une batterie de biomarqueurs de perturbation de leur physiologie. Cette batterie de tests a reposé sur l'étude de la perturbation de paramètres globaux tels que la perméabilité membranaire et la photosynthèse, mais aussi sur l'étude de marqueurs plus spécifiques liés à des enzymes jouant un rôle essentiel dans le métabolisme des algues : les estérases et les phosphatases. La pertinence de l'utilisation de ces biomarqueurs a été démontrée *via* une comparaison avec une batterie de bioessais monospécifiques standardisés, classiquement utilisés en écotoxicologie, basés sur trois niveaux trophiques différents : les producteurs primaires, les consommateurs primaires et les consommateurs secondaires détritivores.

Des biomarqueurs du stress oxydant (e.g. peroxydation lipidique, catalase) ont également fait l'objet d'une étude préliminaire pour la détection d'effets toxiques des RUTP.

La deuxième partie de notre étude a porté sur l'adaptation de ces biomarqueurs au développement d'outils de mesures de toxicité *in situ*. L'outil développé a permis d'étudier la perturbation de la photosynthèse par l'intermédiaire de l'émission de la fluorescence des chlorophylles. Nous nous sommes particulièrement attachés à travailler sur la mise en contact bio-récepteur (microalgues)/transducteur, qui représente un enjeu majeur pour le développement des biocapteurs. Pour cela, trois axes distincts ont été explorés. Le premier a été consisté à inclure des algues dans des hydrogels d'alginate/silice qui présentent l'avantage de posséder de bonnes propriétés optiques. Le second axe a porté sur l'utilisation de nanofibres de polymères artificiels ou naturels créées par électrospinning afin d'adsorber et/ou de piéger physiquement les microalgues entre les fibres. Cependant, les résultats n'ont donné aucune perspective d'application et, nous n'avons donc pas poursuivi ce développement. Enfin, la réalisation de puces microfluidiques par xurographie a permis de créer des systèmes simples, à bas coût, de très bonnes qualités optiques et avec une bonne répétabilité, et permettant l'analyse de la fluorescence des microalgues insérées par capillarité. Dans ce cas, les algues ne sont pas immobilisées mais circulent librement dans des microcapillaires. L'intérêt de ce dispositif est d'éliminer toute barrière physique entre les algues et les polluants.

Sur l'ensemble des techniques mises en oeuvre, nous avons cherché à valider la faisabilité et sensibilité des capteurs créés en laboratoire, avant de passer à une adaptation au terrain. Finalement, les

biocapteurs microfluidiques se sont révélés être les plus intéressants. Une station portative de terrain a été construite et testée *in situ* à la fois sur RUTP et sur les milieux contaminés par les RUTP. Ce dispositif pourrait être utilisé pour l'étude d'autres matrices contaminées comme les eaux usées ou les rejets de station d'épuration.

Démarche générale de la thèse

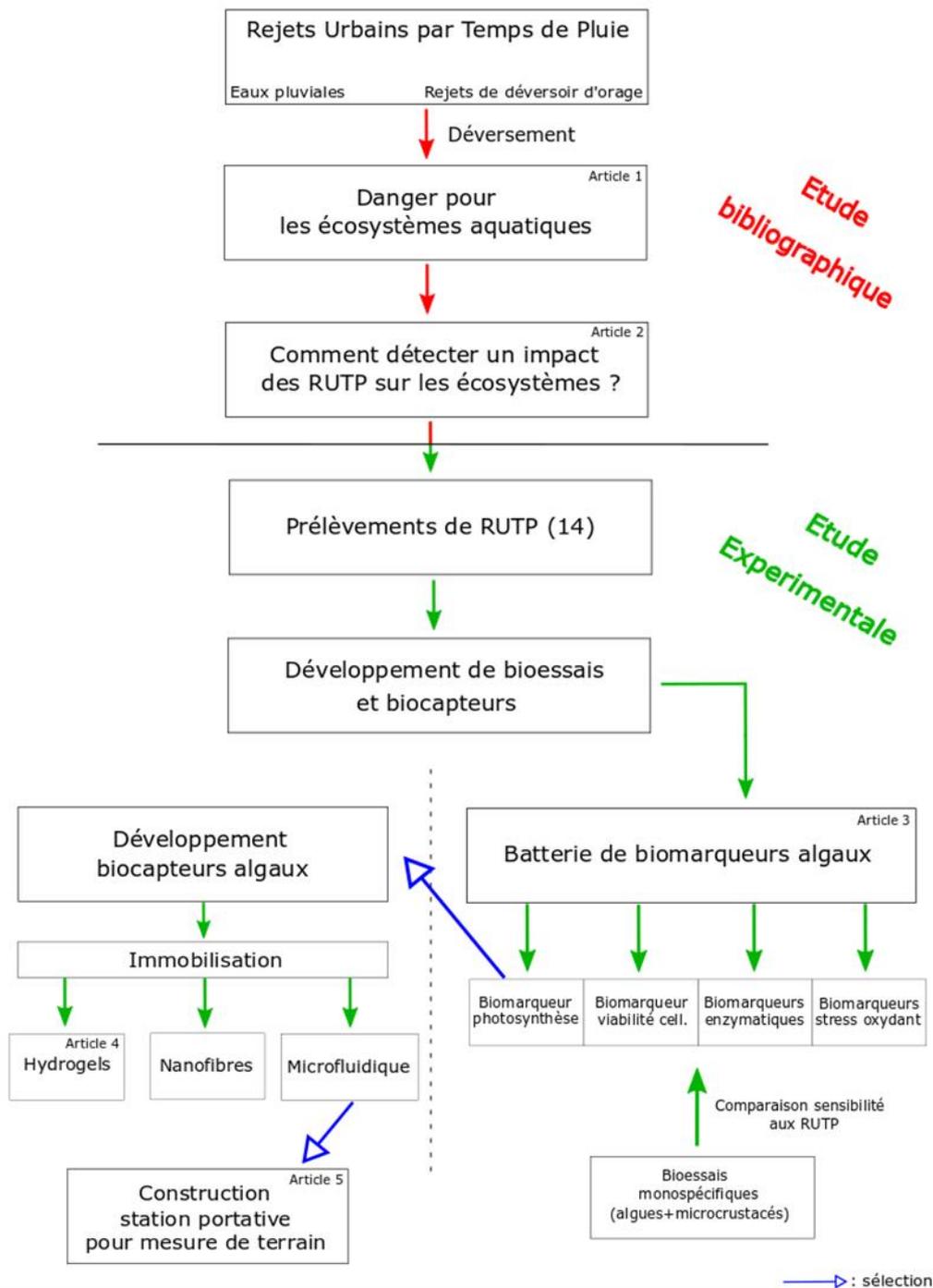


Figure 5 : Schéma général de l'organisation de la thèse

L'objectif de cette partie est de présenter les différents sites d'étude, les organismes modèles et les techniques mises en place pour la réalisation de la démarche exposée ci-dessus. Une présentation plus approfondie des protocoles utilisés lors des expérimentations est détaillée dans chaque chapitre.

2.2 Présentation des sites d'études et des campagnes de prélèvements

La figure 6 présente la localisation des sites de prélèvements des RUTP étudiés.

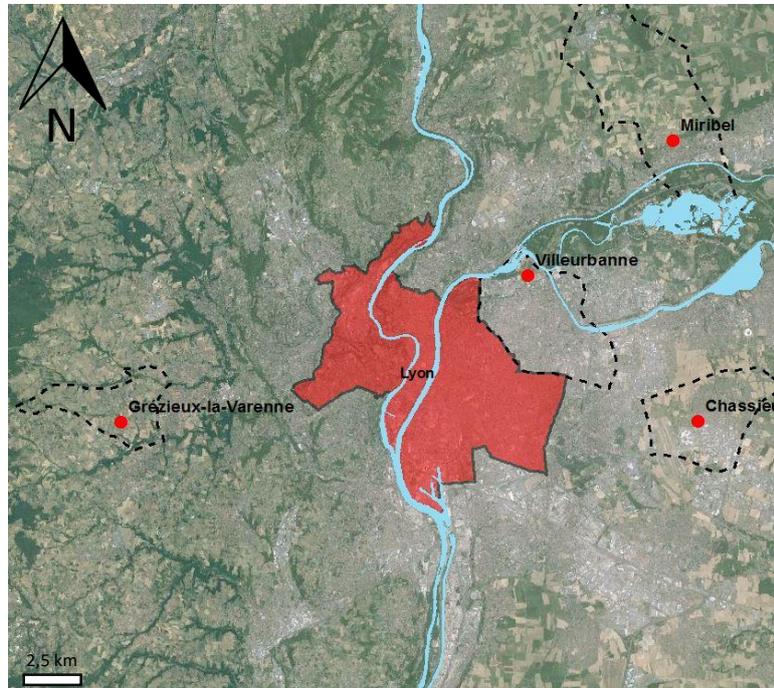


Figure 6 : Cartographie des différents sites de prélèvements des RUTP dans la région Lyonnaise. Les points rouges représentent la localisation des sites d'études.

Les différents sites de prélèvements ont été sélectionnés selon plusieurs critères :

- Leur diversité : nous avons en effet choisi des sites correspondant à des bassins versants de tailles et de natures variées ;
- Leur connaissance : une part importante des sites choisis est étudiée depuis plusieurs décennies et de nombreuses données à la fois pluviométrique, de contamination chimique et biologique, sont disponibles ;
- Leur étude : elle s'appuie sur un partenaire scientifique (OTHU : Observatoire de Terrain en Hydrologie Urbaine) créé spécifiquement pour l'étude des rejets urbains et de leurs impacts.
- Leur proximité avec le laboratoire : cela permet leur traitement rapide afin de ne pas laisser les échantillons évoluer et se détériorer.

Au final, 4 sites ont été retenus autour de l'agglomération Lyonnaise :

- 2 bassins de rétention et/ou infiltration d'eaux pluviales ayant des bassins versants urbains denses et différents (activités tertiaires ou industrielles) ;
- Une surverse de bassin écrêteur en zone péri-urbaine à forte pression agricole ;
- Un déversoir d'orage en zone péri-urbaine rejetant un mélange d'eaux usées non traitées et d'eaux pluviales.

Les différents sites et les caractéristiques de leurs bassins versants, responsables de la contamination des RUTP, sont détaillés ci-après.

2.2.1 Bassin d'infiltration de l'IUT de la Doua - Villeurbanne



Figure 7 : Bassin d'infiltration du campus de la Doua – Villeurbanne (Photographie : Marie Morin). La flèche rouge indique le lieu de prélèvements des rejets.

Le bassin d'infiltration de l'IUT de biologie de l'Université de Lyon 1 (Claude Bernard) est situé sur le campus de la DOUA, au sein de la commune de Villeurbanne. Construit il y a une trentaine d'années, ce bassin draine un bassin versant d'une superficie de 2,5 hectares avec un taux d'imperméabilisation très important (comparant surface imperméable et surface totale du bassin versant) de 90 % (Datry et al., 2003). Ce dernier est principalement composé de bâtiments d'enseignement supérieur et de recherches, d'entreprises, de parkings, routes et zones enherbées. Le bassin d'une superficie de 750 m² permet de contenir jusqu'à 4 000 m³ de volume de précipitations. Les eaux pluviales du campus sont drainées directement par des canalisations se déversant à travers plusieurs entrées dans le bassin. La flèche sur la figure 7 montre la sortie de la canalisation utilisée pour collecter les eaux pluviales. Cette canalisation draine 75 % de la surface du bassin versant.

Ce bassin est situé sur la nappe alluviale du Rhône. Dans la région, la nappe phréatique est située à une profondeur qui varie entre 2,5 et 3,5 mètres sous la surface du sol. En revanche, au niveau de ce bassin, la nappe phréatique est haute avec d'importantes fluctuations. Par conséquent, la nappe peut parfois se situer à moins d'un mètre de profondeur du sol. Cette caractéristique la rend d'autant plus fragile aux risques de contamination.

2.2.2 Bassin de rétention/infiltration Django-Reinhardt de Chassieu

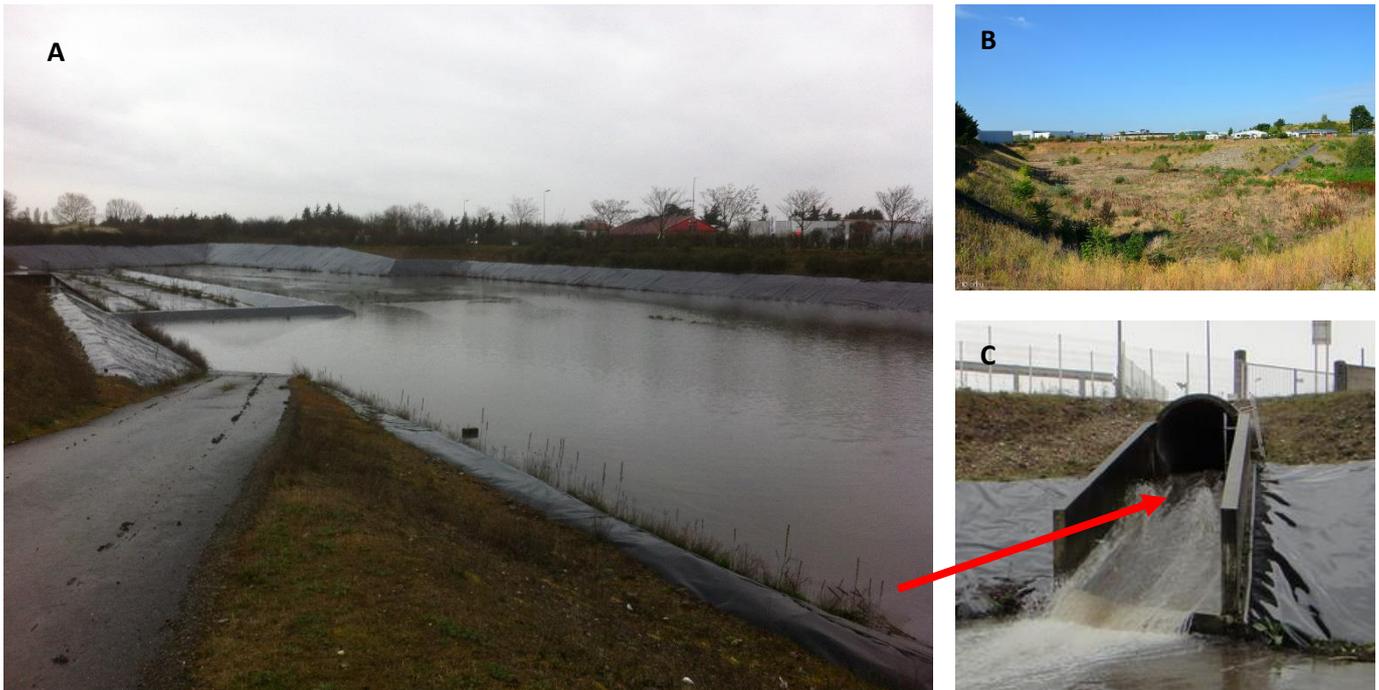


Figure 8 : Bassin de rétention/infiltration Django-Reinhardt – Chassieu (Photographie personnelle). A : Bassin de rétention ; B : Bassin d'infiltration ; C : entrée des eaux pluviales dans le bassin de rétention. La flèche rouge indique le lieu de prélèvements des rejets.

Ce double bassin de rétention/infiltration est l'exutoire principal de rejets d'eaux pluviales strictes (réseau séparatif) lessivant un bassin versant d'environ 185 hectares, fortement industriel (industrie de type traitement des papiers/cartons, production d'enrobés bitumineux, etc.) avec une circulation routière dense (plus de 10 000 véhicules par jour). Il est localisé sur la commune de Chassieu dans l'Est Lyonnais. Le bassin versant lessivé possède un taux d'imperméabilisation des sols très important, de 75 % (El-Mufleh et al., 2014), avec un coefficient d'apport de 0,4, qui implique une surface active de 74 % de la superficie totale (Becouze-Lareure, 2010). Si ce bassin versant est fortement urbanisé, il faut noter que 28 % de sa surface correspond à des espaces végétalisés (parcs), agricoles ou naturelles. Ce site est composé de deux bassins : le premier est un bassin de rétention (Figure 8 A) d'une surface de 11 300 m² et d'un volume de 32 000 m³ qui est connecté à un bassin d'infiltration (Figure 8 B) de 61 000 m³. La zone de prélèvements des échantillons se situe à l'entrée du bassin de rétention comme montré en figure 8 C.

Contrairement au bassin précédent (IUT), la nappe phréatique présente sous le bassin d'infiltration est plus profonde (- 13 mètres).

N.B. : Ce bassin reçoit, en plus des eaux pluviales, des rejets dits de temps sec (RUTS) déversés en continu. Ces rejets issus des industries situées sur le bassin versant correspondent à des eaux de refroidissement ou de lavage déversées dans les réseaux d'eaux pluviales et qui peuvent donc contribuer à la contamination des RUTP.

2.2.3 La surverse du torrent du Couloup à Miribel

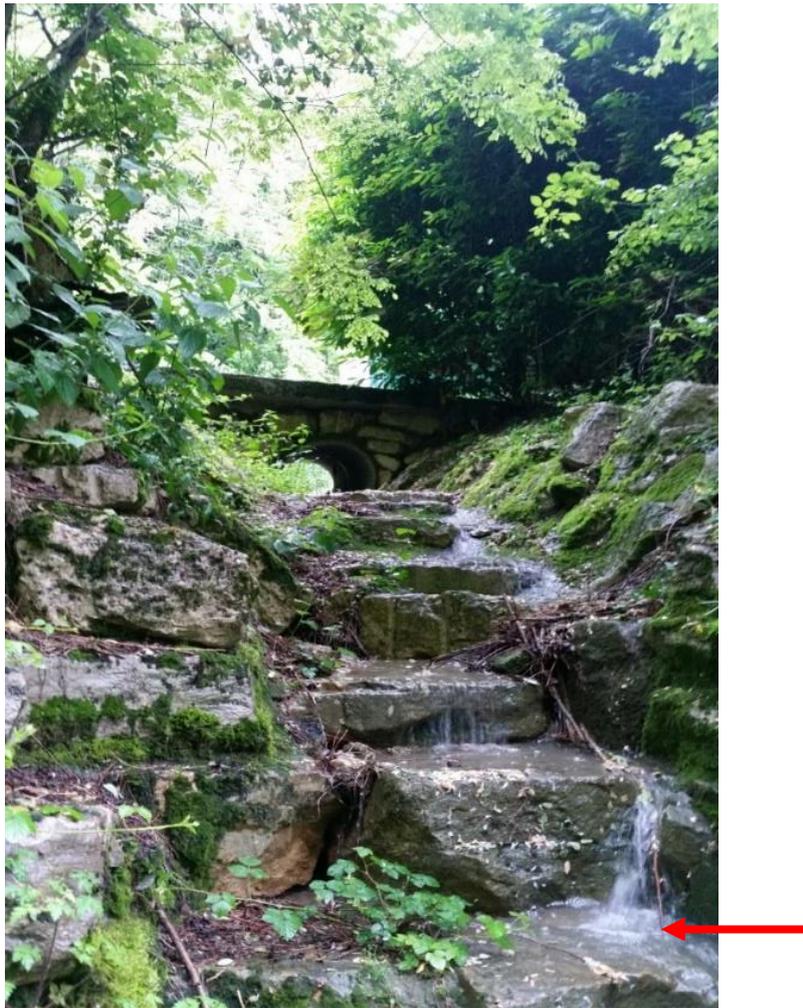


Figure 9 : Surverse du Couloup, de bassins écrêteurs à Miribel (Photographie : Marie Morin). La flèche rouge indique le lieu de prélèvements des rejets.

Le « Torrent le Couloup », est situé sur la commune de Miribel, dans l'Ain, à la périphérie de Lyon (Figure 9). Il est alimenté par la surverse de 3 bassins d'infiltration interconnectés sur un plateau, dans les hauteurs de la ville de Miribel, afin de lutter contre les problèmes de crues, d'éboulements et d'inondations accentués par les fortes pentes de la ville. Au total, le plateau possède une capacité de rétention des eaux pluviales de 1 800 m³. Le lit du torrent du Couloup suit une ligne de pente dans la partie centrale du territoire de Miribel et rejoint directement les canalisations d'eaux pluviales, pour aller se jeter directement dans le Rhône situé en contrebas de la ville. Cette surverse draine par conséquent un bassin versant de 11 hectares, dont 8,5 ha appartiennent à la zone de la Cotière, de nature péri-urbaine avec une forte pression agricole. Si aucune donnée précise concernant ce bassin versant n'existe, le taux d'imperméabilisation de la commune de Miribel n'est que de 21 %, ce qui est beaucoup plus faible que pour les autres sites d'étude.

2.2.4 Le déversoir d'orage à Grezieux-la-Varenne



Figure 10 : Déversoir d'orage situé à Grezieux-la-Varenne (A) et de la Chaudanne dans laquelle les rejets se déversent (B) (source : OTHU). Les flèches rouges indiquent les lieux de prélèvements des rejets.

Le déversoir de Grezieux-la-Varenne (situé au lieu-dit du Pont de la Barge) est localisé au niveau du bassin versant de l'Yzeron, rivière de l'Ouest Lyonnais (Figure 10 A). Le bassin versant total de l'Yzeron représente 129 km². Le déversoir d'orage se situe en zone-péri urbaine résidentielle, avec présence sur le bassin de quartiers pavillonnaires mais aussi de quelques entreprises et commerces. Ce déversoir d'orage se situe au niveau du ruisseau de « la Chaudanne », de 4,4 km de long, se déversant directement dans le Rhône (Marti et al., 2017) (Figure 10 B). La Chaudanne est également un affluent de l'Yzeron, dont le bassin versant péri-urbain représente une superficie de 3,7 km², soit environ 370 hectares. Le déversoir se situe exactement à 2,96 kilomètres de la source de ce ruisseau et est à l'origine d'importantes modifications physico-chimiques (e.g. pH, conductivité, oxygène dissous, présence de contaminants) de ce dernier par temps de pluie. En effet, si le débit moyen de la Chaudanne avoisine les 30 L/s, celui-ci peut augmenter jusqu'à 1500 L/s en cas de très forte pluie. En revanche, durant plusieurs semaines en été, le ruisseau est à sec et la seule source d'eau provient du déversoir en cas d'évènements pluvieux.

Compte tenu de sa facilité d'accès, il nous a paru intéressant de prélever à la fois des rejets directement au niveau du déversoir d'orage, mais aussi des échantillons dans le ruisseau immédiatement en aval du déversoir.

2.2.5 Méthodologie de prélèvement et conservation des échantillons

Deux campagnes de prélèvements ont été effectuées. La première, en 2015, composée de 7 rejets, a servi à la validation d'une série de biomarqueurs algaux pour étudier leur sensibilité aux RUTP. La seconde, en 2016, également composée de 7 rejets, a permis de développer de nouveaux biomarqueurs (du stress oxydant) et de valider le biocapteur algal que nous avons conçu. Un résumé des différentes caractéristiques des événements pluvieux associés aux rejets prélevés est présenté dans le Tableau 1. On peut noter la disparité des événements associés aux prélèvements, tant en termes de durée, de volume, d'intensité que durée de la période sèche antérieure aux événements.

Tableau 1 : Résumé des campagnes de prélèvements des RUTP en 2015 et 2016.

Date	Site	Durée (hh:min)	Hauteur (mm)	Intensité de la pluie (mm/h)	Période sèche avant l'évènement (jj:hh:min)
2015					
25/03/2015	Chassieu - Django Reinhardt	20:06	17,2	0,86	03:03:48
05/05/2015	Chassieu - Django Reinhardt	00:06	0,4	4,00	01:09:48
27/02/2015	Miribel - Torrent du Couloup	09:42	13,2	1,36	02:01:54
19/04/2015	Miribel - Torrent du Couloup	04:30	5,4	1,2	01:09:48
19/04/2015	Villeurbanne - IUT La doua	04:24	4,6	1,04	01:08:35
27/02/2015	Villeurbanne - IUT La doua	11:36	11,8	1,02	02:01:18
25/03/2015	Villeurbanne - IUT La doua	19:00	16	0,84	03:04:24
2016					
13/04/2016	Chassieu - Django Reinhardt	17:24	22,6	1,3	04:06:42
09/05/2016	Grezieux - Déversoir d'orage /Aval (Rivière) (2 échantillons)	20:00	17,2	0,86	00:13:00
19/05/2016	Grezieux - Déversoir d'orage /Aval (Rivière) (2 échantillons)	21:00	12,4	0,59	03:17:00
02/06/2016	Miribel - Torrent du Couloup	06:48	10,2	1,5	01:08:30
02/06/2016	Villeurbanne - IUT la Doua	05:06	9	1,76	01:11:06

Les échantillons (sauf ceux provenant du déversoir d'orage) ont été prélevés manuellement, au cours des événements pluvieux, à l'aide de flacons de 2 L en polyéthylène haute densité (PEHD) au préalable rincés 3 fois avec l'échantillon prélevé afin de diminuer les phénomènes d'adsorption des contaminants sur le contenant. Les flacons ont été remplis à ras-bord afin d'éviter toute oxydation et échange avec l'air en surface. Les échantillons provenant du site de Grezieux-la-Varenne ont été prélevés à l'aide d'un échantillonneur automatique durant l'évènement, puis ensuite moyennés sur la durée de l'évènement pluvieux, afin d'obtenir un échantillon représentatif de l'épisode pluvieux. Dans le cas des autres sites, aucun équipement en état de fonctionnement au moment des campagnes de prélèvement n'a permis d'effectuer ce même travail. Dans tous les cas, les échantillons ont été immédiatement ramenés en glacière au laboratoire, filtrés ou selon le besoin, aliquotés et congelés afin d'éviter toute évolution dans le temps et toute dégradation des polluants.

2.3 Présentation des organismes d'étude

Les microalgues sont des organismes modèles largement utilisés en écotoxicologie et, par conséquent, intéressants pour la détection de la toxicité des RUTP. Ces organismes font partie des producteurs primaires jouant un rôle clef dans les réseaux trophiques des écosystèmes aquatiques. En effet, ils constituent la base des réseaux trophiques car ils sont la source alimentaire du zooplancton, lui-même consommé par les poissons et les oiseaux. Des impacts sur ces communautés algales peuvent donc avoir des répercussions sur les espèces situées plus haut dans la chaîne trophique, que sont les consommateurs primaires et secondaires et, *in fine*, sur l'écosystème entier (Nyholm et Peterson, 1997). Les trois espèces que nous avons étudiées sont présentées ci-dessous. Elles possèdent toutes des caractéristiques différentes qui en font des algues modèles intéressantes pour notre travail :

2.3.1 Les microalgues

- *Chlorella vulgaris* (Figure 11) appartient à la famille des Chlorellaceae. Les microalgues *Chlorella* se retrouvent de manière ubiquiste dans les habitats aquatiques d'eau douce ou salée. *C. vulgaris* est une algue verte unicellulaire eucaryote d'eau douce, de forme ronde ou ellipsoïde, et de 5 µm de diamètre en moyenne (Clement-Larosière, 2012). Elle possède une membrane (bicouche lipidique) ainsi qu'une paroi cellulosique ayant une forte résistance mécanique. *C. vulgaris* se multiplie par reproduction



Figure 11 : Photo de *Chlorella vulgaris* (source : Clement-Larosière, 2012).

asexuée (mitose), c'est à dire par la division d'une cellule mère en 2, 4, 8 ou 16 cellules filles (spores) identiques à la cellule initiale. Aucune reproduction sexuée n'a encore été mise en évidence pour le moment. Cette microalgue possède un chloroplaste pariétal contenant des pigments photosynthétiques tels que les chlorophylles ou les caroténoïdes, et représentant 50 % du volume cellulaire. Cette algue possède de plus des enzymes membranaires relativement sensibles aux polluants telles que des phosphatases alcalines et des estérases.

- *Pseudokirchneriella subcapitata* (famille des Chlorophyceae) (Figure 12) est une microalgue planctonique unicellulaire dulçaquicole non motile (sans flagelle), vivant dans les écosystèmes d'eaux douces, rivières, lacs, étangs et zones humides. *P. subcapitata* est autotrophe. Elle se développe soit dans la colonne d'eau, soit dans les sédiments quand les conditions sont favorables. Elle mesure en moyenne 5 à 6 µm et possède une forme de croissant. Le cytoplasme de ces organismes unicellulaires

renferme de nombreux pigments chlorophylliens tels que la chlorophylle a et b et des caroténoïdes. Sa reproduction s'effectue uniquement par zoo-sporulation de 2, 4 ou 8 autospores issues d'une cellule mère. Elle se trouve généralement isolée ou sous forme de paquets difformes dans son environnement. Le test d'inhibition de la croissance chez cette algue fait partie des essais standardisés régulièrement mis en œuvre pour évaluer la toxicité chronique d'une matrice environnementale (Weyers et al, 2000).



Figure 12 : *Pseudokirchneriella subcapitata* (source : www.mokkka.hu).

- *Chlamydomonas reinhardtii* (Figure 13) est une algue ovoïde de la famille des Chlamydomonadaceae, mesurant 10 à 15 µm de diamètre en moyenne durant sa phase exponentielle de croissance. Si les algues du genre *Chlamydomonas* vivent dans de nombreux biotopes différents (eaux douces, salées, chaudes, froides, ...), *C. reinhardtii* vit principalement en eau douce ainsi qu'à la surface des sols. Elle possède un unique chloroplaste prenant environ les deux tiers de son volume cellulaire. Son organisation cellulaire interne est très similaire à celle de *C. vulgaris*. Contrairement aux deux précédentes, cette microalgue possède la particularité d'avoir deux flagelles antérieurs d'environ 12 µm de long qui lui permettent de se déplacer dans son environnement, à une vitesse de plusieurs fois son diamètre par seconde. Par ailleurs, elle se distingue des précédentes par ses deux modes de reproductions : asexuée (mitose) (la majorité du temps) et sexuée en cas des stress important (Hallmann, 2011). Cette algue se reproduit relativement vite puisqu'elle peut se diviser en l'espace de 8 heures en conditions optimales. Cette algue a particulièrement été utilisée comme espèce modèle pour étudier la photosynthèse, le métabolisme carboné et le stress oxydant.

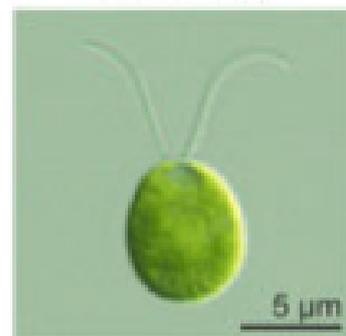


Figure 13 : *Chlamydomonas reinhardtii* (source : Hallmann, 2011).

Quelle que soit l'algue présentée ci-dessus, de nombreuses études font état de leur sensibilité respective pour les polluants classiquement retrouvés dans les RUTP. Elles possèdent de nombreuses enzymes et/ou paramètres physiologiques (e.g. photosynthèse) qui peuvent être facilement étudiés pour la détection d'espèces toxiques dans les milieux aquatiques (voir partie 2.4).

2.3.2 Les microcrustacés

Afin de réaliser notre batterie de bioessais en laboratoire, nous avons donc utilisé deux organismes très classiquement employés en écotoxicologie, représentant deux types trophiques différents, et permettant d'effectuer des bioessais monospécifiques communs en écotoxicologie : les consommateurs primaires (daphnies) et les consommateurs secondaires détritivores (ostracodes) :

- *Daphnia magna* (famille des Daphnideae) (Figure 14) est une espèce de microcrustacé zooplanctonique dulçaquicole. Les daphnies sont des organismes relativement répandus en Europe. Leur taille ne dépasse en général pas 6 millimètres de longueur. Elles sont principalement retrouvées dans les eaux douces à faible courant, comme les lacs, étangs, mares, etc. relativement riches en matière organique. Ces organismes possèdent un œil unique ainsi que deux paires d'antennes, dont les plus grandes lui servent d'appareil locomoteur et les plus petites à se nourrir. Les daphnies possèdent deux modes de reproduction. En conditions favorables, les daphnies femelles vont effectuer une reproduction parthénogénétique donnant ainsi naissance à une nouvelle génération de femelles. En conditions défavorables, un certain nombre d'œufs produits vont engendrer des mâles qui vont alors féconder les femelles. Les œufs ainsi obtenus resteront en dormance jusqu'à ce que des conditions favorables les autorisent à donner naissance à des femelles.

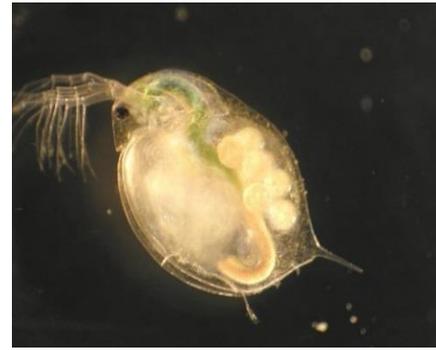


Figure 14 : *Daphnia magna* (source : www.vustah.cz).

Les daphnies sont des organismes utiles pour l'évaluation environnementale de substances/matrices de par leur facilité de culture en laboratoire, leur ubiquité et leur niveau trophique (intermédiaire entre production primaire et consommation secondaire) (Adema, 1978). Ces organismes sont de plus couramment utilisés dans l'évaluation écotoxicologique du traitement des effluents en raison de leur forte sensibilité, simplicité et rapidité de reproduction (Mohavedian et al., 2005). C'est de plus, en France, le seul bioessai réglementaire pour l'établissement de la redevance pollution des rejets en milieu dulçaquicole (eaux douces) (www.legifrance.gouv.fr).

- Les ostracodes (*Heterocypris incongruens*) (Figure 15) sont de très petits arthropodes communs faisant partie des crustacés. Ils mesurent en général entre 150 micromètres et 2 millimètres. Ils possèdent une carapace formée de deux valves composées de calcite (CaCO_3) emprisonnant leur corps. Ils possèdent de plus 7 appendices permettant leur locomotion, nourriture, enfouissement, etc. Leur reproduction s'effectue en général par parthénogénèse et mène à la formation de cystes pouvant être récupérés et conservés du fait de leur grande résistance à la dessiccation, jusqu'à utilisation. Ces organismes vivent à la surface ou à l'intérieur des sédiments marins ou continentaux. Les ostracodes sont des consommateurs secondaires détritivores se nourrissant de fines particules en suspension. Ils se développent grâce à des mues successives (environ 8 stades de développement). Les carapaces se régénèrent en quelques heures à quelques jours (Ruiz et al., 2005). Il est donc possible de suivre leur rythme de croissance.

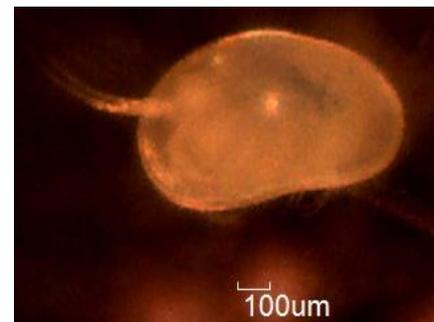


Figure 15 : *Heterocypris incongruens* (source : Photographie personnelle).

Du fait de leur taille réduite, les bioessais effectués sur ces organismes nécessitent une faible place en laboratoire. Les ostracodes sont relativement sensibles aux polluants tels que les herbicides,

pesticides, hydrocarbures et métaux lourds, pouvant être retrouvés dans les RUTP. Ils sont autant voire plus sensibles que les amphipodes, bactéries ou copépodes (Ruiz et al., 2013). Toutes ces caractéristiques en font des organismes de choix pour effectuer des tests de toxicité.

2.4 Présentation générale et justification des techniques utilisées pour les différentes étapes de notre démarche

Les différentes techniques et essais employés au cours de ce travail sont présentés ici ainsi que les arguments qui ont justifié leur choix. Les protocoles sont détaillés dans les parties spécifiques aux expérimentations effectuées.

2.4.1 Les biomarqueurs algaux

Les biomarqueurs peuvent être définis comme des paramètres biochimiques, cellulaires, physiologiques ou comportementaux mesurés dans les tissus ou les fluides d'un organisme ou sur l'organisme entier, afin de mettre en évidence l'exposition à un ou plusieurs contaminants, ou les effets de ceux-ci. (Depledge, 1994). Les raisons de l'utilisation des microalgues ont été développées précédemment dans le manuscrit. Notons que chez les microalgues, des perturbations de paramètres physiologiques ou cellulaires peuvent apparaître rapidement après une exposition à une pollution. Ainsi, les biomarqueurs cellulaires constituent de bons outils d'alerte précoce de la pollution. Au cours de notre étude, nous avons travaillé sur une batterie de biomarqueurs à la fois généraux et spécifiques de la pollution chez *Chlorella vulgaris*. Quatre biomarqueurs ont été particulièrement ciblés :

- La perméabilité membranaire. Ce biomarqueur souvent assimilé à la viabilité cellulaire est pertinent pour l'étude de la toxicité des RUTP car la membrane est la première barrière cellulaire rencontrée par les polluants, avant d'entrer dans la cellule. Une perturbation de la perméabilité membranaire peut être mesurée à l'aide d'un fluorochrome mis en contact avec les algues. Celui-ci va pénétrer dans la cellule uniquement si la membrane cellulaire a subi des dommages et est devenue perméable. Dans ce cas, il se fixe au noyau et devient fluorescent.

- La perturbation de la photosynthèse, *via* l'étude de l'autofluorescence chlorophyllienne (A-Chl). La photosynthèse convertit l'énergie lumineuse en énergie chimique (ATP) utilisable par la cellule. Elle reflète donc directement l'activité métabolique des cellules algales. La perturbation de la photosynthèse par des polluants s'effectue au niveau des chaînes photosynthétiques localisées dans les membranes thylakoïdiennes, constituant des chloroplastes.

En temps normal, l'énergie lumineuse est captée sous forme de photons par le photosystème II pour ensuite être transformée en énergie chimique (ATP) (Figure 16). Cette transformation s'effectue grâce à l'apparition d'un flux d'électrons entre les différents centres réactionnels (e.g. Quinone B) au sein des chaînes photosynthétiques jusqu'à un accepteur final. De nombreuses familles de polluants peuvent alors agir sur cette chaîne. Par exemple, certains herbicides comme le Diuron, vont venir bloquer le centre réactionnel de la Quinone B. Le processus de transfert d'électrons est alors bloqué et l'énergie est dissipée par l'émission d'une énergie lumineuse fluorescente. Selon les familles et les modes de toxicité des polluants sur les algues, il va alors apparaître une augmentation ou une diminution de la fluorescence par rapport à une cellule saine. Généralement, la perturbation de la fluorescence chlorophyllienne est mesurée par fluorimétrie avec une longueur d'onde d'excitation de 460 nm (correspondant aux chlorophylles) et une longueur d'onde d'émission de 680 nm environ. Ce biomarqueur rapide et simple à mesurer peut-être facilement adapté pour la création de biocapteurs algaux. De plus, la perturbation de la photosynthèse est un marqueur non spécifique répondant à de nombreux polluants (e.g. métaux lourds, herbicides, HAP, alkyphénols). Cela en fait donc un marqueur peu discriminant mais très sensible à la pollution et, par conséquent, pertinent pour détecter la présence de polluants dans des eaux contaminées. C'est pourquoi il a été utilisé au cours de notre étude.

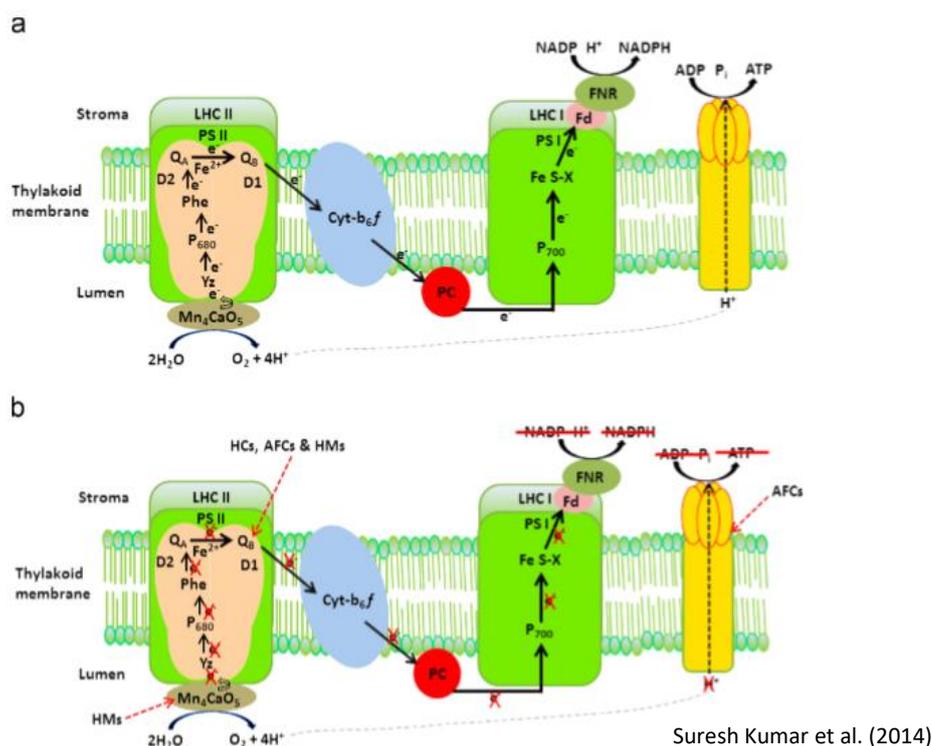


Figure 16 : Exemple de représentation de la réaction d'une chaîne photosynthétique en absence (a) ou présence (b) de polluants toxiques perturbant leur bon fonctionnement. (Suresh Kumar et al., 2014). Les quatre complexes protéiques servant à la photosynthèse sont représentés : photosystème II (PSII), cytochromes B6, photosystème I (PSI) et ATP synthase. Les flèches noires continues indiquent un transport d'électrons. Les sites d'actions des métaux lourds (HM), herbicides (HC) et composés antifouling (AFC) et leur mode de perturbation de la photosynthèse des algues sont représentés en figure 16b.

- Les activités enzymatiques des estérases et phosphatases alcalines. Contrairement aux deux précédents marqueurs, ces activités enzymatiques sont plus spécifiques et sensibles à certaines familles de polluants (respectivement pesticides et éléments traces métalliques). Les enzymes sont des catalyseurs de réactions chimiques au sein des cellules vivantes. Chez les microalgues, les estérases sont des enzymes impliquées dans le processus de photosynthèse. Les phosphatases alcalines ont quant à elle un rôle sur la gestion du stock interne de phosphore des algues, indispensable à leur développement. Ce sont donc deux enzymes qui sont impliquées dans des processus cellulaires cruciaux pour les microalgues. Elles sont, de plus, des enzymes membranaires dont l'activité est facilement et rapidement détectable et perturbée par les polluants. Ces caractéristiques en font de bons indicateurs de la toxicité des RUTP. Elles sont, toutes deux, des enzymes michaeliennes régies par une équation de réaction enzymatique représentée sur la Figure 17 A. Les polluants, interagissant avec ces enzymes, vont venir se fixer, soit sur leur site actif (site où le substrat se fixe et où la réaction se produit) à la place du substrat dans le cas d'une inhibition compétitive, soit sur le complexe substrat/enzyme dans le cas d'une inhibition non compétitive, entraînant une diminution de la vitesse de réaction de ces enzymes.

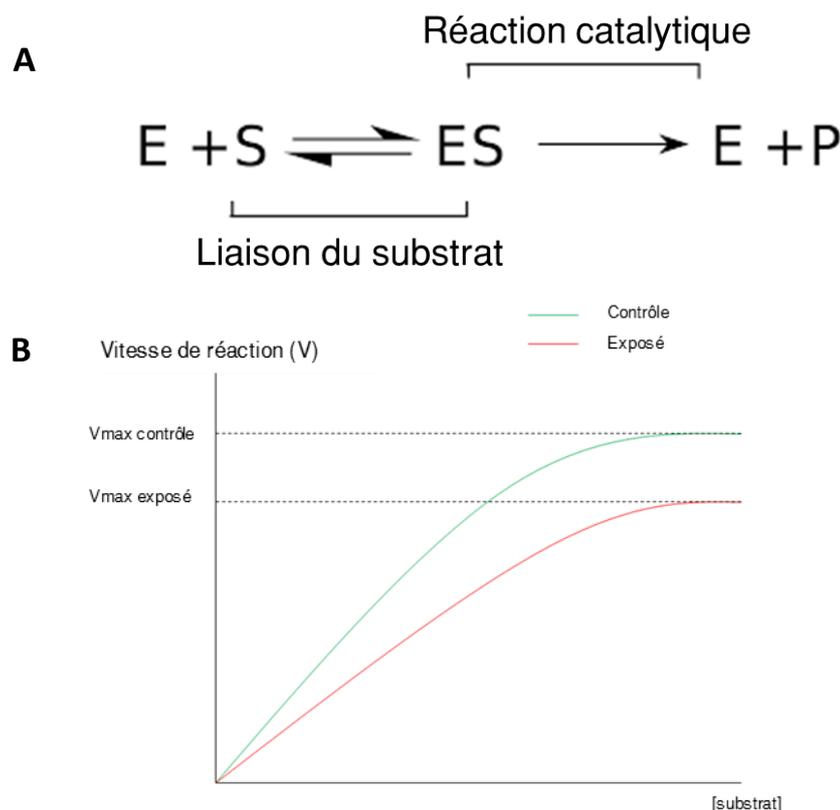


Figure 17 : (A) Equation simplifiée d'une réaction enzymatique (E : Enzyme, S : Substrat, P : Produit) (B) Exemple de perturbation d'une cinétique enzymatique de type Michaëlis-Menten après exposition à un polluant quelconque.

En laboratoire, une perturbation de leur activité enzymatique peut alors être suivie à l'aide d'un substrat artificiel dont la réaction, avec l'enzyme concernée, le transforme en un produit fluorescent.

Après une contamination des algues aux polluants, elles sont mises en contact avec une gamme de concentration en substrat, durant une période donnée (dans notre étude, 30 min), et l'apparition du produit par fluorescence est alors observée. Il est ensuite possible de faire un suivi de la vitesse de réaction de l'enzyme au cours du temps en fonction de la concentration en substrat, et de déterminer, pour des algues exposées ou non, une vitesse maximum de réaction enzymatique (Figure 17 B). Une perturbation de leur réaction peut alors être déterminée après exposition aux RUTP par exemple.

Par ailleurs, nous avons également initié un travail préliminaire concernant l'analyse du stress oxydant chez *Chlamydomonas reinhardtii*. Les biomarqueurs du stress oxydant permettent le suivi d'un stress toxique en présence de certains polluants, qui engendrent la création de ROS (reactive oxygen species), qui dégradent le matériel cellulaire. De nombreuses réactions enzymatiques vont alors très rapidement se mettre en place afin de les éliminer. Une augmentation de l'activité de ces systèmes de défense permet donc de détecter la présence de polluants au sein des microalgues. Nous avons mené un premier travail sur 4 biomarqueurs classiques du stress oxydant chez les microalgues (perturbation des activités enzymatiques de la catalase, l'ascorbate peroxydase, la superoxide dismutase et l'apparition d'une peroxydation lipidique). L'étude de ces biomarqueurs repose sur une mesure par absorbance des cinétiques d'apparition ou disparition de substrats/produits de la réaction de ces enzymes. Dans le cas de la peroxydation lipidique, une modification de la structure des acides gras (dégradation) présents sur les membranes cellulaires va libérer des résidus (Malondialdehyde). Ceux-ci sont repérés *via* leur mélange avec un réactif (acide thiobarbiturique), suivi d'une détection par absorbance du produit de la réaction entre le malondialdehyde et l'acide.

Une vue d'ensemble des différents biomarqueurs algaux étudiés au cours de la thèse est présentée en figure 18.

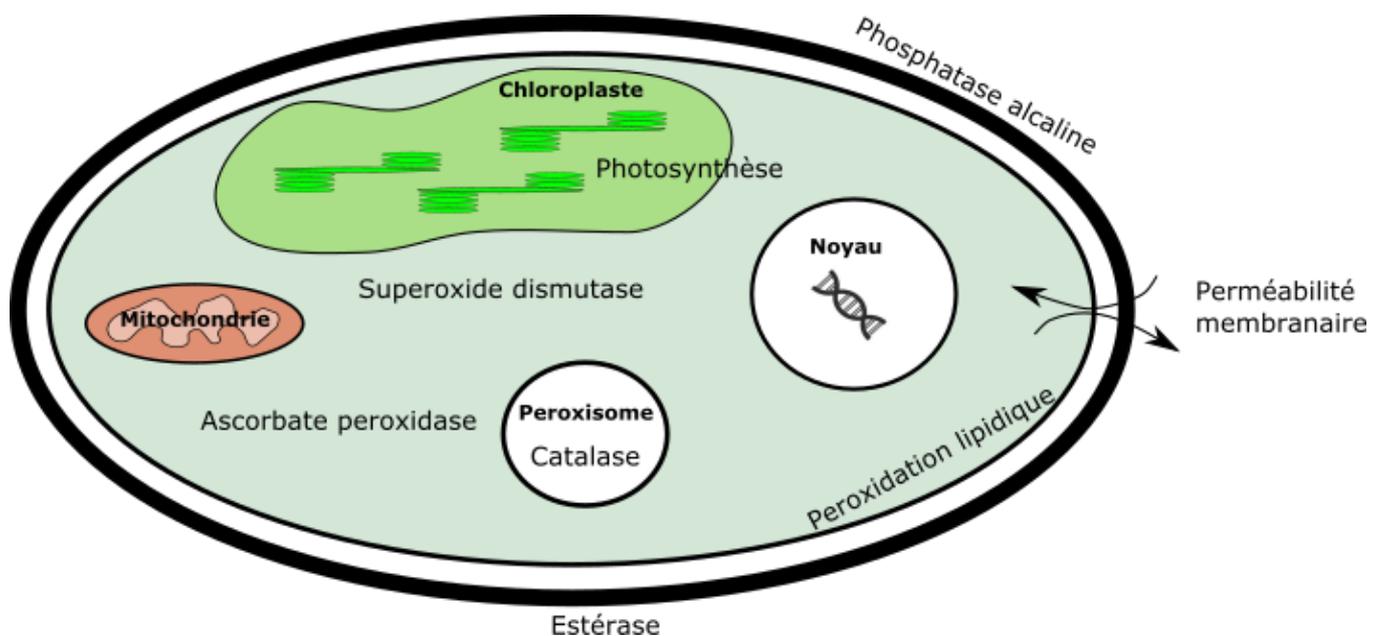


Figure 18 : Vue d'ensemble des différents biomarqueurs étudiés sur *C. vulgaris* et *C. reinhardtii*.

2.4.2 Les bioessais monospécifiques réalisés

Nous avons réalisé, en parallèle des biomarqueurs sur microalgues, une batterie de bioessais dits monospécifiques sur différents niveaux trophiques. Ces essais ont été les suivants :

- L'essai d'inhibition de la croissance des populations de *C. vulgaris* et *P. subcapitata*, des microalgues (producteurs primaires). Cet essai est basé sur l'exposition d'une population d'algues à une concentration donnée, laissée en culture durant 72 heures sous des conditions de lumière et température particulières, avec un rejet liquide (RUTP) à tester ou non (contrôle). A la fin de l'essai, la concentration des populations témoins et exposées est déterminée, et le taux d'inhibition de la croissance est ainsi établie.

- L'essai d'inhibition de la mobilité de *Daphnia magna* est basé sur l'exposition de daphnies néonates (<1 jour) à une solution toxique (RUTP) durant 24 et/ou 48 heures dans des tubes à essais, dans le noir à une température contrôlée. A la fin de l'essai, le nombre de daphnies encore mobiles dans les témoins et les tubes exposés est décompté, afin de calculer le pourcentage d'immobilisation.

- L'essai d'inhibition de la croissance d'*Heterocypris incongruens*. Cet essai est basé sur l'exposition d'ostracodes néonates (<2 jours) durant 6 jours à une matrice toxique (RUTP) dans le noir à une température contrôlée. Au début et à la fin de l'essai la taille des individus exposés ou non (contrôle) est mesurée afin de calculer l'inhibition de la croissance des individus mis en contact avec les échantillons testés.

Ces essais ont été choisis car ils concernent différents niveaux trophiques des écosystèmes. De plus, ils permettent d'évaluer la toxicité des RUTP sur matrice filtrée ou non, et enfin de prendre à la fois en compte des effets toxiques aigus et chroniques.

2.4.3 Développement des biocapteurs algaux optiques

Dans l'objectif d'aboutir à la mise au point de biocapteurs, nous avons travaillé sur une étape essentielle : la mise en contact bio-récepteur (microalgues)/transducteur.

2.4.3.1 Immobilisation des algues via des hydrogels

L'utilisation de la double encapsulation des algues dans des hydrogels pour créer des biocapteurs a déjà été initiée par plusieurs auteurs dont Ferro (2013). Cette partie de notre travail s'est inscrite dans la continuité des expérimentations de ce dernier. Elle a visé à améliorer l'élaboration et la sensibilité des biocapteurs à hydrogel, afin de développer des outils capables d'être déployés sur le terrain pour le suivi *in situ* des RUTP. La technique d'immobilisation utilisée est basée sur un processus en 2 étapes : la première a consisté à inclure des microalgues dans des billes d'hydrogel alginate ; la deuxième à emprisonner les billes dans un hydrogel de silice vitreuse (Figure 19). Cette technique permet

de protéger l'alginate d'agents chimiques (comme les ions phosphates) qui pourraient entraîner sa dissolution. L'alginate permet quant à lui de protéger les algues de la toxicité du procédé de fabrication (sol-gel) de l'hydrogel de silice. Une double encapsulation est donc nécessaire. Les avantages de cette technique nous ont amenés à l'utiliser pour la création de biocapteurs. Ces derniers sont ainsi durables dans le temps (puisque'épargnés du problème de dissolution de l'alginate classiquement utilisé seul pour l'immobilisation des algues), et de bonne qualité optique grâce à la grande transparence des hydrogels. La création des deux hydrogels repose sur un procédé dit de « sol-gel » basé sur une hydrolyse puis une condensation de composés colloïdaux (silice ou alginate) présents dans les solutions utilisées pour la création des hydrogels poreux. Dans notre cas, les pores siliceux sont de diamètre plus faible que les microalgues, empêchant leur libération.



Figure 19 : Illustration du principe d'immobilisation des microalgues *via* l'utilisation d'hydrogels. (A) Billes d'alginate contenant des microalgues immobilisées, (B) Hydrogel de silice, (C) Principe de double encapsulation des algues dans des micropuits.

2.4.3.2 Immobilisation des algues à l'aide de nanofibres

Les nanofibres peuvent être utilisées pour immobiliser les microalgues de deux façons différentes : par d'adsorption et colonisation de nanofibres (Eroglu et al., 2012), ou par piégeage dans une cavité fermée à l'aide d'un tapis de nanofibres (Lee et al., 2009). L'utilisation des nanofibres s'avère ainsi intéressante pour créer un piège physique (rétention/adsorption) suffisamment lâche pour permettre aux solutions (RUTP, effluents, ...) à tester d'être en contact avec les microalgues, de manière optimale, sans barrière trop importante. C'est pourquoi ces deux types d'immobilisation ont été envisagés au cours de ce travail. Afin de créer ces nanofibres, nous avons utilisé une technique appelée électrospinning. Cependant, les résultats obtenus n'étant pas fructueux, ils n'ont pas été abordés dans les sections résultats suivantes.

2.4.3.3 Insertion des microalgues dans des systèmes microfluidiques

Les grands principes de la microfluidique et ses atouts ont été présentés au paragraphe 1.3 de la partie bibliographique. Ici, il s'agit d'introduire plus particulièrement la technique de xurographie utilisée pour la création de puces microfluidiques. Cette technique a été pour la première fois utilisée par Bartholomeusz et al. (2005) dans le cadre des microtechnologies en général. La xurographie peut être définie comme l'utilisation d'outils de découpe de feuilles polymères, adhésives ou non (Figure 20 A). Dans notre cas, une feuille adhésive double face est découpée avec la forme des microcanaux, puis les parties non désirées (qui seront les canaux) sont retirées à la pince. La feuille est ensuite collée entre deux lames de verre. Cette technique présente de nombreux avantages qui ont mené au choix de son utilisation pour la conception de biocapteurs : elle est peu chère (1500 euros pour une machine de découpe, une trentaine d'euros pour un couteau de découpe et de l'ordre d'un euro le mètre d'adhésif), rapide à réaliser (environ 1 minute par puce) et sans contrainte de salle blanche, contrairement à la technique de lithographie molle très majoritairement employée. Un exemple de puce ainsi créée est illustré dans la figure 20 B. Il est alors possible d'insérer des microalgues, exposées sans contrainte de barrière physique avec les polluants, par capillarité à l'intérieur de ces systèmes afin d'évaluer la perturbation de la fluorescence chlorophyllienne, au travers des lamelles de verres transparentes, de manière sensible et rapide.

Deux étapes ont été réalisées afin de valider l'intérêt de cette technique pour la conception de biocapteurs algaux. La première « en laboratoire » a consisté à fabriquer des puces simples, pour démontrer la sensibilité des réponses obtenues avec des algues insérées dans ces puces à l'aide d'une fibre optique mise au contact des algues. La seconde étape a abouti à la création d'un fluorimètre portatif capable de lire la fluorescence des microalgues introduites dans des puces. Le dispositif ainsi obtenu permet d'effectuer des mesures de terrain.



Figure 20 : (A) appareil de xurographie (GRAPHTEC CE 5000 – source : <http://www.promattex.com>). (B) puce microfluidique à canal unique réalisée par xurographie (photographie personnelle).

Résultats et Discussion

3 Bioessais et biomarqueurs en laboratoire

3.1 Contexte et enjeux

L'objectif de cette étude est de démontrer/observer l'utilité des biomarqueurs algaux pour la détection précoce de toxicité en comparaison aux divers bioessais classiquement menés en laboratoire. En effet, certains bioessais monospécifiques (croissance algale, mobilité des daphnies) manquent de sensibilité pour détecter la toxicité de RUTP. Ces rejets présentent la particularité d'une pollution de concentrations variables dans le temps et l'espace, et de la présence de polluants très souvent à l'état de traces. La discrimination d'un biomarqueur sensible à la pollution permettra d'adapter ce dernier à la réalisation de biocapteurs de pollution *in situ*. Nous avons donc réalisé une batterie classique de bioessais sur divers organismes représentant plusieurs niveaux trophiques (détritivores, producteurs primaires, consommateurs primaires) : inhibition de la mobilité à 24 et 48 h sur *Daphnia magna*, inhibition de la croissance à 6 jours d'*Heterocypris incongruens* et inhibition de la croissance à 72 h de *Pseudokirchneriella subcapitata* et *Chlorella vulgaris*). En parallèle nous avons mené sur *Chlorella vulgaris* une batterie de tests comprenant 4 biomarqueurs ayant chacun leur spécificité : perméabilité membranaire (souvent associée à une viabilité cellulaire), activité phosphatase, activité estérase et activité photosynthétique, après 2 et 24 h d'exposition (exposition de courte durée afin d'observer des effets précoces).

3.2 Article 3 - Microalgal whole-cell biomarkers as sensitive tools for fast toxicity and pollution monitoring of urban wet weather discharges

Article en cours de finalisation pour soumission dans Chemosphere.

Microalgal whole-cell biomarkers as sensitive tools for fast toxicity and pollution monitoring of urban wet weather discharges.

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Abstract

Urban wet weather discharges (UWWD) management is an important issue. UWWD represent most of the time an important source of untreated pollution for all aquatic bodies. This pollution is complexe to predict due to the variety of storm events and urban watershed leached by the rain. Previous studies tried to demonstrate ecotoxic impact of UWWD. However, most of them point out the limit of *classic monospecific bioassays due to the high dilution of micropolluants or the presence of nutrients masking toxic effects*. Overcoming this problem is therefore of strong interest. In this study, we demonstrated the utility to use a battery of biomarkers (Membrane permeability, chlorophyll fluorescence, esterase and alkaline phosphatase activities) on the microalgae *Chlorella vulgaris* to detect toxic effect of 7 UWWD samples after short exposures (2 and 24 hours). These biomarkers are linked to the microalgal life-traits. Complementarily, monospecific bioassays were carried on *Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Daphnia magna* and *Heterocypris incongruens* to compare their sensitivity to the UWWD samples. No toxic effect was obtained with all the bioassays. At the opposite, algal biomarker pointed out a disturbance of microalgae physiology, and particularly a perturbation of chlorophyll fluorescence was observed for all the sampled tested. While algal membrane permeability was affected by only one UWWD, the two enzymatic activities were stimulated or inhibited depending of the sample. Finally, this study demonstrates the sensitivity of algal biomarkers and generally the need to develop new fruitful approaches to characterize UWWD toxicity.

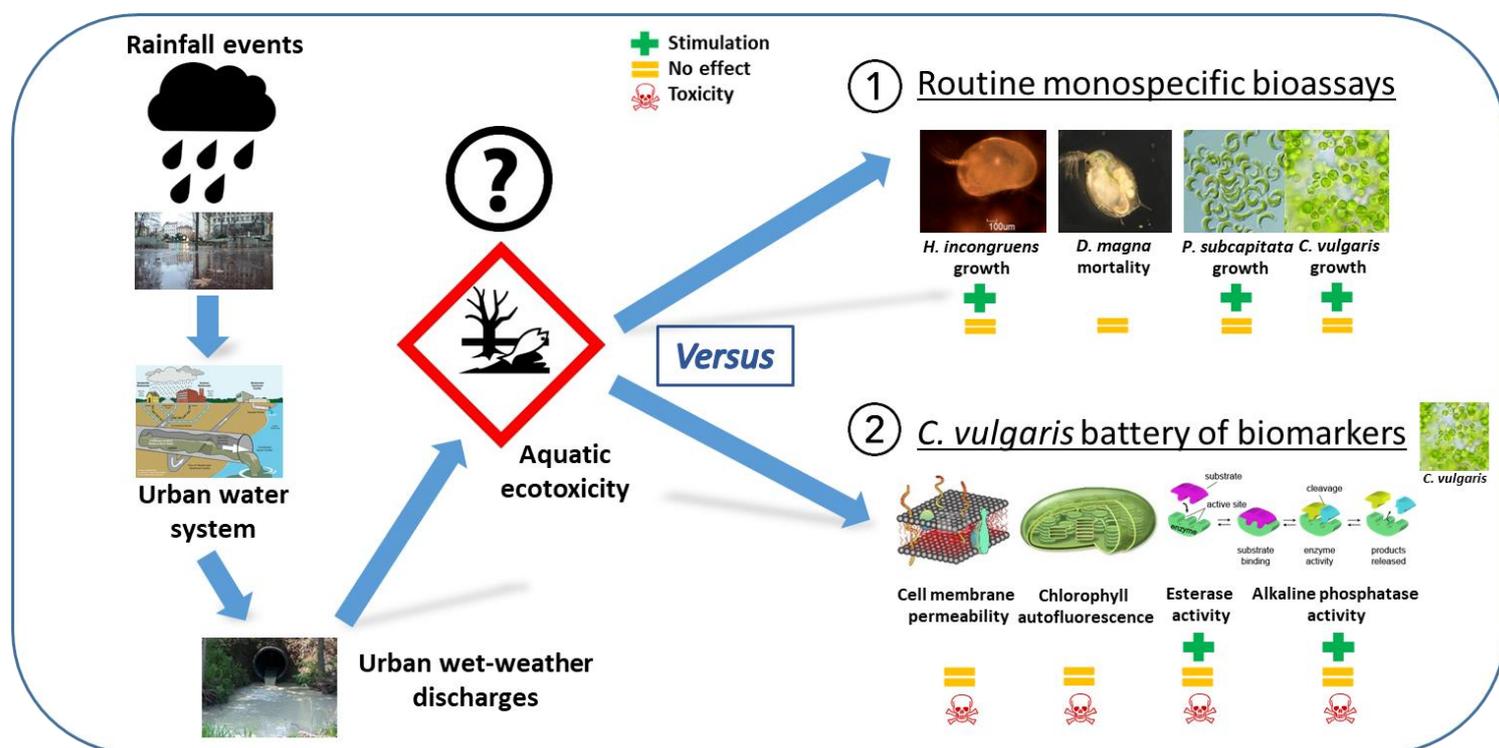
Keywords

Urban wet weather discharge, Ecotoxicity, Bioassays, Biomarker, Microalgae, Microcrustacean

Highlights

- Urban wet weather discharges often contain residual micropollutant concentrations.
- A new developed algal biomarker battery was applied to UWWD samples.
- Algal Autofluorescence appeared to be particularly sensitive.
- These biomarkers could be employed for UWWD *in situ* monitoring (biosensors).

Graphical abstract



1. Introduction

Urban wet weather discharges (UWWDs) (stormwaters/combined sewer overflows) represent an important source of pollution for aquatic mediums. Their treatments are often limited in the case of stormwater to simple infiltration ponds (Gosset et al., 2016), or inexistent in the case of combined sewer overflows (CSO). Consequently, many pollutant families have been detected in these effluents, with low concentrations however. For instance, Polycyclic Aromatic Hydrocarbons (PAHs) (Hwang et Foster, 2006 ; Terzakis et al., 2008; Zgheib et al., 2011; Kalmykova et al., 2013), (alkyl-)phenols (Bjorklund et al., 2009; Zgheib et al., 2011; Cladière et al., 2013); heavy metals (copper, lead and zinc, etc.), polychlorobiphenyls (PCBs), pesticides, and phthalates (Gasperi et al., 2008; Terzakis et al., 2008; Kalmykova et al., 2013; GooRé Bi et al., 2015; Becouze-Lareure et al., 2016) can be found in these effluents. They represent an ecotoxicological hazard already demonstrated by Marsalek et al. (1999), Tang et al. (2013), and Wu et al. (2014).

In the aim to preserve suburban water bodies as imposed by the European Urban Wastewater Directive (91/271/EEC), it is necessary to develop monitoring tool to detect chemicals from the lowest concentrations. These tools have to be sensitive, cost effective, fast, reliable and easy to performed bioassays (Wieczerzak et al., 2016). Many classical monospecific bioassays are currently used to assess the ecotoxicity of urban stormwaters. 24-h *Daphnia magna* immobilization, 6-d *Heterocypris incongruens* or 7-d *Lemna minor* growth assays are among the most common ones. However, some

studies already showed the difficulty to detect urban wet weather discharges toxicity, mainly due to their low contamination level, and/or to the presence of nutrients interfering with measured endpoints (Angerville et al., 2013; Sebastian et al., 2014; Gosset et al., 2016) with these monospecific bioassays. As noted by Wigh et al. (2016), urban treated wastewaters can contain residual concentrations of micropollutants and standardized monospecific bioassays might not be sensitive enough to detect ecotoxicity. This can be applied to urban wet weather discharges, since Tang et al. (2013) demonstrated that stormwater toxicity could be compared to secondary treated urban effluents. Consequently, it is necessary to development more sensitive ecotoxicity assays.

Among organisms used for UWWDs ecotoxicity assessment, microalgae are particularly interesting. Microalgae are considered to be a good model for ecotoxicity bioassays because they represent the first level of the trophic chain and any disturbance have effect on upper levels (Chouteau et al., 2004; Machado et Soares, 2012). Moreover, they are very sensitive to many toxicants (e.g. heavy metals, pesticides, pharmaceuticals), easy to culture (low cost organisms), and as unicellular organisms present a good reproducibility, reliability and robustness for ecotoxicity tests (Silva et al., 2009). Microalgae have a short life cycle and are able to quickly respond to the environmental changes over a number of generations (Kaczala et al., 2011). Algal growth assay is also commonly used for stormwater ecotoxicity assays. However, there are some limitations of using microalgae for ecotoxicity assessment. Among them, we can particularly retain: the inability of commonly used automatic counting techniques to distinguish between live and dead cells or particulate matter, the inability to measure more than one test endpoint in one single species at a time (Stauber et al., 2002), and interference occurred with nutrients in the samples for ecotoxicity detection. As expressed by Machado and Soares (2014), algal growth bioassays (i.e. *P. subcapitata* growth inhibition) can detect slow responses to toxic substances and polluted matrices, e.g. Kaczala et al. (2011) with stormwater runoff samples.

However, quickly toxic effect can be detected by using early warning systems based on the observation of metabolic perturbations (distress signals), toxicity biomarkers, using short time of exposure, little amounts of toxic matrices and automatized reader (Torres et al., 2008; Machado and Soares, 2015).

In our previous works specific bioassays were developed to studying response of micro phytoplankton after exposure of different chemicals. Some of them are more or less specific to certain toxicant compounds families. For example, Durrieu and Tran-Minh (2002), Chouteau et al. (2004, 2005) and Guedri and Durrieu (2008) demonstrated that alkaline phosphatase enzyme activity, linked to vital algal phosphorus metabolism, was more inhibited by heavy metals than by other chemicals families (with inhibitions detected for heavy metals concentration (e.g. Lead) at around 10 µg/L after only 30 min exposure). Esterase enzymes activities are also used as a biomarker of microalgae's viability. These enzymes are directly linked to photosynthesis metabolism. Their sensitivity to pesticides has been demonstrated by Prado et al. (2009). Chouteau et al. (2005) previously observed organophosphates ecotoxicity on microalgae after few hours/days exposure. Moreover, this marker can be affected by

heavy metals (Yu et al., 2007; Machado and Soares, 2013), or organic toxicants (Gilbert et al. 1992). Since these enzymatic biomarkers efficiency have been proof with pure substances, they were also applied for natural samples monitoring (Gilbert et al., 1992; Blaise and Menard, 1998; Regel et al., 2002; Durrieu et al., 2004). Non enzymatic biomarkers as membrane permeability present also a great interest since cellular membrane is the first structure in contact with pollutants, after extracellular wall. Consequently, membrane integrity is directly linked to the normal functioning of the microalgae and is used to assess microalgae viability (Machado and Soares, 2012). Moreover, since also previous enzymatic biomarker specified before are located on the external membrane of microalgae, membrane integrity is particularly important to control. All these discussed enzymatic (or not) biomarkers are easy to monitor in laboratory, using non fluorescent probe let in contact with organism and degraded in fluorescent product.

Algal growth and photosynthesis disturbance are the basic reflex of the toxic effects of pollutants on microalgae (Franqueira et al., 2000; Torres et al., 2008). A-chlorophyll autofluorescence is a biomarker directly linked to as photosynthesis disturbance in ecotoxicological studies. This wide used can be linked to the fact that this biomarker is impacted less specifically by a huge number of pollutant's families as herbicides, biocides (e.g. Tributyltin), heavy metals (Suresh Kumar et al., 2014), alkylphenols (Gao and Tam, 2011), aromatic polycyclic hydrocarbons (Aksmann and Tukaj, 2008) or phthalates (Melin and Egnéus, 1983) able to impact different part of the two photosystems (e.g. herbicides on Photosystem-II Quinone B). Microalgal autofluorescence disturbance was applied to various polluted matrixes as raw sewages, wastewater treatment plant effluents, wastewaters, tap waters, surfaces waters, and one time to stormwaters samples (Tang et al., 2013).

Development of new tools (here biomarkers) for fast assessment of the ecotoxicological risk caused by contaminant discharges in aquatic mediums is of great interest for UWWDs management (Moore et al., 2004). The purpose of this study was to conduct an ecotoxicological *Chlorella vulgaris* short-exposure biomarker battery (membrane permeability, esterase, phosphatase and chlorophyll emission measurement) performed on several campaigns of UWWDs sampled in the aim to demonstrate their early sensibility. A comparison was performed with classical monospecific ecotoxicological acute and sub-chronic bioassays, carried on in various trophic levels (*D. magna* immobilization inhibition, *H. incongruens* growth inhibition, *P. subcapitata* and *C. vulgaris* growth inhibition assays). Our objective is to demonstrate the utility and early sensitivity of biomarkers compared to these assays often time consuming and not enough sensitive to detect potential toxicity of these rejects.

2. Materials and Methods

2.1 Sampling sites and procedure

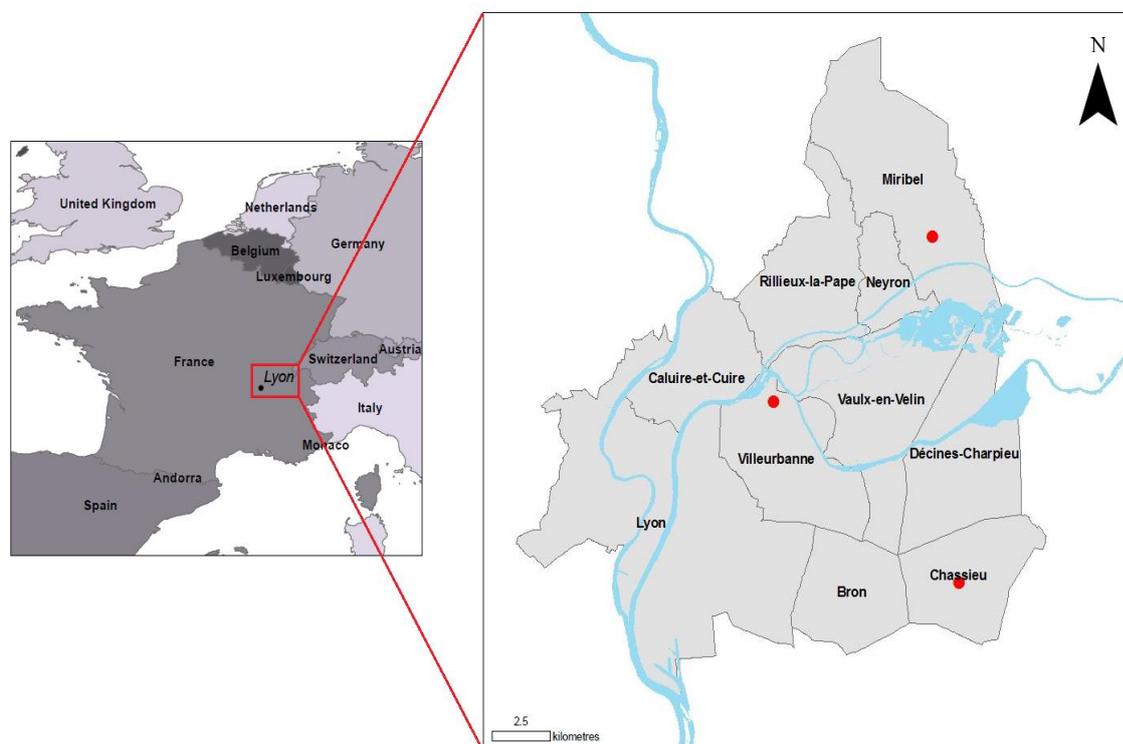


Figure 1 - Sampling site location of the stormwaters. Sampling sites are highlighted in red.

The seven discharges studied here were sampled between February and May 2015. Three types of stormwaters linked to their watershed were studied (Table 1). The first one corresponds to discharges collected in the stormwater outfall of a retention–infiltration basin located in Chassieu, a sub-urban zone on Lyon agglomeration (Figure 1). The watershed is a highly urbanized industrial area of 185 ha, with an imperviousness rate of 75 % (El-Mufleh et al., 2014). The second one (Torrent du Couloup) corresponds to discharges collected in the overflow of three interconnected retention ponds, in the city of Miribel. The watershed of 11 ha drained is sub-urban with an important agricultural activity. Finally the third one (IUT La Doua) correspond to stormwaters collected in the outfall of a 750 m² infiltration basin located in Villeurbanne a high-urbanized zone on Lyon agglomeration. The watershed at the origin of the discharges is around 2.5 ha, with many tertiary activities (teaching and research building, companies, car parks, lawns, roads) (Datry et al., 2003).

Grab stormwater samples were collected manually with 2 L high-density polyethylene flasks previously washed three times with these discharges. All the samples were immediately transferred to the laboratory after collection. Then, aliquots were prepared and stored at -20 °C.

Table 1: Characteristics of the rain events for stormwater samples collected in this study.

Date	Site	Duration (hh:min)	Rain height (mm)	Rain intensity (mm/h)	Dry period before rain event (jj:hh:min)
03-25-2015	Chassieu - Reinhardt	Django 20:06	17.2	0.86	03:03:48
05-05-2015	Chassieu - Reinhardt	Django 00:06	0.4	4.00	01:09:48
02-27-2015	Miribel - Couloop	Torrent du 09:42	13.,2	1.36	02:01:54
04-19-2015	Miribel - Couloop	Torrent du 04:30	5.4	1.2	01:09:48
04-19-2015	Villeurbanne - doua	IUT La 04:24	4.6	1.04	01:08:35
02-27-2015	Villeurbanne - doua	IUT La 11:36	11.8	1.02	02:01:18
03-25-2015	Villeurbanne - doua	IUT La 19:00	16	0.84	03:04:24

2.2 Physico-chemical analyses of wastewater samples

Classical physico-chemical parameters (as pH, Conductivity, Dissolved Organic Carbon (DOC), Total suspended Solids (TSS) and ion chemical's concentrations (e.g. Na⁺, NH₄⁺, K⁺, Mg₂⁺, Ca₂⁺, Cl⁻, NO₂⁻, PO₄³⁻, NO₃²⁻, SO₄²⁻) were measured according to European standards as described by Angerville et al. (2013).

A total of 105 organic compounds were analyzed by an accredited (COFRAC accreditation by the French Ministry of Ecology and Sustainable Development) laboratory (CARSO, Chassieu, France) according to European standards (34 pesticides, 15 Polycyclic Aromatic Hydrocarbons (PAHs), 22 Volatile Organic Compounds (VOCs), 19 Benzene derivatives, 12 Phenol derivatives and 3 others) on samples previously filtered at 0.45 µm. The methods and standards used for chemical analyses of organic pollutants were provided and detailed by Gosset et al. (*in press*). Additionally, seven major metals (Pb, Cr, Ni, Cd, Cu, Fe, and Zn) were also analyzed by ICP-AES (PinAAcle 900T Perkin Elmer).

2.3 Test organisms culture

Daphnia magna original culture was obtained from the National Research Institute in Science and Technology for Environment and Agriculture (IRSTEA). Organisms were cultured in two litter glass jar, under a light intensity of around 500 lux (± 200) and a temperature of 25 (± 2) °C, with a 16 h light/8 h dark photoperiod. *Daphnia magna* were cultured in normalized AFNOR medium. *D. magna* were weekly transplanted to permit to keep healthy cultures.

The algal strains were purchased from the French National Museum of Natural History (Paris, France). *P. subcapitata* and *C. vulgaris* were cultured in Lefebvre-Czarda (LC) medium (AFNOR, 1980). Algae cultures were transplanted weekly under sterile condition, using sterilized glassware and medium (autoclaved during 20 min, at 130 °C, under 1.3 bar), with the aim of using fresh algae in their exponential growth phase. Algae were maintained at 21 °C in a culture room under a 16 h/8 h light/dark cycle (5000 lux illumination). In the case of algal phosphatase activity, *C. vulgaris* solutions were cultured during 3 weeks in LC medium depleted in phosphate (starvation process), in the same conditions than described before, in the aim to remove all the internal stocks of phosphate in algae and lead to a maximum phosphatase activity for enzymatic assays (Chouteau et al., 2005). At the end of the starvation period, algae were stored one month maximum at 4 °C in the dark for the assays.

2.4 Daphnia (*D. magna*) mobility inhibition test

This mobility inhibition bioassay is a 24/48 h acute toxicity test conducted following the European norm (AFNOR 2012a). Briefly, four individual replicates (containing 5 neonates) were exposed to different concentrations of a non-filtered stormwater at 25 °C in 10 mL test tubes, in the dark. After 24 h and 48 h, the tubes were gently shaken and the number of mobile individuals per well was counted. EC₂₀ 24 h/48 h values were calculated using the Hill probit model (Regtox®, E. Vindimian, <http://normalesup.org/~vindimian/>).

2.5 Ostracode (*H. incongruens*) growth inhibition test

This monospecific bioassay is a 6-days chronic toxicity test, conducted following the European norm NF ISO 14371 (AFNOR 2012b), using the OSTRACODTOXKIT F®. Ostracode cysts obtained from the kit were hatched in standard freshwater (created from the kit) during 52 hours before to start the assays, at 25 °C, under 3000-4000 lux. Ten neonates were sacrificed to measure the initial ostracod neonate length. After that, six replicates (in 6 wells-plates) of ten hatched neonates were exposed to a 50 % concentration of each non-filtered wastewaters following the normalized protocol and six replicates were also exposed to a 95 % concentration of each non-filtered wastewater which was the highest possible exposure concentration due to a 5 % concentration of algal suspension added as a food source. After 6 days of exposure in the dark, ostracodes mortality and growth were compared to a control group. For that, mortality was noted, and surviving individuals anaesthetized in lugol and their body length measured with the Motic® computer program.

2.6 Algal (*P.subcapitata* and *C. vulgaris*) growth inhibition test

Briefly, after 3 days of algae preconditioning under the test conditions climatic chamber (Aralab Fitoclima D 1200), at 21 °C under a continuous lighting of 10 000 lux in 48 well plates, growth inhibition assays were performed following the OECD (2006) guidelines, exposing algae (initial concentration of 1.10⁴ cell/mL) to a range of (filtered or unfiltered) stormwater concentration under continuous light

during 72 hours. These assays have been done in triplicate. At the end of the assay, algae exposed in plates were re-suspended and algal concentration determined using a Thoma cell and an optic microscope (magnification x400). EC₂₀ 72 h values were calculated using the Hill probit model (Regtox®, E. Vindimian, <http://normalesup.org/~vindimian/>).

2.7 Algal (*C. vulgaris*) toxicity biomarkers assays

2.7.1 Algal exposure to stormwaters

For all algal biomarkers assays, exposure have been done in 48 well plates. Aliquot of 500 µL of microalgae in growing medium (final concentration of 5.10⁵ cell/mL) were mixed with 500 µL of 0.45 µm filtered or unfiltered stormwater sample, and exposed during 2 and 24 hours. For all the biomarkers assays using fluorescent probe, negative controls were done with samples without algae. Consequently, some of the data are lacking for enzymatic analyses because of the biodegradation of fluorescent substrates consumed by endogenous microbial organisms initially present in samples. They were noted “not determined” (N.D.). Moreover, since probes used for enzymatic activities are photodegradable, negative control with probe only was used to correct data.

2.7.2 Algal cells membrane permeability

For cell viability assays, protocol used was adapted from (Machado and Soares, 2012). 48-well plates were centrifuged in the aim to remove exposure medium and algae were suspended in 250 µL of ultrapure water to avoid any interference of medium with probes. 100 µL of algal solution (final concentration of 1.10⁶ cell/mL) exposed or not to UWWDs were put in contact with 100 µL of Sytox Green probe (final concentration of 0.25 µM) in dark 96-wells plate, let in contact 40 minutes in the dark and fluorescence signal was read with a microplate fluorimeter (Fluostar, BMG) (480/538 nm ex./em. Couple). Positive control was done in the same conditions exposing algae at 65 °C during one hour in the aim to kill them to optimize fluorescence signal (100 % of cells with permeable membranes), and calculate the percentage of algal cells with permeable membranes. Assays were performed in 4 replicates.

2.7.3 Esterase activity

Experiments were done following the same principle that in Chouteau et al. (2005) and Machado and Soares (2014). Briefly, microplates were centrifuged and exposure medium (samples or control) was removed. Algae (final concentration of 1.10⁶ cell/mL) were exposed to a range of dilution of enzyme substrate (0, 2, 4, 6, 8, 12, 16, 20 µM of Fluoresceine Diacetate) in 96 well microplate. Microplate were immediately placed in a microplate reader fluorimeter (Fluostar, BMG), and fluorescence of created product (fluoresceine, 480/538 nm ex./em. couple) was read during 30 min. After that, maximum speed reaction (V_{max} , in arbitrary Units/min) was determined analyzing Michaelis and Menten kinetics

obtained for exposed and control and enzymatic activity inhibition was assessed comparing V_{\max} using the following equation 1:

$$\text{Eq. (1): Ares (\%)} = \frac{V_{\max S}}{V_{\max C}} * 100$$

Where $V_{\max S}$ was the maximum reaction speed of the algae exposed to sample and $V_{\max C}$ the maximum reaction speed of the control algae. Assays have been done in triplicates.

2.7.4 Alkaline phosphatase activity

This biomarker assay was also carried out following the experimental procedure described in Chouteau et al. (2005), and previously described for esterase activity, but using starved microalgae. The principle of this assay was the same than for esterase activity, with another substrate (MUP: 4-Methylumbelliferyl phosphate, with a 0, 2, 4, 6, 8, 12, 16, 20 μM concentration range) transformed in a fluorescent product (MUF: Methyl-umbelliferone) detected with the excitation/emission couple 365/460 nm. For these activity, Tris-HCl (final concentration: 0.01 M, pH = 8.5) buffer medium was added in the wells. Assays have been done in triplicates.

2.7.5 A-Chlorophyll autofluorescence

Microalgae A-chlorophyll autofluorescence assays were performed using the method of Védrine et al. (2003), and Machado and Soares (2015). After stormwater exposure, 100 μL of microalgae solution was put in 96-well plate and A-Chl autofluorescence was read using a microplate reader fluorimeter (Fluostar, BMG), with a 460/680 nm ex./em. Couple. Fluorescence increase/decrease compared to control was calculated using the following equation:

$$\text{Eq. (2): Fluorescence enhancement (\%)} = \frac{(A\text{-Chl fluo } S - A\text{-Chl fluo } C)}{A\text{-Chl fluo } C} * 100$$

With A-Chl fluo S the autofluorescence of algae exposed to stormwaters and A-Chl fluo C autofluorescence of control algae. Assays have been done in 4 replicates.

2.8 Statistical analyses

Statistical analyses were done using GraphPad Prism version 7 for windows (GraphPad software, San Diego, CA, USA). Kruskal-Wallis followed by Dunn's post-tests were used to test significant differences for algal and ostracod growth bioassays and membrane permeability biomarker since data were not normally distributed. For A-Chlorophyll autofluorescence assays, one-way ANOVA followed by Dunn's post-tests, because of parametric conditions validation. Concerning other biomarkers (esterase and alkaline phosphatase activities), Mann-whitney tests were performed since each exposed algae to sample was compared to their own control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and discussion

3.1 Physico-chemical analysis of stormwater samples.

Physico-chemical analyses are presented in Table 2. Concerning routine water quality parameters, pH values were included between 7.03 and 8.49, and so neutral to slightly alkaline. Conductivity was ranged from 39.5 to 338 $\mu\text{S}/\text{cm}$, TSS from 12.2 to 256.14 mg/L. COD was comprised between 1.96 and 23.5 mg/L. One of the seven stormwater was significantly different in term of anions and cation concentrations, mainly due to the small rainfall event: Chassieu (05/05/2015), explaining the high conductivity of 338 $\mu\text{S}/\text{cm}$. Nevertheless, all the values are consistent with the huge amount of literature about UWWd physico-chemistry (e.g. Zgheib et al., 2011). However, any comparison of UWWd pollutants concentrations with literature studies would be unfruitful because of their variability dependent on the leached watershed activities.

Then, chemical analyses highlighted that for the seven stormwaters, several pollutants were detected, the chemical pattern depending on the characteristics of the corresponding watershed. Six heavy metals (Cr, Cu, Pb, Ni and Zn) were detected in most samples, except for Cd and Pb only present in respectively two and four of 7 stormwater samples. In all of them, Zn and Cu concentrations were important (up to their PNEC value). On the contrary, low concentrations of Pb was detected (or not) everywhere. The presence heavy metals can be easily explained by the vehicle brake emissions for (Cu), tire wear (Zn) atmospheric deposition (Cu and Pb) (Davis et al., 2001; Zgheib et al. 2012).

Regarding organic micropollutants, some pesticides were observed in the microgram per liter range concentration. For example, chemical analyses exhibited in Chassieu (03/25/2015 and 05/05/2015) a concentration of respectively 2.6 and 0.5 $\mu\text{g}/\text{L}$ of Fosetyl-aluminium fungicide. Glyphosate herbicide was measured in three samples, until 69.25 $\mu\text{g}/\text{L}$ in Miribel (04/19/2015) (significant concentration in comparison with the PNEC value). Finally, one of its degradation by-product, AMPA (acide 2-amino-3-(5-méthyl-3-hydroxy-1,2-oxazol-4-yl)propanoïc) was detected three samples also, until 20.15 $\mu\text{g}/\text{L}$ in Miribel (04/19/2015) too. Presence Glyphosate and AMPA in the runoff may be related to the agricultural activities (Miribel), the road and private garden maintenance, and the municipal uses (Zgheib et al., 2011). Fosetyl-aluminium detection in Chassieu samples is unclear.

Polycyclic aromatic hydrocarbons compounds sought, only one was detected in all samples: Fluorene with relative important concentrations compared to PNEC values (between 0.85 and 6.04 $\mu\text{g}/\text{L}$). Its presence is mainly explained by the activities related to vehicles (e.g. road traffic, gas stations, maintenance, garages) (Zgheib et al., 2012). Concerning alkyphenols, Nonylphenol, a molecule used biocides production as chemical adjuvant, was also detected in one sample (Miribel 02/27/2017: 0.158 $\mu\text{g}/\text{L}$) at low concentration. Finally, for others compounds, Tetrachloroethylene was detected in Villeurbanne (02/27/2015) at 0.99 $\mu\text{g}/\text{L}$, Tributyl phosphate in three samples until 1.15 $\mu\text{g}/\text{L}$ and Benzyl

chloride in 4 samples (max. 4.4 µg/L). Their presence could result from plastics production, and wood industry.

All the other molecules sought in stormwaters analyzed were under their limit of quantification and consequently not detected. In term of ecotoxicological hazard, pollutants considered as dangerous (Measured Concentration up to the PNEC) are highlighted in Table 2. Copper, Zinc, and Fluorene concentration observed would so theoretically lead to ecotoxic adverse effects on aquatic organisms exposed to these 7 discharges. Depending of the stormwater, Glyphosate, Benzyle chloride and other metals (Cd, Cr and Ni) presence also participate to the ecotoxic effect. These results are consistent with those obtained by Gosset et al. (2017), point out their contribution on the dangerousness of UWWD, and demonstrate the presence here of therorically hazardous effluents (substance approach) for aquatic ecosystems.

Table 2: Physico-chemical analyses of the 7 stormwater samples collected in 2015, perfomed on the filtered part of the discharges. Aquatic PNEC value associated to the different pollutants detected were taken from INERIS and AGRITOX international databases. Concentrations up to the PNEC values are written in bold.

Parameters	Chassieu 03/25/2015	Chassieu 05/05/2015	Villeurbanne 02/27/2015	Villeurbanne 03/25/2015	Villeurbanne 04/19/2015	Miribel 02/27/2015	Miribel 04/19/2015	Freshwater aquatic PNEC (µg/L) (source: INERIS (2005) / AGRITOX databases)
pH	8,01	8,49	7,23	7,03	8,33	7,12	7,18	/
Conductivity (µS/cm)	103,6	338	39,5	59,5	113,1	69,4	105,1	/
TSS (mg/L)	82	256,14	12,2	17,34	15,4	221	56,26	/
COD (mg/L)	7,1	23,5	1,96	3,74	15	2,71	6,53	/
Na ⁺ (mg/L)	4,8	12,0	1,6	5,6	1,6	1,0	2,4	/
NH ₄ ⁺ (mg/L)	1,1	6,9	0,09	0,2	0,10	0,6	0,3	/
K ⁺ (mg/L)	1,7	10,3	0,7	1,6	4,7	2,8	3,3	/
Mg ²⁺ (mg/L)	0,7	4,7	0,2	0,3	0,40	0,5	0,6	/
Ca ²⁺ (mg/L)	17,1	45,0	9,3	12,9	12,0	15,1	25,4	/
Cl ⁻ (mg/L)	0,7	21,1	0,9	0,8	8,9	0,2	1,6	/
NO ₂ ⁻ (mg/L)	<0,01	0,4	0,02	0,02	0,08	0,01	0,05	/
NO ₃ ⁻ (mg/L)	1,6	29,4	1	4,6	4,9	0,5	7,0	/
PO ₄ ³⁻ (mg/L)	0,01	0,02	<0,01	<0,01	0,01	0,01	0,3	/
SO ₄ ²⁻ (mg/L)	0,3	33,7	0,66	1,2	2,1	0,4	3,4	/
<u>Pesticides (µg/L)</u>								
Glyphosate	N.D.	N.D.	N.D.	N.D.	13,975	2,265	69,25	28
AMPA	0,975	0,783	N.D.	N.D.	N.D.	N.D.	20,15	80
Fosetyl-aluminium	2,6	0,573	N.D.	N.D.	N.D.	N.D.	N.D.	590
<u>Alkylphenols (µg/L)</u>								
Nonylphenol	N.D.	N.D.	N.D.	N.D.	N.D.	0,158	N.D.	0,33
<u>PAHs (µg/L)</u>								
Fluorene	3,449	5,367	0,854	2,573	4,796	6,035	5,934	0,25
<u>Others (µg/L)</u>								
Tributyl phosphate	0,118	1,154	N.D.	N.D.	N.D.	0,065	N.D.	82
Tetrachloroethylene	N.D.	N.D.	0,99	N.D.	N.D.	N.D.	N.D.	51
Benzyle chloride	N.D.	N.D.	1	4,4	2,6	N.D.	0,5	1
<u>Metals (µg/L)</u>								
Cadmium (Cd)	0,09	0,24	N.D.	N.D.	N.D.	N.D.	N.D.	0,19
Chromium (Cr)	0,63	8,54	10,13	0,24	0,59	0,28	0,31	4,7
Copper (Cu)	12,48	10,61	6,89	6,99	18,39	2,31	4,38	1,6
Lead (Pb)	0,1	0,38	1,39	N.D.	N.D.	0,19	N.D.	2,1
Nickel (Ni)	1,23	3,95	8,01	2,93	4,95	0,64	0,59	1,7
Zinc (Zn)	80	210	170	30	40	20	20	7,8

3.2 *D. magna*, *P. subcapitata*, *C. vulgaris* and *H. incongruens* monospecific bioassays

Looking for *D. magna* immobility inhibition bioassays, no toxicity was detected regardless the samples for all exposure conditions (until 90 % of stormwater) at 24 and 48 hours of exposure, leading to the calculation of an EC₂₀ value above 90 % for each of them. This absence of acute toxicity on *D. magna* is in accordance with tendencies shown in previous studies (Marsalek et al., 1999; Waara and Färm, 2008; Angerville et al., 2013, Becouze-lareure et al., 2016; or Gosset et al., *in press*). Some of these studies suggested the lack of sensitivity of *D. magna* bioassay concerning this kind of discharges.

In the Figure 2 are presented algal (*P. subcapitata* and *C. vulgaris*) growth inhibition bioassays results. With *P. subcapitata* growth inhibition bioassays, no toxicity was observed until 90 % of stormwater samples. For concentration for each one no significant toxicity was observed ($p > 0.05$), conducting to EC₂₀ up to 90 %. However, it was possible to observe slight toxicity effect tendencies for 4 samples, e.g. Chassieu 05-05-2015, and Miribel 04-19-2015 (respectively 17.50 ± 2.07 % and 12.38 ± 4.23 % with filtered samples). Few stimulation (but not significant) was observed in some samples like Chassieu 03-25-2015. Concerning *C. vulgaris* assays, same tendencies appeared for the different samples, but for one of them (Chassieu 03-25-2017), a significant stimulation after 72 h exposure (-8.85 ± 2.75 % ($p = 0.046$) and -10.93 ± 1.04 % ($p = 0.025$)) was measured after unfiltered and filtered sample exposure.

Looking for *H. incongruens* bioassay, mortality observed for all the samples and control (50 and 95 % of sample exposure) was less than 10 %. Figure 3 illustrates *H. incongruens* growth inhibition results at 50 and 95 % of stormwater sample after 6 days' exposure. After contact to a 50 % stormwater concentration, as recommended in the standardized bioassay, ostracod growth was not significantly affected regardless of the sample. With 95 % of stormwater in the exposure medium, a more representative concentration of the samples for toxicity evaluation, three samples (Villeurbanne 03-25-2015 and 04-19-2015 and Miribel 04-19-2015) presented a slight growth stimulation compared to control ($p < 0.05$). On the contrary, for one of them, ostracod growth was significantly slightly inhibited (11.53 ± 11.13 %, $p = 0.022$). Compared to others monospecific chronic standardized tests, *H. incongruens* growth inhibition bioassay did not suggest a biological significance threshold. However, several authors such as Becouze-Lareure et al. (2016) placed this threshold at the same level than the other chronic standardized *Brachionus calyciflorus* reproduction test: 30 % of toxic/stimulation effects. Here, we used so the same limit. Consequently, regarding *H. incongruens* assays results, toxicity observed in the Chassieu 05-05-2015 sample cannot be considered as biologically significant.

In brief, these two sub-chronic bioassays appeared to be useless to detect stormwater ecotoxicological effects. Many authors as Sterner (1997), Adams and Stauber (2004), Elser et al. (2007), Waara and Färm (2008), Xin et al. (2010) or Civitello et al. (2014), have pointed out that nutrient enrichment such as with phosphorus and nitrogen can stimulate the growth and reproduction of primary producers and consumers. They could consequently hinder ecotoxicological effects (Adams and

Stauber, 2004; Waara and Färm, 2008; Gosset et al., *in press*). In our case, nutrients (e.g. between 0.5 and 29.4 mg/L of NO_3^- in stormwaters samples) could have participated to stimulate the algal growth. In *H. incongruens* bioassay, algal population supplied would have been stimulated and consequently enhanced the amount of available food for primary consumers (Gosset et al., *in press*).

To conclude, as expressed by Burton et al. (2000) and Griffin et al. (1991), traditional ecotoxicity testing seems to be unappropriated to study the impact of urban runoffs and others UWWD, notably due to the exposure design of the bioassays (e.g. constant exposure to the same discharge concentration during a finally short time). Sub-chronic bioassays employment to UWWD study remains questionable.

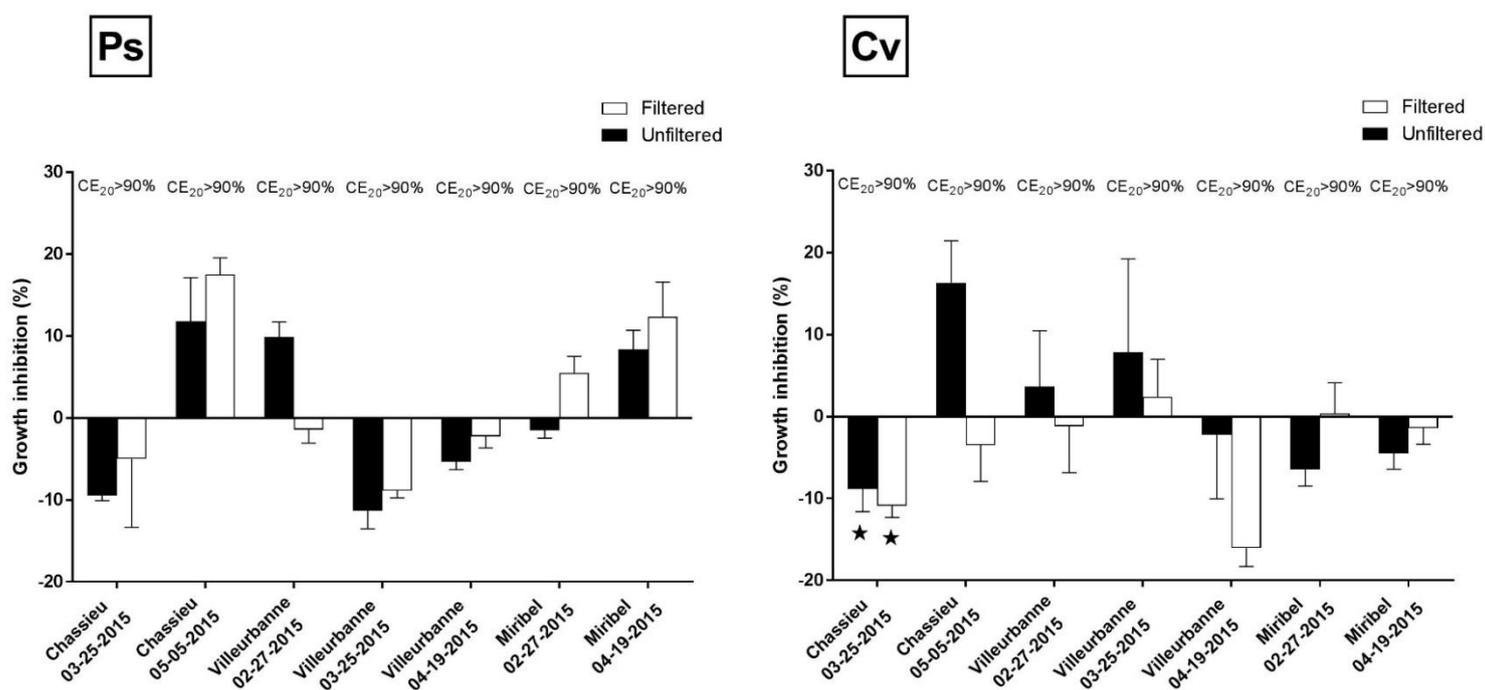


Figure 2: *P. subcapitata* (Ps) and *C. vulgaris* (Cv) growth inhibition at 90 % of sample exposure, after 72 hours exposure to the 7 filtered or unfiltered stormwater samples collected from February to May 2015, and CE_{20} associated. Bars show standard deviation (n=3). Asterisks indicate a significant difference from the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3 Algal biomarker's battery

Biomarkers battery was performed with filtered and unfiltered stormwater samples for two principal reasons: to understand the part of toxicity due to dissolved and particle fractions of the discharges, and because in some case bacteria or particles could interfere with used probes and lead to mistakes.

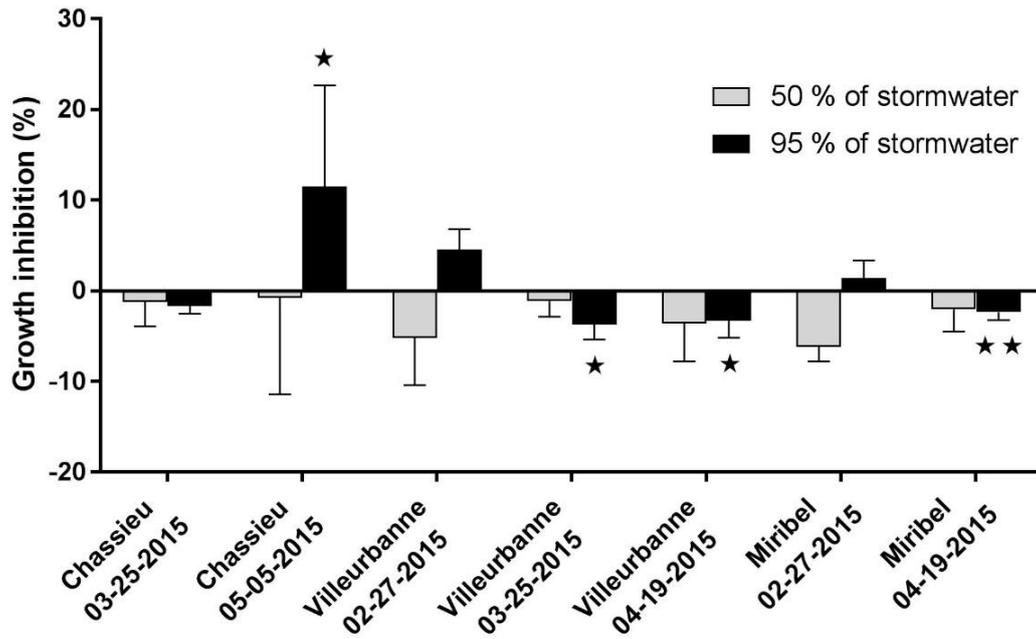


Figure 3: Ostracod growth inhibition compared to control after 6 days exposure to 50 and 95 % of 7 unfiltered stormwaters samples collected from February to May 2015. Bars show standard deviation (n=6). Asterisks indicate a significant difference from the control (* p<0.05, ** p<0.01, *** p<0.001).

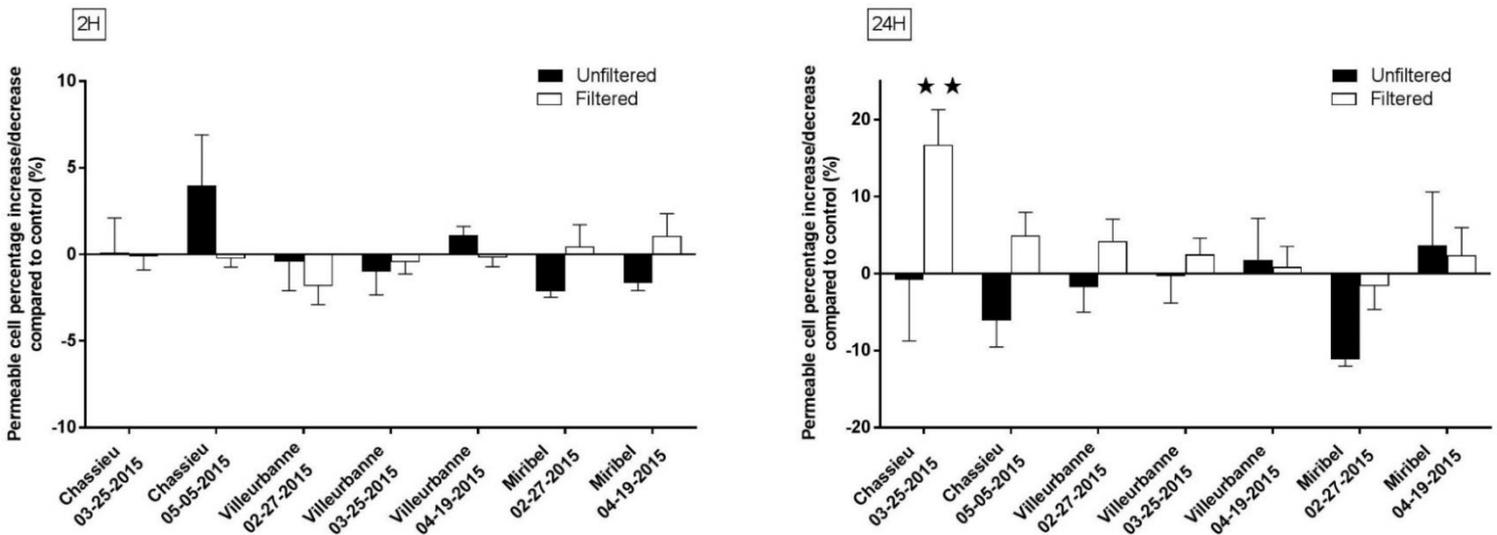


Figure 4: *C. vulgaris* membrane cell permeability (identified to cell viability) after 2 and 24 hours exposure to 7 filtered or unfiltered stormwater samples collected from February to May 2015. Bars show standard deviation (n=4). Asterisks indicate a significant difference from the control (* p<0.05, ** p<0.01, *** p<0.001).

3.3.1 Cell membrane permeability (cell viability)

C. vulgaris cells membrane permeability (Figure 4) exposed to stormwaters did not statistically differ from the control after 2 hours exposure whatever the sample. However, after 24 hours exposure, a toxicity appeared for filtered sample from Chassieu (03-25-2015), with a slight but significant ($16.72\% \pm 4.49$, $p = 0.0095$) increase of cell permeability compared to control. For all other samples, no statistically differences were observed with this biomarker. Membrane permeability is often associated to cell viability, the ability to divide (Machado et al., 2012), and the algal growth. The absence of cell death in most of the cases is in coherence with the growth assays results which did not show any toxic effects of the tested stormwaters.

3.3.2 A-Chl autofluorescence

A-Chlorophyll fluorescence modification compared to control is directly linked to a photosynthesis process disturbance. No chlorophyll fluorescence disturbance was observed after 2 hours of contact with *C. vulgaris*, regardless of the stormwater samples, except for a filtered one (Chassieu 05-05-2017), which highlighted a significant decrease of fluorescence ($-9.08\% \pm 3.26$, $p=0.044$) (Figure 5A). After 24 hours' exposure, results pointed out an increase of chlorophyll fluorescence for all the 7 filtered samples (until a value of $24.14\% \pm 4.76$ for Chassieu 05-05-2015, $p = 0.0001$). On the contrary, tendency was unclear for unfiltered samples with increase (Chassieu 05-05-2017, $13.4235097\% \pm 0.31$, $p=0.0001$) or decrease (Miribel 02-27-2017, $-10.93\% \pm 2.17$, $p=0.0001$) of fluorescence depending of the sample.

These results are consistent with Tang et al. (2013) who observed that photosynthesis disturbance (in their case using a Phyto-PAM measuring photosynthetic efficiency) was a most sensitive parameter than algal growth assay to assess urban stormwater toxicity. It has been also expressed by several authors that growth and photosynthesis disturbance are the basic reflex of the toxic effects of pollutants on microalgae (Franqueira et al., 2000; Torres et al., 2008).

In our study, experiments with filtered sample seemed to lead to better responses than with whole effluents (7 filtered samples impacting fluorescence, against 2 whole stormwater samples). This point is in contradiction to previous study (Angerville et al., 2013; Becouze-lareure et al., 2016) that observed with microcrustaceans (*H. incongruens*). Particle phase of UWWD was the most toxic for organisms. In our case, we suggest that humic substances present in particle phase can interact with pollutant such as heavy metals and reduce their bioavailability for microalgae. Moreover, humic substances can interact with algal cell (adsorption), and lead to transfer of labile phosphorus, carbon or nitrogen towards cells (Koukal et al., 2003), masking toxic effects. Finally, these substances can represent a physical barrier to toxic small (e.g. nano) particles, bacteria or virus and represent a protection against UV (Campbell et al., 1997).

Regarding substances responsible of the observed toxic effects, herbicides are often associated to the toxic effects observed on photosynthetic apparatus (Tang et al., 2013; Suresh Kumar et al., 2014). Nevertheless, in many case, multiple compounds can disturb it. Fluorene concentrations could explained these results. Poor number of studies concern the impact of PAH on algal chlorophyll fluorescence. However, Aksmann and Tukaj (2008) proved effect of anthracene on *C. reinhardtii* autofluorescence, or also Chung et al. (2007) of Pyrene, Naphtalene and Phenanthrene on *P. subcapitata*. Presence of heavy metals in the seven samples may have also an impact on the fluorescence disturbance, as it has been already evidenced with pure solution of copper on *P. subcapitata* (Machado et al., 2015), and *C. vulgaris* (Hadjoudja et al., 2009). Finally, the delay observed in toxic response (in most cases after 24 h than 2 h), have deeply observed by Hadjoudja et al. (2009), or could be easily explained by a bioconcentration of pollutants along the time.

3.3.3 Esterase and alkaline phosphatase activities

Toxicity of stormwater samples was also observed at the biochemical level with enzymatic biomarkers. The enzymatic activities were modified when *C. vulgaris* was exposed to the different samples. However, these changes were not significantly different (using Mann-whitney test), due to the low number of replicates (3). Nevertheless, looking for the Figure 5B, it can be observed that three of the 7 samples led to an important esterase activity inhibition after 2 hours' exposure (Chassieu 03-25-2015, 05-05-2015 and Miribel 04-19-2015) compared to their respective control. For two on them, important stimulation of this activity was observed (Villeurbanne 03-25-2015 and Miribel 02-27-2015). After one day of exposure, most of the sample studied led to an important stimulation of esterase activity. However, one unfiltered sample (Chassieu 03-25-2015) was at the origin of a consequent inhibition (residual activity of $56.11 \% \pm 7.18$).

Esterase activities have been demonstrated as relatively specific of organophosphate pesticides (Chouteau et al., 2004, 2005). However, in most case, inhibition of enzymes is not really specific (Durrieu et al., 2004). This nonspecific effect has been already observed for example on freshwater creeks samples (Regel et al., 2002)

Stimulation of esterase activity have been observed in many studies with exposition of algae to low pollutant concentrations (Francklin et al., 2001; Regel et al., 2002; Hadjoudja et al., 2009; Prado et al., 2009; Melegari et al., 2013). Stimulation observed for those samples can be interpreted in several ways. First of all, an explanation would consist to say that stimulation is due to nutrients (nitrogen, phosphorus, and organic matters). Notwithstanding, various studies suggested that stimulation of esterase enzyme activity in presence of low pollutants concentration would be explained by either: (1) Augmentation of membrane permeability due to membrane hyperpolarization favoring an FDA uptake (esterase substrate employed) in the cells (Francklin et al., 2001; Hadjoudja et al., 2009). However, as observed previously (3.3.1), this way seems to be false because of absence of stormwater effects on *C. vulgaris* membrane permeability. (2) FDA and Fluorescein (FDA enzymatic product) pH dependence.

FDA and Fluorescein fluorescence is enhanced with pH increase (Lage et al., 2001). Some pollutants such as heavy metals (e.g. copper, zinc) can affect ionic regulations algal systems (Sodium/Hydrogen channels), leading to a pH increase and consequently an increase of Fluorescein fluorescence. (3) A defense mechanism consisting in an adaptation of their physiology to stressful conditions created by toxic compounds and a help to repair cell damages (Prado et al., 2009).

Results of residual alkaline phosphatase activities assays are presented in Figure 5C. Non filtered Chassieu 03-25-2015 exposed group exhibited an important inhibition after 2 hours (residual activity $15.99 \% \pm 4.59$). For other samples, not huge toxicity, and sometimes stimulation was observed. The algal response pattern was quite different after 24 hours exposure: if important phosphatase activity appeared for 3 samples, 2 unfiltered samples present a substantial reduction of enzymatic activity compared to control (respective residual activities of $81.49 \% \pm 1.18$; $50.97 \% \pm 4.70$ for Villeurbanne and Miribel 04-19-2017). Ecotoxic effects observed here could be principally explained by heavy metals presence in stormwaters, as alkaline phosphate is relatively specific to these pollutant family. Enzymatic inhibition was demonstrated after *C. vulgaris* 240 minutes' exposure to $10 \mu\text{g/L}$ of Cadmium, Zinc or and Lead (Chouteau et al., 2005), concentrations in coherence with those detected in these stormwaters. Few studies highlighted influences of some organic compounds too, such as glyphosate, detected in our samples (Singh and Darmwal, 2004). The observed stimulation causes widely observed notably after 24 h exposure are similarly the same than for esterase activity, except for the defense mechanism that would consist here in an stress that increase of alkaline phosphatase activity explained by the depletion of intracellular phosphate (essential for algal growth) due to pollutants (e.g. glyphosate), as demonstrated by Singh and Darmwal (2004).

To conclude, Although, changes in the esterase and alkaline phosphatase enzyme activities of *C. vulgaris* were evident, no significant statistical differences between control and exposed algae were detected. Nevertheless, we obviously proved here the sensitivity of algal biomarkers compared to monospecific bioassays after a short exposure, with a huge number of toxic responses depending of the sample and the biomarker studied, showing the importance to use a battery of biomarkers.

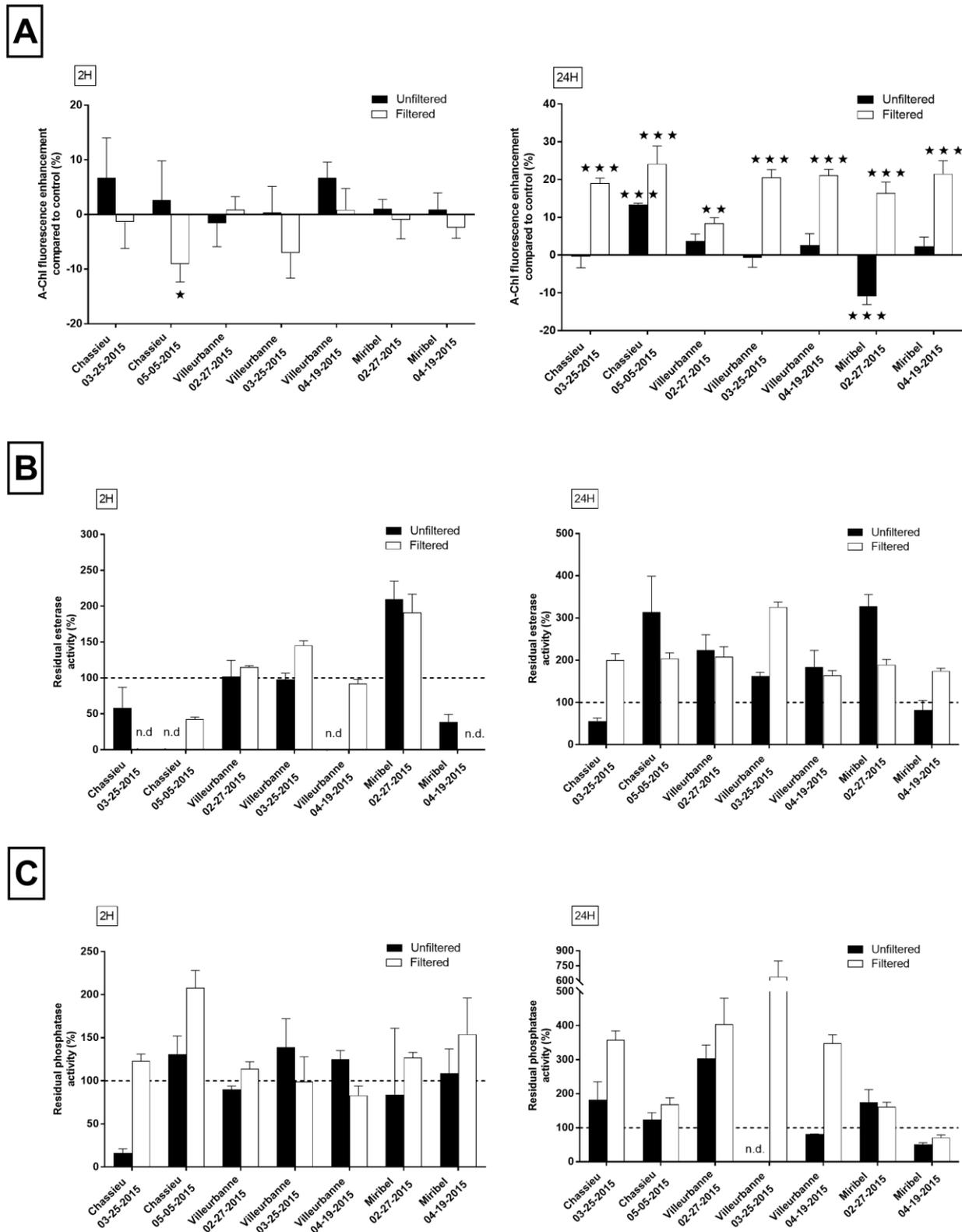


Figure 5: *C. vulgaris* A-Chl autofluorescence disturbance (A), esterase (B) and alkaline phosphatase (C) residual activities after 2 and 24 hours exposure to 7 filtered/unfiltered stormwater samples collected from February to May 2015. Bars show standard deviation (n=4 for photosynthetic activities and 3 for the 2 others). Asterisks indicate a significant difference from the control (* p<0.05, ** p<0.01, *** p<0.001).

3.4 Limits of links between physico-chemical analyses and ecotoxicity results

Many ecotoxicological studies tried to highlight relationships between pollutants concentrations detected in urban discharges and ecotoxicity results. However, it is often difficult to obtain direct links between them and especially when matrixes are low polluted (Becouze-Lareure et al., 2016). This finding is explained by several facts: (1) The ecotoxicological bioassays are taking into account all pollutants present in discharges, when physico-chemical analyses can only find what they are researching and can so forget hundreds of molecules; (2) The biodisponibility for the aquatic organisms employed of the micropollutants is unknown (due to interactions between chemicals and particles sorption/desorption phenomenon); (3) As evocated before (3.2), nutrients can also masked the toxicity of discharges; (4) Chemical analyses do not take into account indirect ecotoxic effects such as bioconcentration and bioaccumulation which can have a strong influence on the toxicity of many pollutants such as herbicides (Fahl et al., 1995); (5) As suggested by Wigh et al. (2016), biomarker response (as bioassays) sensitivity is influenced by many parameters. Among them, the sample characteristic itself, the exposure design (e.g. time), and metabolism of exposed organisms. Consequently, the absence of toxicity may be due only to the study design and not the absence of sublethal toxic effects. Finally, for all these reasons it is often, as in our case, useless to realize in deep statistical analyses to link chemistry and toxicity.

4. Conclusion

In this study, the utility of the employment of a microalgal biomarker battery for the toxicity assessment of urban wet weather discharge samples has been studied. Physico-chemical analyses revealed a very variable mixture of residual and important concentration of various micropollutants families, depending of the UWWD watershed. The set of classical monospecific bioassays (including algal growth inhibition bioassay) did not reveal any toxic effect for the different aquatic compartment studied, suggesting that they are not well adapted to assess ecotoxicity of this type of contamination. However, the microalgal biomarker battery permitted to detect toxic disturbance of algal metabolism after short exposure (2 and 24 h) to the samples, with an important response variability between samples. A-chlorophyll fluorescence disturbance seems to be the most sensitive biomarker among the four studied (sensitivity order: A-chlorophyll fluorescence > esterase activity = phosphatase activity > membrane permeability). So, these markers should consequently be considered for toxicity evaluation of UWWD. Next experiments should be consisting of a transposition of these biomarkers in *in situ* experiments directly in UWWD outfall and receiving waters (e.g. by the deployment of algal biosensor) to create early warning systems for water body's management and ecological good status preservation.

Conflict of interest

The authors declare that they have no conflict of interest.

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3.3 Synthèse et discussion

Au cours de ce travail, nous avons tout d'abord mis en évidence la présence de composés inorganiques (métaux lourds) et organiques (pesticides, hydrocarbures ou des phénols) dans les RUTP étudiés, à des concentrations susceptibles d'impacter les organismes aquatiques (concentrations supérieures à leur valeur de PNEC). Nous avons montré un manque de sensibilité des bioessais monospécifiques (standardisés ou non) pour mettre en évidence une toxicité des RUTP, confirmant ainsi les tendances observées dans la littérature de ces deux dernières décennies. Par ailleurs, nous avons démontré l'utilité des biomarqueurs chez les microalgues (ici *C. vulgaris*) pour identifier et mesurer des effets toxiques liés à la présence de polluants dans les RUTP. Ces biomarqueurs ont permis de détecter des perturbations après des expositions très courtes des organismes (2 et 24 heures). La perturbation de la fluorescence chlorophyllienne est particulièrement intéressante car, en plus d'être un paramètre sensible, son observation ne nécessite pas l'usage d'additifs chimiques (substrat, solvant, etc.).

Ces biomarqueurs algaux sont des outils rapides et pertinents pour la détection de la toxicité des RUTP. Toutefois, leur usage se limite à des essais *ex situ*, impliquant le transfert des échantillons depuis le terrain au laboratoire, et leur stockage. Cette chaîne de gestion des échantillons peut altérer les échantillons et conditionner la réponse aux bioessais, les analyses sont alors effectuées *a posteriori* de l'évènement pluvieux. C'est la raison pour laquelle nous nous sommes intéressés par la suite à la conception et l'étude de biocapteurs algaux susceptibles d'être mis en œuvre *in situ* (partie 4 suivante).

Mais avant cela, nous présentons ci-dessous (3.4) une étude préliminaire relative au développement de biomarqueurs algaux du stress oxydant qui semblent avoir un fort potentiel pour observer la toxicité des RUTP.

concentrations en ROS dans la cellule. Une augmentation de la concentration des ROS va généralement engendrer une augmentation de leur activité.

La production de ROS est stimulée par des facteurs de stress environnementaux tels que les Ultraviolets (UV), les températures extrêmes, les métaux lourds, les pesticides ou d'autres polluants organiques (Marshall et Newman, 2002). Le stress oxydant provoqué par ces conditions modifie rapidement le métabolisme des organismes vivants. La mesure des activités des enzymes du stress oxydant ou le dosage des molécules non-enzymatiques nous donnent une indication sur le niveau de stress subi par les organismes. Il est donc intéressant d'étudier les biomarqueurs du stress oxydant pour évaluer la toxicité des milieux aquatiques. Le suivi de ces molécules représente ainsi un intérêt certain en écotoxicologie. Certaines enzymes sont utilisées comme biomarqueurs du stress oxydant chez des organismes exposés à des environnements pollués.

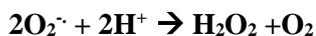
Au cours de notre étude de la toxicité des RUTP, nous nous sommes particulièrement attachés à considérer 4 biomarqueurs du stress oxydant, couramment cités dans la littérature car ils présentent un intérêt pour la mise en évidence de la toxicité :

- **La catalase (CAT)** : la catalase est une enzyme anti-oxydante présente chez les organismes eucaryotes dans les peroxysomes. Elle fait partie de la famille des enzymes oxydoréductases et catalyse la dismutation du peroxyde d'hydrogène (qui provient de la photorespiration dans les cellules végétales) en eau et oxygène (Figure 21) selon la réaction suivante :



Cette enzyme est un biomarqueur intéressant car elle dégrade rapidement le peroxyde d'hydrogène.

- **La superoxyde dismutase (SOD)** : les superoxydes dismutases appartiennent au groupe des métallo-isoenzymes. Ces enzymes catalysent la décomposition de l'anion superoxyde (O_2^-) en Oxygène et Peroxyde d'Hydrogène :



Gill et al. (2010) désignent cette enzyme comme la plus efficace du système antioxydant car elle est présente dans tous les organismes aérobies et dans tous les compartiments subcellulaires. Cette enzyme est un biomarqueur potentiellement sensible aux stress abiotiques.

- **L'ascorbate peroxydase (APX)** : l'ascorbate peroxydase intervient dans la réaction de transformation du Peroxyde d'Hydrogène en eau (*cf.* Figure 21). Elle possède une affinité importante avec le Peroxyde d'Hydrogène, dix fois supérieure à celle de la catalase (Gill et al., 2010). Cette forte affinité permet de déceler des variations faibles de Peroxyde d'Hydrogène (ROS), ce qui fait de l'APX un biomarqueur sensible.
- **La peroxydation lipidique** : la peroxydation lipidique n'est pas un mécanisme de défense contre les ROS, mais il est un bon indicateur de dommages créés par les ROS. La peroxydation

lipidique se produit en présence d'Oxygène singulet 1O_2 qui appartient à la famille des ROS. L'oxygène moléculaire dans son état fondamental, activé par les radicaux libres devient très réactif (Cillard J. et Cillard P., 2006). Il se fixe ensuite sur les acides gras insaturés présents dans la plupart des membranes plasmiques et mitochondriales et modifie leur structure. Il en résulte une altération des fonctions membranaires : la perméabilité ionique et la synthèse d'ATP dans les membranes sont modifiées.

Couplée avec l'étude des biomarqueurs enzymatiques, la peroxydation lipidique permet d'ajouter une information sur l'impact provoqué sur les cellules par le stress oxydant.

Le tableau 2 présente quelques études menées sur la catalase, la SOD, l'APX et la peroxydation lipidique comme biomarqueurs algaux. Les activités enzymatiques et la peroxydation lipidique répondent à une gamme assez large de substances toxiques : les métaux lourds tels que le Cuivre et le Zinc, les fongicides, les alkyphénols ou encore les composés pharmaceutiques et détergents. Tout comme pour la perturbation de la photosynthèse ou la perméabilité membranaire, il semble que ces indicateurs puissent être des bons marqueurs de pollution globale, non spécifiques à une famille de polluants en particulier. Les études menées sur ce type de biomarqueurs concernent essentiellement des bioessais sur substances pures. Dans le cadre de notre projet de recherche, nous nous sommes intéressés à développer de nouveaux protocoles pour les tester sur des rejets complexes tels que les RUTP.

Tableau 2 : Exemple d'études sur la perturbation du métabolisme du stress oxydant chez plusieurs espèces de microalgues exposées à des perturbations.

Perturbation étudiée	Biomarqueur du stress oxydant	Conditions d'essai	Espèce utilisée	Résultats	Références
Stress oxydatif Irradiance, température	CAT	Broyats de cellules	<i>Cladophora glomerata</i> , <i>Enteromorpha ahlnneriana</i>	Forte irradiance et température basse induisent une stimulation de l'activité de la catalase	Choo et al., 2004
Fongicide (fluodioxonil) et Cuivre	CAT, APX	Extraits enzymatiques	<i>Scenedesmus obliquus</i>	Stimulation de l'activité de la CAT et APX induite par la présence de Cuivre et du fluodioxonil	Dewez et al., 2005
Zinc et Cuivre	CAT, APX, Peroxydation lipidique	Broyats de cellules	<i>Pavlova viridis</i>	Stimulation de CAT, APX et augmentation de la peroxydation lipidique par le Cuivre et le Zinc	Li et al., 2006
Zinc et Cuivre	CAT, Peroxydation lipidique	Broyats de cellules	<i>Pseudokirchneriella subcapitata</i>	Le Cuivre et le Zinc induisent une augmentation de l'activité de la catalase et de la peroxydation	Soto et al., 2011

Glyphosate	CAT, SOD, Peroxydation lipidique	Broyats de cellules	<i>Chlorella kessleri</i>	Stimulation de la CAT et SOD, augmentation de la peroxydation lipidique	Romero et al., 2011
Cuivre, Irgarol et Aatrazine (pesticides)	SOD, CAT, APX, Peroxydation lipidique	Broyats de cellules	<i>Cylindrotheca closterium</i>	Augmentation de la peroxydation en présence de Cuivre et stimulation la SOD en présence d'atrazine	Lozano et al., 2014
			<i>Phaeodactylum tricornutum</i>	Augmentation de l'activité de CAT et inhibition de l'APX pour toutes les substances testées	
			<i>Rhodomonas salina</i>	Augmentation de l'activité de CAT et SOD pour une concentration faible de Cuivre (5 µg/L)	
Phytohormones et métaux lourds	SOD, CAT, APX, Peroxydation lipidique	Broyats cellulaires	<i>Chlorella vulgaris</i>	Stimulation des activités enzymatiques en présence d'un cocktail de phytohormones et de métaux	Piotrowska- Niczyporuk et al., 2012
Acide Trichloroisocyanurique et Ciprofloxacine	CAT	Extraits enzymatiques	<i>Chlorella vulgaris</i>	Stimulation de l'activité après exposition aux deux molécules	Nie et al., 2008
Nonylphénol	CAT	Extraits enzymatiques	<i>Chlorella vulgaris</i> et <i>Selenastrum capricornutum</i>	Stimulation de l'activité enzymatique après 24 heures d'exposition chez les 2 microalgues	Gao et Tam, 2011

Nous avons donc cherché à développer et appliquer cette sélection de biomarqueurs du stress oxydant (catalase, ascorbate peroxidase, superoxide dismutase et peroxydation lipidique) sur *Chlorella vulgaris*, afin de pouvoir détecter rapidement des effets toxiques dus à une courte exposition lors d'une campagne d'échantillons de RUTP prélevés durant l'année 2016 (Voir partie démarche générale, paragraphe 2.2.5). Toutefois, aucun effet n'a pu être démontré à l'aide des trois biomarqueurs enzymatiques (soit à cause de la perturbation de bactéries présentes dans les rejets, ou d'une trop grande variabilité des résultats). C'est la raison pour laquelle seuls les résultats positifs avec le biomarqueur d'effet de la peroxydation lipidique seront présentés par la suite.

3.4.2 Matériels et méthodes

3.4.2.1 Exposition des algues aux RUTP

Les algues *Chlorella vulgaris* ont été mises en contact avec les rejets urbains de la manière suivante :

50 mL d'algues (concentration initiale de $5 \cdot 10^6$ cellules/mL) ont été introduits dans des erlenmeyers de 100 mL (Figure 22) avec 50 mL de rejets non filtrés, et exposés 24 heures. Les expositions ont été faites en 5 réplicas et des contrôles négatifs sans algues ont été réalisés en parallèle afin de s'assurer que les



signaux observés n'étaient pas des interférences dues aux particules ou microorganismes présents dans les rejets. Les algues utilisées étaient âgées de 3 à 4 jours (phase exponentielle de croissance). Les échantillons étaient en agitation permanente à température contrôlée (20 °C) et soumise à un rythme 16h jour/8 h nuit avec une luminosité de 2700 Lux. La verrerie utilisée pour les expositions a été préalablement autoclavée et les manipulations ont été faites en conditions stériles.

3.4.2.2 Préparation des échantillons avant mesure

Les 100 mL d'algues exposées ont été centrifugés à 5000g, à 4 °C pendant 10 minutes, le culot a ensuite été rincé avec 50 mL de tampon phosphate 50 mM, pH 7. Les algues ont à nouveau été centrifugées, le culot a été remis en suspension dans 3 mL de tampon phosphate puis introduit dans deux micro-tubes de 1,5 mL. Les micro-tubes ont été centrifugés, un culot remis en suspension dans un tampon d'extraction des enzymes du stress oxydant (1 mM EDTA, 1 mM MgCl₂, 50 mM Tris-HCl pH 7,8, 1 % Polyvinylpyrrolidone) puis congelé pendant 20 h à -20 °C.

3.4.2.3 Mesure de la peroxydation lipidique

Le suivi de la peroxydation lipidique s'est effectué par mesure de la concentration en Malondialdéhyde (MDA) présent dans les algues. Le MDA est formé lors de la dégradation des acides gras insaturés. Il est donc considéré comme un produit de la réaction de peroxydation lipidique (Buege et Aust, 1978).

La concentration en MDA est déterminée indirectement en mesurant l'absorbance du produit de réaction entre le MDA et l'acide Thiobarbiturique (TBA). La lecture de l'absorbance s'est effectuée par spectrophotométrie à un instant t donné et à trois longueurs d'ondes fixes : 440, 532, 600 nm. Le réactif utilisé comprenait : 15 % w/v d'acide trichloroacétique (TCA), 0,375 % d'acide Thiobarbiturique (TBA), 0,25 N d'acide chlorhydrique.

Les essais ont été réalisés à partir des culots d'algues obtenus après extraction. Les culots ont été remis en suspension dans 400 μL de tampon phosphate à 50 mM, pH 7. 200 μL ont été introduits dans des tubes en verre HACH avec 1,8 mL de solution TCA-TBA-HCl et 200 μL ont été insérés dans 1,8 mL de solution sans TBA (uniquement TCA – HCl). Les échantillons ont ensuite été placés dans un bloc chauffant (HACH) à 90 °C pendant 25 minutes, puis refroidis à température ambiante. Les échantillons ont enfin été centrifugés 10 minutes à 12900 g à 20 °C. Le surnageant a été analysé au spectrophotomètre.

Le calcul de la concentration en MDA équivalent a été obtenu à l'aide de la méthode de Hodges et al., (1999) à l'aide de l'équation (1) suivante :

$$(1) \quad MDA \text{ équivalent } (nmol.mL^{-1}) = \left(\frac{A-B}{157000} \right) * 10^6$$

$$\text{Avec : } A = Abs_{532+TBA} - Abs_{600+TBA} - Abs_{532-TBA} - Abs_{600-TBA}$$

$$B = (Abs_{440+TBA} - Abs_{600+TBA}) * 0.0571$$

Les concentrations des algues ont été déterminées, avant la mesure de peroxydation lipidique, par comptage au microscope optique à l'aide d'une cellule de Thoma afin de déterminer si un effet des RUTP sur la croissance algale avait eu lieu. Cette observation a pour but de rapporter les valeurs de MDA obtenues au même nombre de cellules. A la fin de l'essai, la concentration en MDA présentée est exprimée en nmol/mL et par million de cellules.

3.4.2.4 Traitement statistique des résultats

Les résultats sont soumis à des analyses statistiques à l'aide du logiciel XLSTAT. Une vérification de la normalité des distributions de l'ensemble des échantillons est réalisée. Des tests ANOVA suivi de tests post-hoc ont été réalisés afin de comparer les résultats témoins et échantillons et de déterminer les différences significatives.

3.4.3 Résultats

3.4.3.1 Effets des RUTP sur la croissance algale

La figure 23 indique que les contrôles présentaient une croissance de 15 à 23 % par rapport à la situation initiale (5 millions de cellules/mL). L'inhibition de la croissance pour le rejet du déversoir d'orage prélevé le 9 mai pouvait être négligée (-0.67 %). La majorité des échantillons traités présentaient une croissance supérieure à celle des contrôles (5-45 %), avec des différences significatives pour les rejets de Miribel (37 %) et de Chassieu (45 %).

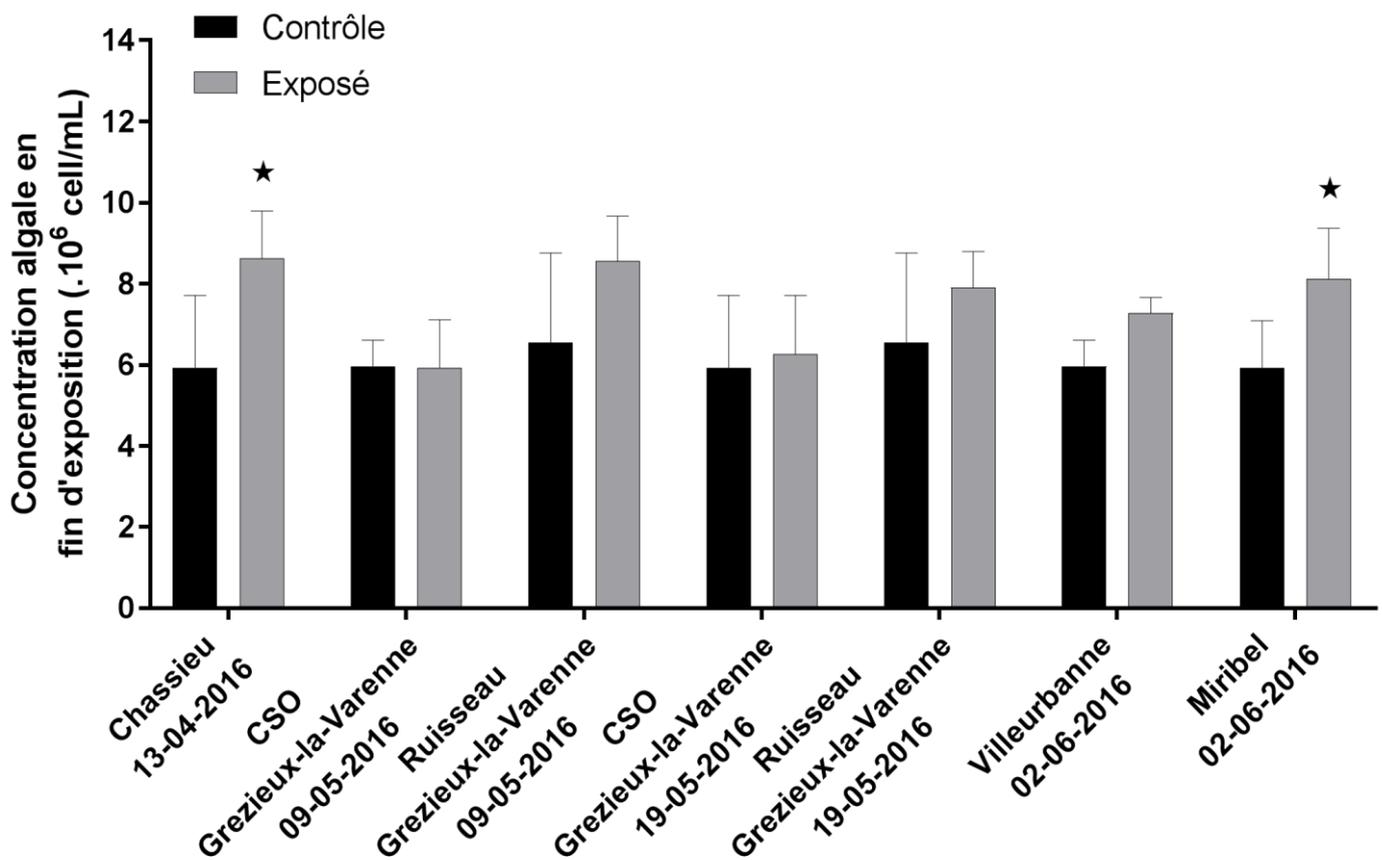


Figure 23 : Concentration des *Chlorella vulgaris* exposées aux RUTP étudiés à la fin des 24 heures de l'essai (en millions de cellules par mL). CSO : Combined sewer overflow (déversoir d'orage); ★ : résultats significatifs ($p < 0.05$).

3.4.3.2 Effets des RUTP sur la peroxydation lipidique des algues

La figure 24 montre une augmentation significative de la concentration en MDA au sein des cellules soumises au rejet du déversoir d'orage (CSO) du 9 mai (+53 %) et à l'eau du ruisseau contaminée en aval du déversoir du 9 mai (+43 %). Pour les autres rejets, une concentration plus faible en MDA en comparaison avec les contrôles (-12 à -26 %) est constatée, mais cette différence n'est pas statistiquement significative.

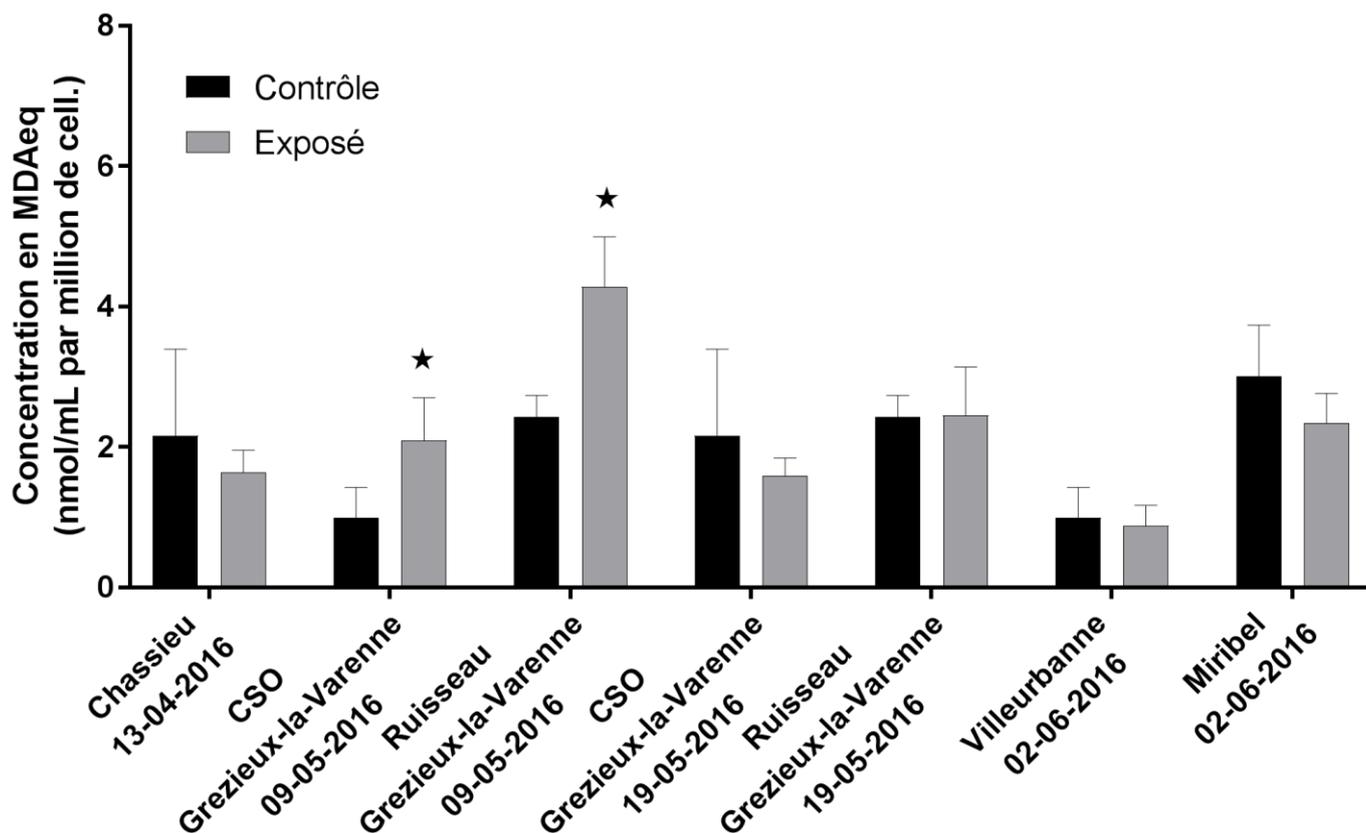


Figure 24 : Concentration MDA équivalent dans les cellules de *Chlorella vulgaris* exposées ou non aux RUTP étudiés à la fin des 24 heures de l'essai (nmol/mL par millions de cellules). CSO : Combined sewer overflow (Déversoir d'orage); ★ : résultats significatifs (p<0.05).

3.4.4 Discussion et perspectives

Comme discuté durant la précédente section liée à l'utilisation des biomarqueurs algaux, nous avons constaté une stimulation de la croissance des algues après exposition à deux échantillons d'eaux pluviales. Les raisons de cette stimulation sont les mêmes qu'évoquées précédemment : présence notamment de nutriments en concentrations non négligeables). Les essais ont confirmé le manque de sensibilité de paramètres tels que l'inhibition de croissance avec des rejets présentant une contamination

résiduelle. A l'inverse, deux des 7 échantillons (un rejet de déversoir d'orage et une eau de surface (ruisseau) dans laquelle celui-ci se rejette) ont montré des effets significatifs sur la peroxydation lipidique des algues, mettant en évidence un impact toxique après 24 heures d'exposition.

Trois points de discussions peuvent alors être amenés à ces résultats. Premièrement, les analyses chimiques de la campagne de 2016 (voir Tableau S1 – partie 4.2.1.2) n'ont pas dévoilé de différences marquées en termes de concentrations de polluants qui pourraient expliquer ces effets toxiques, excepté pour le glyphosate (13,7 et 1,51 µg/L respectivement dans la fraction dissoute pour le rejet de déversoir et échantillon de ruisseau contaminé). Cependant, ces concentrations en glyphosate s'avèrent beaucoup plus faibles que celles ayant montré des effets sur la peroxydation lipidique d'une algue du même genre, *Chlorella kessleri* (seuil d'effet : 50 mg/L) (Romero et al, 2011). Il faut toutefois noter que *C. vulgaris* semble plus sensible au glyphosate (e.g. CE₅₀ d'inhibition de la croissance à 96 heures de 4,69 mg/L pour *C. vulgaris* contre 55,62 mg/L pour *C. kessleri* (Ma et al., 2002 ; Romero et al., 2011)). Par ailleurs une étude réalisée par Tsui et al. (2005) sur *Ceriodaphnia dubia* a montré que le glyphosate pouvait influencer la toxicité des métaux lourds en modifiant leur biodisponibilité (augmentant leur bioconcentration ou la diminuant selon les métaux). Enfin, certains auteurs ont observé que le glyphosate pouvait induire une perméabilité des membranes cellulaires, comme montré chez *Lemna minor* par O'brein et Prendevill (1979), ce qui pourrait favoriser l'accès des polluants aux cellules algales.

Deuxièmement, il est intéressant de noter que la majorité des études ont rapporté une inhibition de la croissance aux concentrations de polluants, pour lesquels un effet toxique apparaît sur les marqueurs du stress oxydant (Nie et al., 2008). Cela n'a pas été le cas ici, attestant que la peroxydation lipidique est plus sensible aux polluants que le paramètre d'inhibition de la croissance des algues exposées aux rejets.

Enfin, on peut aussi relever qu'un autre travail a démontré un potentiel des eaux pluviales à agir sur le stress oxydant. En effet, Tang et al. (2013) l'ont observé en utilisant des biomarqueurs *in vitro* sur cellules humaines génétiquement modifiées (essai sur gène rapporteur « AREc32 »). Seulement, il faut noter que cette étude s'éloigne de par son modèle des études écotoxicologiques classiques sur organismes aquatiques. Il n'est donc pas surprenant que nous ayons également observé dans notre cas des effets sur des organismes aquatiques (microalgues) avec les échantillons que nous avons testés.

Pour conclure, il ne s'agit là que d'une étude préliminaire et il conviendrait maintenant de renforcer ce travail en optimisant le protocole d'exposition (temps d'exposition, filtration des échantillons, algues exposées, etc.). Cependant nous pouvons sans aucun doute avancer que ces biomarqueurs feraient de bons indicateurs de la toxicité des RUTP sur les microalgues.

4 Développement de biocapteurs

Cette partie découle directement des essais écotoxicologiques que nous avons effectués en laboratoire précédemment. Nous avons cherché à adapter les biomarqueurs de toxicité étudiés afin de créer des outils d'alerte précoce, capables de détecter une pollution *in situ*. Nous nous sommes particulièrement focalisés sur la perturbation de la photosynthèse (et plus précisément sur une perturbation de la fluorescence de la chlorophylle a), ce biomarqueur étant le plus facile à mesurer et relativement sensible à la présence de polluants toxiques.

Cette partie se divise en deux volets distincts : l'un ayant trait à la conception et l'optimisation de biocapteurs algaux optiques, étudiant la photosynthèse et basés sur une immobilisation par double encapsulation alginate/silice, et l'autre aux biocapteurs optiques basés sur l'utilisation de puces microfluidiques créées par xurographie.

4.1 Biocapteurs optiques à hydrogels

4.1.1 Contexte et enjeux

Cette étude fait suite à un travail débuté en 2012 par Ferro et al. et poursuivi par Durrieu et al. en 2016. L'immobilisation des microalgues pour la création de biocapteurs est une étape fondamentale afin de permettre un lien entre biorecepteur et transducteur, mais aussi pour permettre une bonne interaction entre molécules (polluants) et organismes bio-indicateurs. Dans le cadre des biocapteurs algaux (mais aussi bactériens), de simples gels d'alginate ou d'agarose ont été souvent utilisés pour immobiliser ces micro-organismes. Cependant, ces gels ont tendance à se dégrader facilement, ou à se dissoudre, notamment au contact avec des ions de type phosphates, présents dans les rejets urbains ou les milieux aquatiques de manière générale. C'est la raison pour laquelle Ferro et al. (2012) ont initié des recherches sur l'immobilisation de microalgues, basée sur une double encapsulation des algues dans une matrice d'alginate, recouverte d'un hydrogel de silice beaucoup plus rigide et qui ne se dissout pas en présence de matrices complexes (voir Article 4 - Figure 1). Cette condition d'immobilisation semble prometteuse et cette recherche a été poursuivie dans le cadre de cette thèse afin d'optimiser la technique et de développer une double encapsulation permettant le maintien des algues immobiles pour leur transport du laboratoire sur le terrain, tout en maintenant/améliorant la sensibilité du système créé. Les objectifs étaient les suivants : 1) Concevoir une double encapsulation de micro-algues (*Chlorella vulgaris* et *Pseudokirchneriella subcapitata*) dans une double matrice alginate/hydrogel de silice (Article 4 - Figure 1), afin d'immobiliser les micro-organismes dans le but de créer des biocapteurs de terrain, 2) Optimiser les paramètres physico-chimiques (e.g. la composition des gels, la porosité et épaisseurs des hydrogels, les propriétés optiques de ces gels) et biologiques (notamment la concentration algale) pour améliorer la sensibilité aux pesticides et, de manière générale, aux polluants présents dans les RUTP afin de détecter une perturbation du métabolisme des algues.

4.1.2 Article 4 - *Development and optimization of an algal double-encapsulation in alginate-silica hydrogel in the aim of biosensor design for herbicide detection in contaminated waters.*

Article en cours de finalisation pour soumission dans Journal of biotechnology.

Development and optimization of an algal double-encapsulation in alginate-silica hydrogel in the aim of biosensor design for herbicide detection in contaminated waters.

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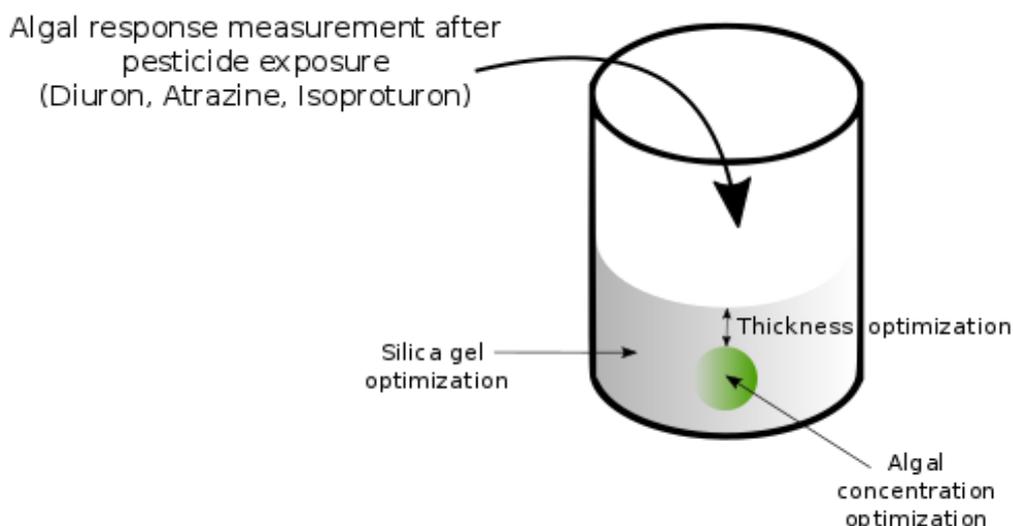
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Graphical abstract



Highlights

- Algal double encapsulation was created for biosensor design.
- Silica hydrogels physicochemical properties were studied for best biosensor design.
- Algal concentration and silica gel thickness parameters were also optimized.
- Best limit detection was obtained for *C. vulgaris* exposed to Diuron.

Key-words

Biosensor; Ecotoxicology; Microalgae; Chlorophyll-a fluorescence; Herbicide; Hydrogel

Abstract

Pesticides are an important source of contamination for urban and suburban surface and ground and seawaters. Since more than 10 years, whole-cell biosensors as algal biosensors were developed to detect them and their toxicity *in situ* and continuously in discharges and receiving ecosystems. Here we proposed to develop and optimize a microalgae double encapsulation immobilization method in alginate beads/silica gels, which have been proof to be safe for algae in previous studies, in the aim of algal biosensor creation. Pesticide detection and perturbation was assessed by chlorophyll-a fluorescence disturbance. Two algae strains were used to create them: *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*. Various parameters were optimized in the aim to obtain the best pesticide detection: silica gels porosity and optical properties, algae concentrations in alginates beads and thickness of silica gel around algal beads. Finally, the best detection limit was obtained with *C. vulgaris* exposed to Diuron (<10ug/L).

1. Introduction

Wide number of anthropic activities are responsible of the release of pesticides in environment, by the production of rejects. Agricultural runoffs (Willis and McDowell, 1982), urban stormwaters (Zgheib et al., 2011; Gasperi et al., 2012) or wastewaters (Gerecke et al., 2002; Köck-Schulmeyer et al., 2013) can be the source of multiple contaminations. Important number of pesticides are currently detected in receiving aquatics ecosystems as rivers, lakes (Konstantinou et al., 2006; Bonansea et al., 2013) or groundwater ecosystems after pesticide soil infiltration, but also in tap water directly consumed by humans. Herbicides represent an important part of the released pesticides in ecosystems, which utilization grew since more than 40 around the world (ex: 48.0 % of the total pesticide consumption in 2005) (Zhang et al., 2011). Herbicides are particularly toxic for plants and microalgae, but also for human health (e.g. Glyphosate recently classed as a probable carcinogenic agent by the World Health Organization).

Maintaining of the good ecological status of water bodies but also tap water quality exposed to herbicides pollution is an important issue around the world, like in Europe with the European Water Framework Directive (WFD, 2000/60/EC). To achieve this, it is necessary to implement on-line monitoring methods to ensure compliance of the quality of aquatic environments. However, as expressed by many authors as Mankiewicz-Boczek et al. (2008), physicochemical analyses alone are not sufficient to evaluate water quality, and a biological monitoring on aquatic organisms is essential. Moreover, pollutant's chemical analyses require samples collection, tranfer to laboratory and storage. Consequently, online *in situ* monitoring is of strong interest for pesticide detection and impact monitoring to prevent any ecosystem degradations.

To achieve this *in situ* monitoring, whole-cell biosensors have been recently developed and applied to studying urban matrixes and aquatic polluted ecosystems (e.g. rivers), as “early warning systems”. Their sensitivity has been demonstrated and, contrary to lab bioassays, biosensors allow a continuous and *in situ* monitoring of water pollutions and impacts (Rogers, 1995). Among their advantages, biosensors are less expensive than chemical analyses and classical ecotoxicological tests, providing good sensitivity, selectiveness and reproducibility (Dennison et Turner., 1995; Rogers, 2006). Moreover, whole-cell biosensors are very useful tools as they do not only determine the presence and even the concentrations of chemical pollutants in an aquatic sample, but they also can be used to observe a biological response, in particular the toxicity of aquatic contaminants (Rodriguez-Mozaz et al., 2005).

Algal biosensors consist of suitable tools for on-line detection of pollutants and a metabolism perturbation response, due to their sensitivity in particular for herbicides. Many authors are already described development of algal biosensors to detect herbicides (Campanella et al., 2001; Chouteau et al., 2005; Shitanda et al., 2009; Zamaleeva et al., 2011). Among them, algal optical biosensors based on photosynthesis disturbance (with chlorophyll-a fluorescence disturbance as target) were widely studied for pesticides analysis in contaminated liquid media (Naessens et al., 2000; Védrine et al., 2003, Ferro

et al., 2012; Lefevre et al., 2012), with low detection limits (until less than 0.1 µg/L of Diuron for example). Herbicide's families mostly are triazines, triazinones, phenylureas, etc., studied of their effect on the primary producers Photosynthem II (Rioboo et al., 2002) (often studied with A-chlorophyll fluorescence).

One of the major step for whole-cell algal biosensors building is the immobilization of living organisms and its effect on cell's viability, development, etc. (Lagarde and Jaffrezic-Renault, 2011; Gosset et al., 2016). The main objective is to maintain biosensor sensitivity (Eltzov and Marks, 2011) or reproducibility (Gu et al., 2004). Many (in-)organic materials have been already used for algal immobilization, as for example alginate, silica, agarose, chitosan, or polyvinyl (Mallick, 2006; Moreno-Garrido, 2008). Among them alginate hydrogels are probably the most suitable for biosensors design. Their macroporous structure do not decrease the toxicity of many pollutants as observed by Abdelhamid et al. (1996). However, their utilization is time-limited because of alginate dissolution with phosphate solutions present in many environmental samples (surface water, wastewaters, stormwaters, etc.) (Taqieddin and Amiji, 2004). From 10 years until now, new (hybrid or not)-silica materials using sol-gel process were developed for cells immobilization (Nassif et al., 2003; Nguyen-Ngoc et Tran-Minh, 2007a,b; Perullini et al., 2007; Pena-Vazquez et al., 2009; Pannier et al., 2014). Besides, silica-materials have good optical properties, and create a solid barrier against cells release. However, they can be harmful for living micro-organisms, because of products releases, aggressive pH, high ionic strength, and lead a rapid decrease of the biosensor signal (Coradin and Livage, 2007; Perullini et al., 2008). Ferro et al. (2012) investigated a new technique to design a new generation of biosensors: double encapsulation of algae in alginate and silica sol-gel. They demonstrated that double encapsulation with silica gel did not inhibited algal growth and was not harmful for them. This work confirmed results from Perullini et al. (2007) of a long term growth of plant cells in these configuration. This technic was so reused by different authors as Pannier et al. (2014).

The present work contributes to the development of double encapsulation of algae in alginate and silica sol-gel initiated by Ferro et al. (2012), Perullini et al. (2014a) and Durrieu et al. (2016). These authors have already demonstrated the viability and utility for algal biosensors building and pesticides detection in environmental samples as stormwaters. The current issue is focused on the sensitivity statement of these systems. Physicochemical and biological parameters as porosity/adsorption, light attenuation, silica gel thickness or algal concentration are important to control. Truly, as demonstrated by Perullini et al. (2014b), silica hydrogel could lead to an adsorption of positively charged molecules (dye). Consequently, this parameter is important to study because some of pesticides are also positively charged (e.g. glyphosate). Moreover, Sicard et al. (2011) observed that silica hydrogel composition influenced light diffusion through silica gel, and consequently the light arriving to the algae (directly linked to the photosynthetic response). Finally, Védrine et al. (2003) observed that algal concentration was fundamental because of pollutant availability for algae and also for algal light reabsorption.

The goal of the present study was to work on these different physicochemical and biological parameters of these algal immobilization techniques in the aim of development new biosensors and obtain the better sensitivity to design finally biosensor ready to use *in situ*. For that, we used two different algae strains: *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*, classically found in freshwater aquatic ecosystems. We realized a double encapsulation of both of them, and previously described essential parameters were studied for signal optimization of our immobilization technique before to evaluate the sensitivity of the created systems, using 3 model pesticides: Diuron, Atrazine and Isoproturon.

2. Materials and methods

2.1 Chemical products and solution preparations

For alginate and silica gels preparations: Sodium alginate (Biochemica) powder was purchased from AppliChem GmbH (Darmstadt, Germany). Calcium chloride dihydrate was obtained from Merck (Darmstadt, Germany). Hydrochloric acid was bought from VWR (Radnor, USA). Potassium dihydrogen phosphate and Dipotassium hydrogen phosphate for phosphate buffer were purchased respectively from CHIMIE-PLUS (Saint Paul de Varax, France) and Merck (Darmstadt, Germany). Sodium silicate solution (purum, $\geq 10\%$, NaOH basis, $\geq 27\%$ SiO₂ basis) and LUDOX® (HS-40 colloidal silica, 40wt % in H₂O) for silica gels were purchased from Sigma–Aldrich (Saint-Louis, USA). All stock and work solutions used were prepared with ultrapure water filtered at 0.22 μm (see section 2.3, 2.4 and 2.5).

For pesticides preparations: Absolute ethanol ($\geq 99.5\%$) was obtained from VWR (Radnor, USA). Atrazine (PESTANAL®) and Isoproturon (PESTANAL®) were purchased from Riedel-de-Haën (Seelze, Germany). Diuron ($\geq 98\%$) was bought from Sigma–Aldrich (Saint-Louis, USA). Diuron and atrazine pesticides stock solutions were prepared in pure ethanol solution at 10 g/L (1 g of pesticide in 100 mL of absolute ethanol). 100 μL of these solutions were mixed just before experiment in final 100 mL of ultrapure water to create 10 mg/L pesticide work solution used for experiments. For Isoproturon, direct 10 mg/L solution was created mixing 10 mg of pesticide with 1 L of ultrapure water, due to the high solubility of this compound (70 mg/L at 20 °C).

2.2 Algal cultures

Two microalgal strains were used during this work: *Chlorella vulgaris* (*C. vulgaris*) and *Pseudokirchneriella subcapitata* (*P. subcapitata*). These algal strains were purchased from the French National Museum of Natural History (Paris, France). *C. vulgaris* and *P. subcapitata* were grown in the Lefebvre-Czarda medium (AFNOR, 1980). These stains were transplanted weekly under sterile condition, using sterilized glassware and medium (autoclaved during 20 min, at 130 °C, under 1.3bar), in the aim to use fresh algae in their exponential growth phase. Algae were maintained in a nycthemeral

16 h/8 h light/dark cycle using 5000lux lighting in special culture room maintained at 21 °C. Culture concentration determination was carried out with Thoma cell counter.

2.3 Principle of the algal double encapsulation

The first step of the double encapsulation presented here (Fig. 1) consist in the creation of Ca(II)-alginate beads. For that, algae solution was mixed with sodium alginate (50:50 v/v) to prepare final solution at 1 wt% of sodium alginate. Then, alginate-algal solutions were added dropwise in a solution of CaCl₂ (0.1 M), let 10 min in the solution under agitation, and finally remove, washed and stocked in Lefebvre-Czarda medium before utilization. Final diameter of alginate beads was around 3.5 mm (63 μL).

Second step consist in a silica sol-gel process, based on preparation of silica matrix solutions in 1.5 mL in Eppendorf tubes, at 20 °C, following the compositions presented in Supplementary data 1, and leading to a nanoporous monolithic structure. For that H₂O, Phosphate Buffer, HCl and Ludox were mixed together.

At the last time, Sodium silicate was added to the preparation. Two parameters were studied in these silica gels: final percentage of silica (SiO₂ water molar relation), and proportion of polymeric to particulate silica precursors (Sodium silicate:Ludox). A total of 16 hydrogels were studied: from 3 to 10 % of total silica and two Sodium silicate:Ludox ratios, 1:10 and 1:20. For next Figures and Tables, hydrogel will be consequently represented as: total silica % (Sodium silicate:Ludox ratio). Finally, firstly created alginate beads were placed in microplate well and then silica matrixes just mixed were disposed in wells just before sol-gel process apparition for final double encapsulation of microalgae (See process in Figure 1).

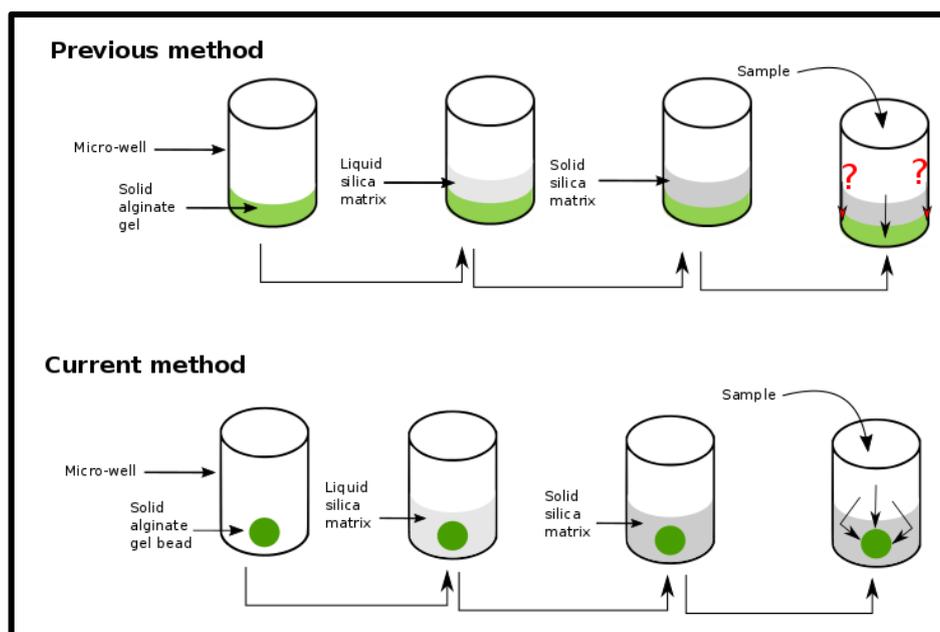


Figure 1: Scheme of the current immobilization method compared to previous studies (Ferro et al., 2012; Perrulini et al., 2014 and Durrieu et al., 2016)

2.4 Physicochemical analyses for suitable silica gel choice

The first step of the experimental work was to study the physico-chemical properties of various silica hydrogels. The objective was to select the more suitable for double encapsulation process in terms of technical feasibility (time of gelation), compatibility for algae (e.g. pH), pesticide diffusion through the hydrogel layer (with a dye and a pesticide diffusion experiments), and optical properties (spectrophotometry analysis).

2.4.1 pH and gelation time determination

Different silica gels were synthesized in 2 mL Eppendorf tubes. For each one, gelation time was determined with a chronometer and pH determined with an Orion star pH meter.

2.4.2 Diffusion experiments

For diffusion study (each silica gel created using composition presented in Table 1, 3 replicates per gel), method from Perullini et al. (2014b) was used. 1 mL of gel was inserted in polystyrene spectrophotometer tanks. After their gelation time, 1 mL of phosphate buffer (pH 7, 0.1 M) was added, and sample gel stored 24 hours in the dark at 4 °C to increase the gel condensation. Afterward, buffer was removed, and 6 µL of Crystal Violet dye at 100 µM was dropped at the top of the gel. Tanks were placed in a desk scanner and image scanned during 2 hours. Images were analyzed using *ImageJ* free software to calculate color intensity profiles. The color intensity values were calculated and restricted to the linear response range determined with a Crystal Violet dye calibration right. Then, color intensity values transformed in dye concentration were used to calculate an apparent diffusion coefficient (D_{app}) at different dye exposure times, using equation (1):

$$(1) \quad C(x, t) = \frac{M}{\sqrt{\pi D_{app} t}} e^{\frac{-x^2}{4 D_{app} t}}$$

Where M is the initial amount of dye seeded in µM, t the time in minutes, and x the distance in mm.

This diffusion coefficient is qualified “apparent” (and not “efficient”) because of the adsorption phenomenon on silica surfaces, as translate the partition of the dye between dye solution and silica hydrogel surface. Consequently, we observed time-decrease of this coefficient for each of the sol-gel created.

To confirm results obtained from this first experiment, we created different silica gel (4, 7 and 10 % Silica; 1:10) to check any pollutant adsorption in silica matrixes. For this purpose, 1 mL of Diuron at 100 µg/L was dropped on the top of each gel, in 48-well plates (8 replicates). Afterward, plates were closed and stored a 4 °C during 24 hours. Finally, 100 µL of algae (final concentration of 1.10⁶ cell/mL) and 100 µL of solution on the top of the sol-gel were placed in 96-well plate (or pure water for control) and a-chlorophyll fluorescence read (ex. 460 nm/em. 680 nm) with a multiplate reader Varian® Cary Eclipse Fluorimeter. Fluorescence enhancement was calculated using eq. (2). Experiments have been done in 8 replicates.

$$(2) \quad \text{Fluorescence enhancement (\%)} = \frac{\text{Fluorescence intensity (exposed)} - \text{Fluorescence intensity (control)}}{\text{Fluorescence intensity (control)}} * 100$$

2.4.3 Optical properties: spectrophotometry analysis

The aim was to observe for our different silica hydrogel studied the light transmission through them until algae. As already explained in section 2.4.2, silica matrixes were prepared in spectrophotometers cells and stocked 24 h at 4 °C in the dark before reading. Because light absorbance and luminescence are nonexistent for silica gel, observed attenuation (attenuance = -log[transmittance]; Sicard et al., 2011) setting tanks in spectrophotometer can be associated to simple scattering. Then, scattering of the different silica gel prepared was observed analyzing gel spectra with Shimadzu UV1800 spectrophotometer between 400 and 800 nm. Experiments have been done in 3 replicates.

2.5 Optimization of biological and physicochemical parameters for double encapsulation

After silica hydrogel selection, optimization of some parameters has been performed in the aim of obtain the best sensitivity of our algal immobilization system for biosensors creation: algal concentration in alginate beads, and silica hydrogel thickness around alginate beads.

2.5.1 Optimization of algal concentration

Algal beads were prepared following the process described before, at different algal concentrations (5, 6, 7, 8, 10, 12, 18 and 20.10⁶ cell/mL final algal concentration in beads). Beads were place in 96-well plate wells. Silica matrix was inserted to create 1 mm thickness silica gel (4 % (1:10)) on the top of the bead. For that, a fixed amount silica solution was added in the well to obtain the same height than the top of alginate bead. After gelation process, another calculated volume of silica solution was placed at the top of the previous gels. Diuron (4mg/L) was then placed at the top of the systems (or ultrapure water for control). After 40 minutes of exposure, a-chlorophyll fluorescence was read and fluorescence increase calculated using eq. (2). Experiments have been done in 8 replicates.

2.5.2 Optimization of silica thickness

Algal beads were prepared in the optimal concentrations determined before (see section 2.5.1). In this case, different volumes of silica gels (4 % (1:10)) were placed in the wells to obtain the following thickness: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mm. Diuron (4mg/L) were placed at the top of the systems (or pure water for control), and after 40 minutes' exposure, algal a-chlorophyll fluorescence was read. Experiments have been done in 8 replicates.

2.6 Determination of algal response with biosensors

Final step of our work consisted to test the sensitivity our system after choosing hydrogel and optimization of parameters influencing response to pesticides. For this purpose, *P. subcapitata* and *C. vulgaris* double encapsulated were built in well plate using optimum algal concentrations and silica gel thickness. Algae immobilized were exposed to a dilution series of 3 models pesticides: Diuron, Atrazine and Isoproturon (0, 1, 10, 100, 1000 and 4000 $\mu\text{g/L}$). Fluorescence increase for each dilution and algae was determined using eq. (2). Experiments have been done in 16 replicates.

3. Results

3.1 Physicochemical parameters study for silica hydrogel selection

As shown on the Figure 2 (left) whatever the conditions a relative stability, pH value of the silica hydrogels was around 8.0 ± 0.5 , corresponding to the optimal pH for microalgal growth and viability. Regarding the gelation time, time decrease was observed with the increase of silica concentration, which is in good agreement with a nucleation and growth mechanism. Moreover, silica gel with a 1:20 ratio present a clear more important gelation time.

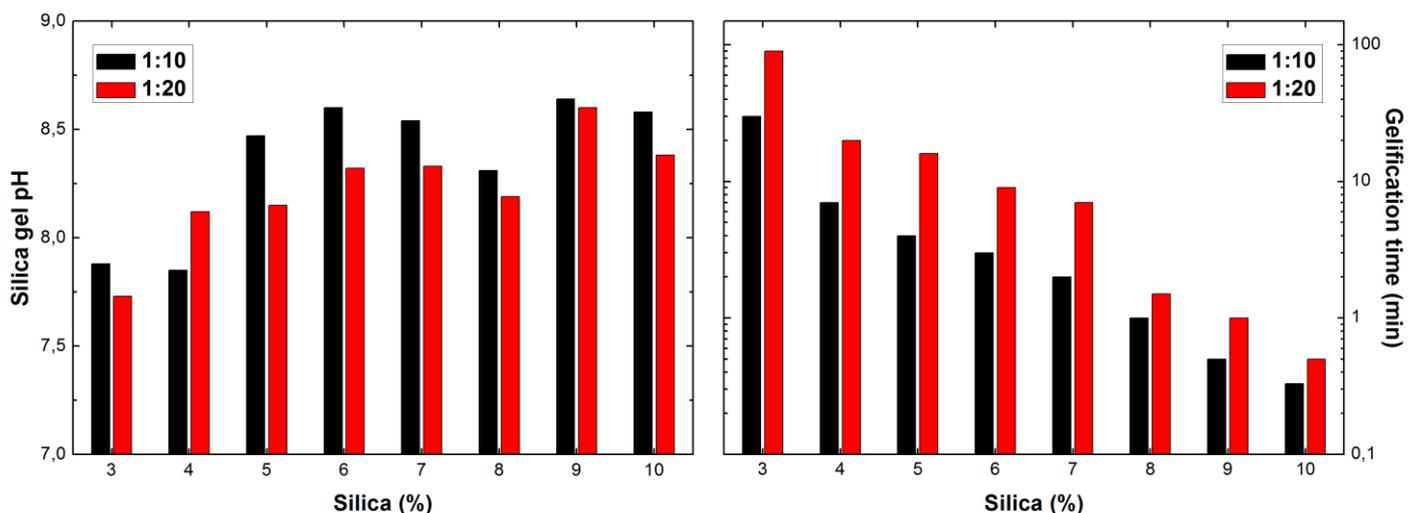


Figure 2: Silica gels pH (left) and gelation time (right) depending of the silica concentration and sodium metasilicate/Ludox ratio (1:10 and 1:20).

Diffusions experiments (see Fig. 3) have been done at pH 7, for which one silica structures surfaces are negatively charged (isoelectric point of silica at around pH 2). Consequently, using a cationic Blue Violet dye, we can observe a decrease of the diffusion coefficient (D_{app}) with time. As highlighted in Figure 3, D_{app} coefficient appeared to be higher with lower silica concentration. Moreover, D_{app} observed appeared slightly lower for silica hydrogels with a ratio 1:20 (e.g. at 8 % of silica and 59 minutes, 1:10 ratio present a D_{app} of $3,71 \cdot 10^{-11} \pm 2,08 \cdot 10^{-12} \text{ m}^2\text{s}^{-1}$ compared to $3,01 \cdot 10^{-11} \pm 1,29 \cdot 10^{-12} \text{ m}^2\text{s}^{-1}$ for 1:20 ratio).

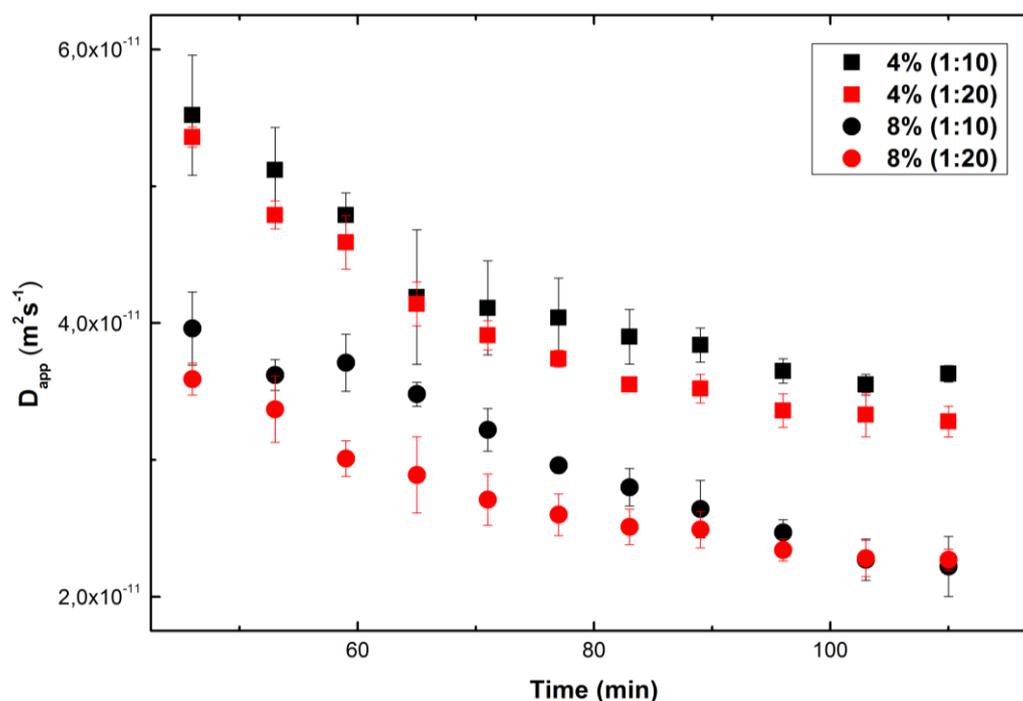


Figure 3: Diffusion profiles of cationique dye BlueViolet in the different silica hydrogel depending of the silica concentration and Sodium metasilicate:Ludox ratio (1:10 and 1:20). The apparent diffusion coefficients (D_{app}) exposed here were obtained from each fitting (dye concentration function of the distance from the top of the hydrogel) for different sampling time and different hydrogel samples tested (here 4 and 8 % of silica with 1:10 and 1:20 Sodium metasilicate:Ludox ratios).

Light scattering study of silica hydrogels 1:10 formulation is illustrated by Figure 4-A. It is complex to distinguish something at the algal a-chlorophyll excitation wavelength (around 460 nm). However, results showed that increase of silica concentration in sol-gel lead to decrease attenuation (and so scattering) at 680 nm (emission wavelength) (e.g. 0.726 u.a. at 3 % of silica, against 0.387 u.a. for 10 % of silica, looking for Figure 4-B). Nevertheless, regarding the gelation time, dye diffusion, and attenuation results, 1:10 Sodium silicate:Ludox ratio hydrogels appeared to the best ratio.

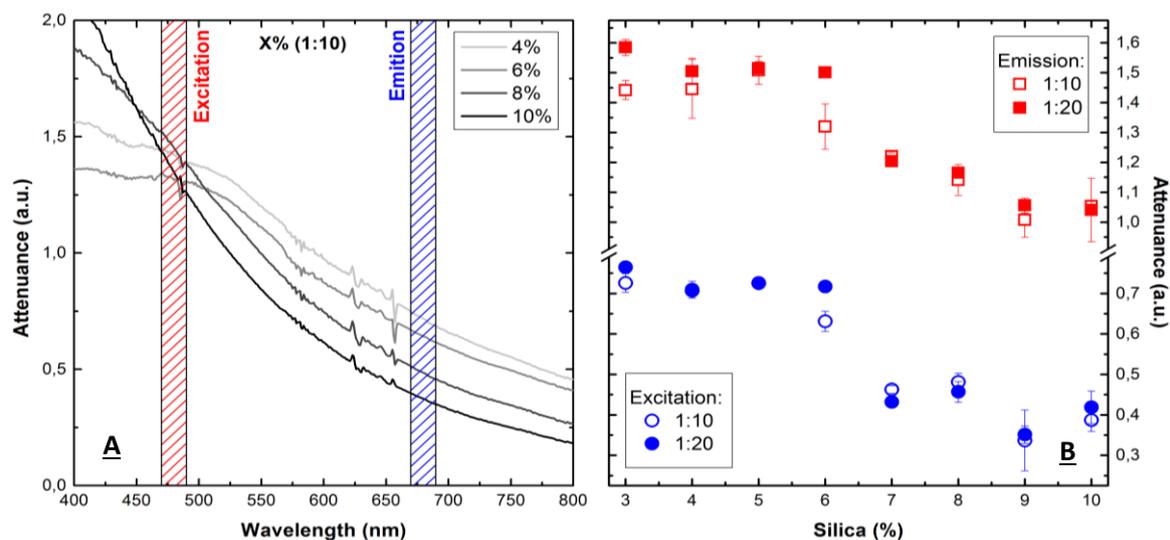


Figure 4: Attenuance spectra (left) obtained for Sodium metasilicate:Ludox ratio and various final silica percentage, and attenuance values (right) measured at 460 nm (a-chlorophyll excitation wavelength) and 680 nm (a-chlorophyll emission wavelength) depending of the silica concentration and sodium metasilicate: Ludox ratio (1:10 and 1:20).

Pesticides/hydrogel contact experiments results are presented in Figure 5. Algae exposed to pesticide solution let in contact with silica hydrogels in the dark (24 h) with more important silica concentration lead to a less important algal fluorescence of algae after 40 min. exposure. 4 % of silica fluorescence increased of *P. subcapitata* and *C. vulgaris* were respectively 131 and 75 % compared to control, against 13 and 35 % for diuron exposed to 10 % silica hydrogels. Looking for all results obtained previously with our set of silica hydrogels, we chose to kept the 4 % (1:10) formulation for encapsulation optimization and sensitivity experiments.

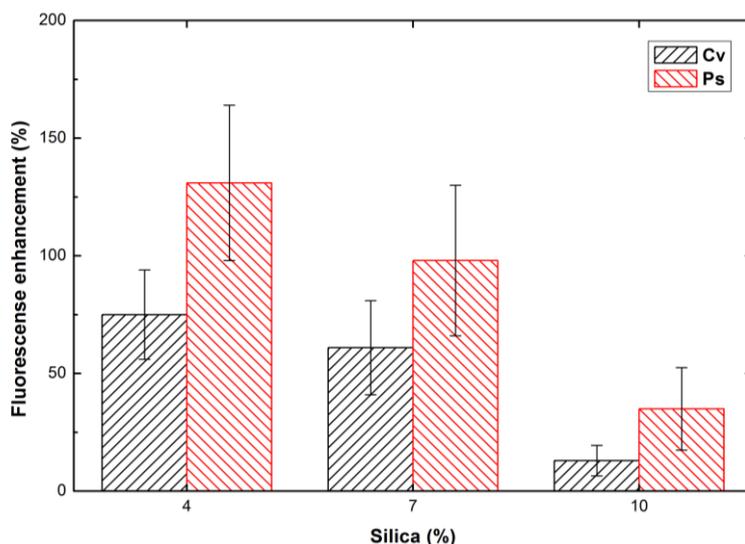


Figure 5: Fluorescence response of *P. subcapitata* and *C. vulgaris* compared to control after 40 min exposure to Diuron in contact on the top of silica hydrogel during 24 hours at 4 °C in the dark. (Sodium silica:Ludox ratio 1:10).

3.2 Algal immobilization parameters linked to sensitivity optimization

Silica thickness optimization was assessed exposing the two algae strains with the double encapsulation with different amount of silica hydrogel on the top of algae beads, at a concentration of 4 mg/L of Diuron. Results of silica thickness study are presented in Figure 6. It was observed that for both *P.subcapitata* and *C. vulgaris* that best response was observed the 1 mm thickness of silica gel up to the algal bead.

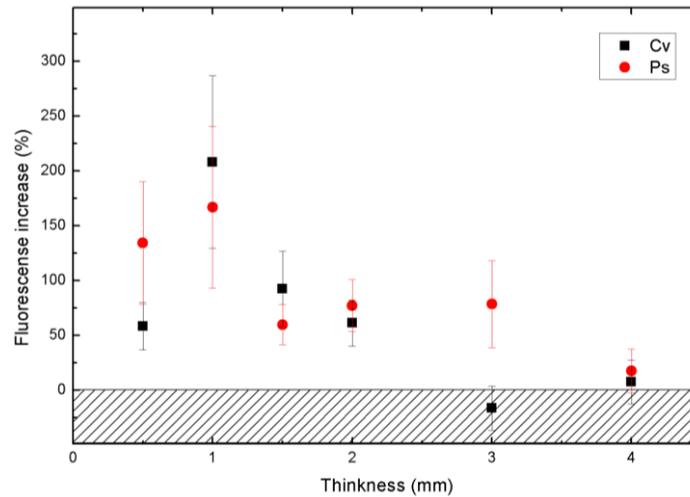


Figure 6: Optimization fluorescence detection sensitivity as a function of silica gels thickness. The response of *P. subcapitata* and *C. vulgaris* encapsulated (silica hydrogel: 4 % (1:10) (a-chlorophyll fluorescence) was measured after exposed 40 min to 4 mg/L of diuron (N° replication=8).

Algal concentration optimization was assessed exposing the two algae strains immobilized with the double encapsulation at different concentrations in Ca-alginate beads, at a concentration of 4 mg/L of Diuron. The results of this experiment are presented in Fig. 7. It was observed for the two strains an increase of the response signal until a maximum, before a fast decrease of the signal stabilized at higher concentration in a second part. Looking for the Fig. 7, *P. subcapitata*, best concentration in Ca-alginate bead was 6.10^6 cell/mL and 7.10^6 cell/mL for *C. vulgaris*.

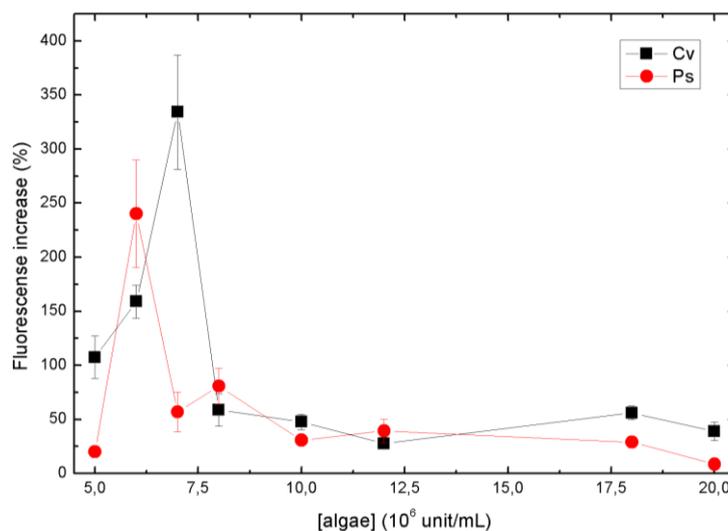


Figure 7: Algal concentration study for the optimization biosensor sensitivity. (N° replication=8).

3.3 Biosensor performance with pesticides solutions

Following previous results, algal dose-response experiments were done exposing the two algae strains immobilized with the double encapsulation, at a final concentration of silica of 4 % in hydrogel, with a Ludox/silica Sodium ratio of 1:10 and respective algal concentrations of 6 (*P. subcapitata*) and 7.10^6 (*C. vulgaris*) cell/mL and a silica thickness of 1mm. Dose-responses curves to pesticides are presented in Figure 8. For all the pesticides and algae used, we observed dose-response curve with the increase of the pesticides concentrations. We can also observe a difference of response between algae and between pesticides. For example, a better response was observed for *C. vulgaris* than *P. subcapitata* exposed to Diuron (e.g. 75 % fluorescence increase for *C. vulgaris* against 65 % for à 1000 $\mu\text{g/L}$), and at the contrary, a better response was observed for *P. subcapitata* than *C. vulgaris* exposed to Atrazine and Isoproturon (e.g. 44 % increase for *P. subcapitata* against 29 % for *C. vulgaris* à 1000 $\mu\text{g/L}$ of Atrazine). Detection limits are for all the pesticides, depending of the algae, less than 10 $\mu\text{g/L}$.

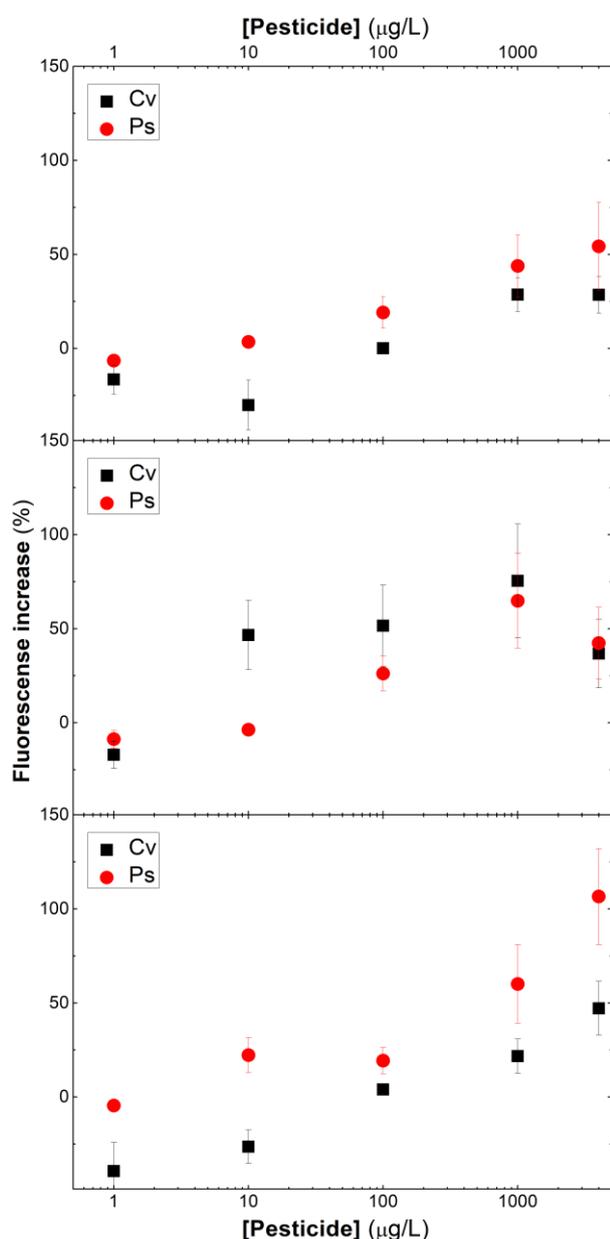


Figure 8: Double Encapsulated (silica hydrogel: 4 % (1:10)) *P. subcapitata* and *C. vulgaris* responses (α -chlorophyll fluorescence enhancement compared to control) for atrazine (upper panel), Diuron (middle panel), and Isoproturon (lower panel) after to expose 40 min (N° replication=16).

4. Discussion

Sol-gel technology as now been demonstrated its capacity to maintain micro-organism's life as algae (Nguyen-Ngoc et Tran-Minh, 2007; Ferro et al., 2012), fungi (Perullini et al. 2010; Ponamoreva et al., 2015) and bacteria (Nassif et al., 2002; Alvarez et al., 2007) along with their immobilization. In the case of double encapsulation of algae for biosensors design, Ferro et al. (2012) and Perullini et al. (2014) have already proven that microalgae could easily grow in the double encapsulation process without significant growth inhibition (e.g. only 2 and 6 % respectively for *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* in Ferro et al., 2012). The aim of this worked was to study biological and physicochemical parameters to improve sensitivity of these systems for biosensors setup.

4.1 Hydrogel selections and optimization

Porosity results demonstrated that with increase of silicate concentration and with increase of silica thickness, porosity tend to decrease. These results have confirmed previous work from Perullini et al. (2014b; 2016). These authors were observed an increase of D_{app} coefficient with pH increase until 7, and SiO₂ finale percentage diminution (until 4 % of silica) in silica hydrogels tested. These experiments have been done with an electrically charged dye. However, as previously mentioned by Perullini et al. (2014b), uncharged compounds should not be adsorbed in hydrogel, but only delayed, with consequently a more important impact at low concentration. This hypothesis is accordance with Ferro et al. (2012), which observed a decrease of A-chlorophyll fluorescence response for *Chlorella vulgaris*, compared to free and alginate encapsulated algae, because of hydrogel barrier leading to a delay in the pesticide transfer to the algae. However, many herbicides are electrically charged at pH 8 (pH of our hydrogels), and in environmental contaminated liquid media (classic pH between 7 and 9). For instance, Glyphosate is known to impact A-chlorophyll fluorescence Naessens et al. (2000). This herbicide has a positively charged amide group (at pH 7) (Tomlin, 1997) that could lead to an adsorption on negatively charged silica particles and so mask toxic effect on microalgae.

Concerning light scattering, this parameter could be partially at the origin of the variability observed. As seen in Figure 3, the light scattering at 460 nm (light excitation wavelength) is relatively high and variable, compared to 680 nm (emission wavelength). This phenomenon was also observed by Sicard et al. (2011). Consequently, this could impact the number of photons absorbed by the algae, and so the final response of chlorophyll fluorescence emitted and captured by the fluorimeter.

4.2 Immobilization parameter optimizations (algal concentration and silica thickness)

Fluorescence response decrease with high algal concentrations, as observed in Figure 7. This can be explained by a partial reabsorption of the fluorescence by neighboring algae (Védrine et al., 2003). Moreover, these fluorescence decrease could be also explained by the number of toxic molecules available for each alga (decreasing with algal concentration), and consequently their response. It is

consequently necessary to pay attention to this parameter to obtain a precise algal concentration and avoid any loss of fluorescence signal. A-chlorophyll fluorescence response decreases with the increase of the silica hydrogel thickness can be explained by a delay of pesticide transfer through the silica toward the algae beads. Truly, pesticides used in this study (Atrazine, Diuron and Isoproturon) are electrically uncharged at pH around 7, and consequently not adsorbed by negatively charged silica particles (isoelectric point at pH \approx 2). These observations are in agreements with Dickson et al. (2013) and Perullini et al. (2014). It is so necessary to use a thin thickness of silica hydrogel in the aim to improve molecules diffusion through microalgae.

4.3 Herbicides monitoring

In term of sensitivity, the best detection limit at 40 min exposure was obtained at a concentration of 10 or less $\mu\text{g/L}$. Compared to previous studies using sol-gel technology, the sensitivity range was the same (Nassif et al., 2003; Nguyen-Ngoc et Tran-Minh, 2007a,b; Perullini et al., 2007; Pena-vazquez et al., 2009; Pannier et al., 2014). This sensitivity was improved compared to the previous configuration of Ferro et al. (2012) which found a detection limit of 23.3 $\mu\text{g/L}$ for Atrazine, due to the optimization parameters done in our study. The decrease of the response at very high concentration of Diuron (4 mg/L) could be explained by an acute toxicity (cytotoxicity) of this herbicide for algae, as previously observed by Ferro et al. (2012). Looking for literature, EC_{50} -72 h growth inhibition was around 12.5 $\mu\text{g/L}$ at 20 °C (Tasmin et al., 2014), and so our maximum concentration used was 320 times up to EC_{50} value. Finally, we also observed that depending of the pesticide, the more sensitive algae was not the same (e.g. *C. vulgaris* for Diuron and *P. subcapitata* for Isoproturon), that confirmed Ferro et al. (2012) results.

4.4 Current limits and further steps

The main limit of our study remains the variability of the signal, as already expressed in section 4.1. This point could be deal with a more “automatized” process of bead creation and silica hydrogel generation around alginate beads. Finally, there will be necessary to use a dedicated instrument for immobilized organism’s studies, compared to microplate reader. For example, Pannier et al. (2014) used a piezoelectric micro-dosage printer to create algae/alginate drops on arrays, with silica hydrogel secondary immobilization and pulse amplitude modulated (PAM) fluorimetry detection, leading to similar responses limits of *Chlorella vulgaris* with atrazine exposure.

5. Main conclusions and perspectives

The aim of this work was to develop and optimize real double encapsulation (alginate/silicate) of algal cells for biosensors development adapted for pesticide detection in liquid media. Silica hydrogels and sol-gel process are suitable techniques to create algae entrapment, and keeping them alive. However, to obtain the best performance and sensitivity, it’s now clear that it’s necessary to make a

compromise between optical qualities and molecules (analytes/pollutants) diffusions potential in hydrogels. Consequently, for algal hydrogel entrapment, several parameters are necessary to optimize, as algal cell's concentrations, alginate beads size, hydrogel's gelation time and pH, hydrogels precursor's concentrations, hydrogel's composition, thickness and light scattering. Nevertheless, as shown in this study, improving the sensitivity is possible for *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*, and detection limits equal of less than 10 µg/L have been reached for Diuron, Atrazine, and Isoproturon, after 40 minutes' exposure. Repeatability could be improved by using a specific fluorimetric transducer dedicated to biosensors, and more automatized immobilization procedures. Moreover, further experiments will need to be oriented toward solidarization between encapsulated algae and optical transducer in the aim to produce a portative biosensor.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary data

Table S1: Silica hydrogel compositions

Silicate gel formulation (Final percentage of silica in hydrogel – Ratio Sodium silicate:Ludox in final gel)	Sodium Silicate (diluted 10 times) in μL	Ludox (pure) in μL	HCl (1.15 M) in μL	Phosphate Buffer (0.5 M, pH 8) in μL	H ₂ O in μL
3 % - 1:10	173	101	45	300	881
3 % - 1:20	87	107	23	300	984
4 % - 1:10	231	135	60	300	774
4 % - 1:20	115	143	30	300	912
5 % - 1:10	288	169	75	300	668
5 % - 1:20	144	178	38	300	840
6 % - 1:10	346	203	90	300	561
6 % - 1:20	173	214	45	300	768
7 % - 1:10	404	236	105	300	455
7 % - 1:20	202	249	53	300	696
8 % - 1:10	462	270	120	300	348
8 % - 1:20	231	285	60	300	624
9 % - 1:10	519	304	135	300	242
9 % - 1:20	260	321	68	300	552
10 % - 1:10	577	338	150	300	136
10 % - 1:20	288	356	75	300	480

4.1.3 Synthèse et discussion

Au cours de notre étude, nous avons réussi à réaliser une double encapsulation de microalgues dans des gels d'alginate/silice. Plusieurs paramètres ont été optimisés tels que : la porosité (maximum à faibles concentrations en silice dans les hydrogels : 3 et 4 % finale), les propriétés optiques (meilleures à forte concentration en silice dans les hydrogels (10 % finale)), l'épaisseur de silice autour des billes d'alginate/algue (1 mm), et les concentrations en algues (6 (*Ps*) et $7 \cdot 10^6$ cell/mL (*Cv*) pour des billes alginate/algues de $63 \mu\text{L}$). Les essais dose/réponse ont permis d'obtenir des limites de détection inférieures à $10 \mu\text{g/L}$, notamment pour *Chlorella vulgaris* avec le Diuron. Il faut également souligner que les premiers essais effectués, par Durrieu et al. en 2016 (Annexe 4), mettant en contact des algues encapsulées avec des eaux pluviales ont contribué à observer des effets, validant l'utilisation de ces systèmes pour la création de capteurs. Enfin, il est aussi important de noter que les biocapteurs à hydrogels pourraient permettre de créer des capteurs sélectifs des polluants présents dans les rejets, du fait des propriétés d'adsorption de certaines molécules des gels de silice. Cependant, cette technique de préparation de biocapteur nécessite de poursuivre son développement pour envisager son utilisation *in situ* (adaptation de la technique). La première étape à franchir sera l'automatisation de la construction des systèmes afin de diminuer la variabilité des réponses observées. De plus, il sera nécessaire de trouver un moyen de solidariser les algues encapsulées à une fibre optique ou un système alimenté, par exemple avec des LED, afin de pouvoir transférer le capteur *in situ*.

C'est pourquoi, au vu du travail de recherche à développer pour une utilisation efficiente des hydrogels dans la conception des biocapteurs, nous avons choisi de nous intéresser également à une autre technologie, comportant de nombreux avantages pour leur création, la microfluidique. L'utilisation de cette technologie est présentée dans la section suivante (4.2).

4.2 Biocapteurs optiques à systèmes microfluidiques

Après avoir étudié l'utilité des procédés sol-gel pour créer des biocapteurs algaux, nous avons choisi de nous orienter vers une utilisation de la microfluidique appliquée à notre problématique de recherche. Dans un premier temps, nous nous sommes intéressés à l'étude de la faisabilité de l'utilisation de puces microfluidiques, créées par xurographie, pour la réalisation de biocapteurs, dans la perspective d'un développement d'outil de terrain.

L'utilisation de la microfluidique pour le développement de biocapteurs est relativement récente, avec une apparition des premières études à la fin des années 90 et une croissance exponentielle au cours de la dernière décennie, comme en atteste la Figure 25. Nous n'avons donc pas été les premiers à nous servir de la microfluidique pour la création de biocapteurs algaux. En effet, depuis quelques années, de premières études ont vu le jour (e.g. Lefevre et al., 2012 ; Tsopela et al., 2014, 2016 ; Tahibegi et al., 2017). Parmi les méthodes de création de systèmes microfluidiques, la xurographie est apparue en 2005 (Bartholomeusz et al., 2005), mais le nombre d'études recourant à cette technique reste relativement faible (Figure 25) ; et aucune ne fait état de son utilisation pour la création de biocapteurs. La xurographie, encore peu employée, sert de technique alternative aux créations de systèmes microfluidiques en salle blanche. La création de systèmes microfluidiques par xurographie présente de nombreux avantages, comparativement aux techniques classiques de salle blanche (e.g. lithographie molle avec PDMS) tels que le faible coût (moins d'un euro par puce), la rapidité de construction (de l'ordre de la minute par puce) ou encore la possibilité de créer des puces multiniveaux en cas de besoin. Tout ce constat traduit bien le caractère novateur du travail qui a été engagé au cours de la thèse.

Dans le contexte d'un travail demandant de très nombreux tests expérimentaux, et comme nous le verrons dans la suite du manuscrit, la première étape de notre étude a été la conception de puces microfluidiques à usage unique peu chères nous permettant d'éviter tout problème de contamination croisée que l'on peut rencontrer en cas de réutilisation multiple de puces microfluidiques classiques (e.g. en PDMS). Au cours de leur fabrication, des algues sont déposées dans une chambre d'exposition et leur milieu de culture retiré par évaporation, avant fermeture des puces. L'idée était que des puces (stockées au froid et à l'obscurité) soient prêtes à l'emploi sur le terrain, avec dépôt direct de l'échantillon du rejet étudié. Seulement, nous avons rapidement pu mettre en évidence (voir résultats préliminaires ci-après) que cette méthode n'était pas satisfaisante, du fait d'une absence de réponse significative des microalgues. C'est pourquoi nous nous sommes orientés ensuite vers une méthode plus simple où les algues sont pré-exposées aux solutions toxiques (dans des microtubes) avant insertion dans les puces et analyse. La perturbation de la fluorescence des algues peut alors être mesurée soit à l'aide d'un fluorimètre de paillasse (fibre optique, dans le cas de la validation en laboratoire), soit avec un fluorimètre portable, utilisable sur le terrain (création d'une station portative dédiée). L'intérêt de cette approche microfluidique est d'assurer un confinement reproductible des algues (cavités de géométrie parfaitement définies, nombre d'algues observées reproductible).

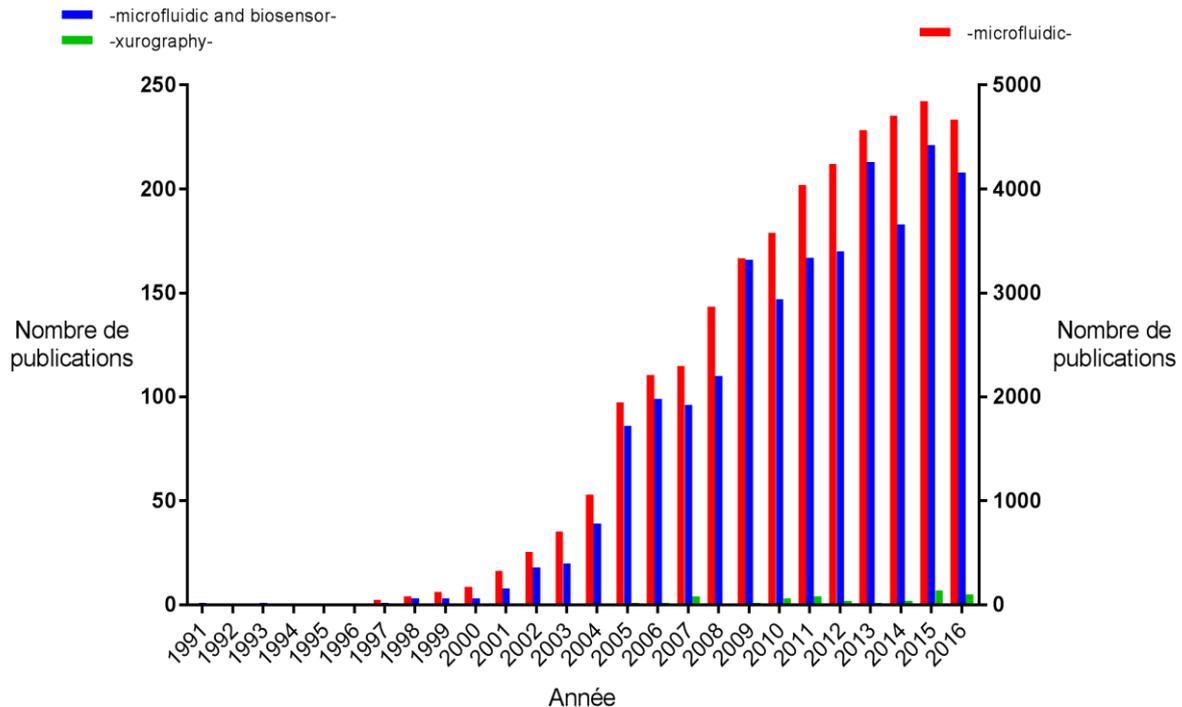


Figure 25 : Evolution au cours du temps du nombre d'articles référencés dans Web of Knowledge pour une recherche comportant les mots clefs : "microfluidic"; "microfluidic" et "biosensor"; et "xurography".

Il est utile de préciser que la xurographie présente quelques inconvénients quand on la compare à d'autres méthodes de fabrication de puces microfluidiques (Yuan, 2016). Le premier est la limite de taille des canaux, de l'ordre de 300 micromètres typiquement (ce chiffre étant fonction de la nature de la feuille adhésive découpée), contre 1 micromètre dans le cas des lithographies molles (cette dimension est en réalité celle des motifs du moule utilisé en lithographie molle). Cependant, dans notre cas, nous ne sommes pas contraints par la nécessité de travailler avec de faibles volumes (cellules algales et échantillons disponibles en quantité). Ainsi, la création de canaux microfluidiques relativement compatibles avec la xurographie convient aux besoins du projet tout en présentant l'avantage de faciliter la circulation des échantillons (algues/eaux polluées) en minimisant le risque de colmatage des canaux. La limitation géométrique de la xurographie n'est donc absolument pas un problème. Une deuxième limite concerne l'étanchéité des canaux qui n'est pas aussi bonne que celle d'un collage plasma PDMS/verre, par exemple. En effet, dans le cas d'un système continu avec pompes en entrée et sortie de la puce microfluidique (complicant son utilisation de terrain), la pression exercée peut conduire à la rupture de la liaison entre adhésif et lames de verre (liaisons non covalentes, ce qui n'est pas le cas dans les systèmes créés en lithographie molle). Dans notre cas, l'emploi des puces en usage unique avec insertion des fluides par capillarité nous affranchit de cette difficulté. Enfin, la dernière limite concerne l'inertie chimique des composés utilisés pour la création des puces. La composition chimique des adhésifs utilisés pourrait entraîner un problème de biocompatibilité avec les microalgues si des polymères ou de la colle venaient à interagir avec l'échantillon liquide auquel les algues sont exposées. Dans notre cas, l'usage unique des puces à des fins de lecture de la perturbation de la fluorescence nous

libère de ce problème éventuel. Les algues étant insérées au dernier moment, quelques minutes au plus avant la lecture de la fluorescence, il est peu probable que des interactions aient lieu.

En résumé, les défauts de la technique fabrication des puces microfluidiques par xurographie (résolution, tenue à la pression et pollution des échantillons), n'en sont pas pour mon travail de thèse. En revanche, les avantages de cette technique (rapidité et coût, principalement) ont été extrêmement favorables pour ce travail : je n'aurais probablement pas été en mesure d'obtenir tous les résultats qui seront présentés dans la suite de ce document avec des puces fabriquées en PDMS car cela aurait tout simplement pris trop de temps. Je propose donc maintenant de présenter ces résultats.

4.2.1 Etude/Validation en laboratoire

4.2.1.1 Introduction

L'objectif de cette première étape est de valider en laboratoire l'utilisation de la xurographie pour le développement de biocapteurs. Nous avons pour cela : pré-exposé des microalgues (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* et *Chlamydomonas reinhardtii*) à un pesticide connu (le Diuron) puis inséré les algues exposées (ou non, témoin) dans des puces conçues pour être compatibles avec la fibre optique d'un fluorimètre de laboratoire (Figure 26). Nous avons cherché à optimiser les réponses obtenues pour ces trois microalgues en testant l'influence de plusieurs paramètres : la concentration algale, le pH et la durée d'exposition. Finalement, la sensibilité du biocapteur a été testée en exposant les microalgues sélectionnées à une gamme de concentration en un pesticide bien connu (Diuron à 1, 10, 50, 100 et 1000 $\mu\text{g/L}$), puis nos résultats ont été comparés à la littérature.

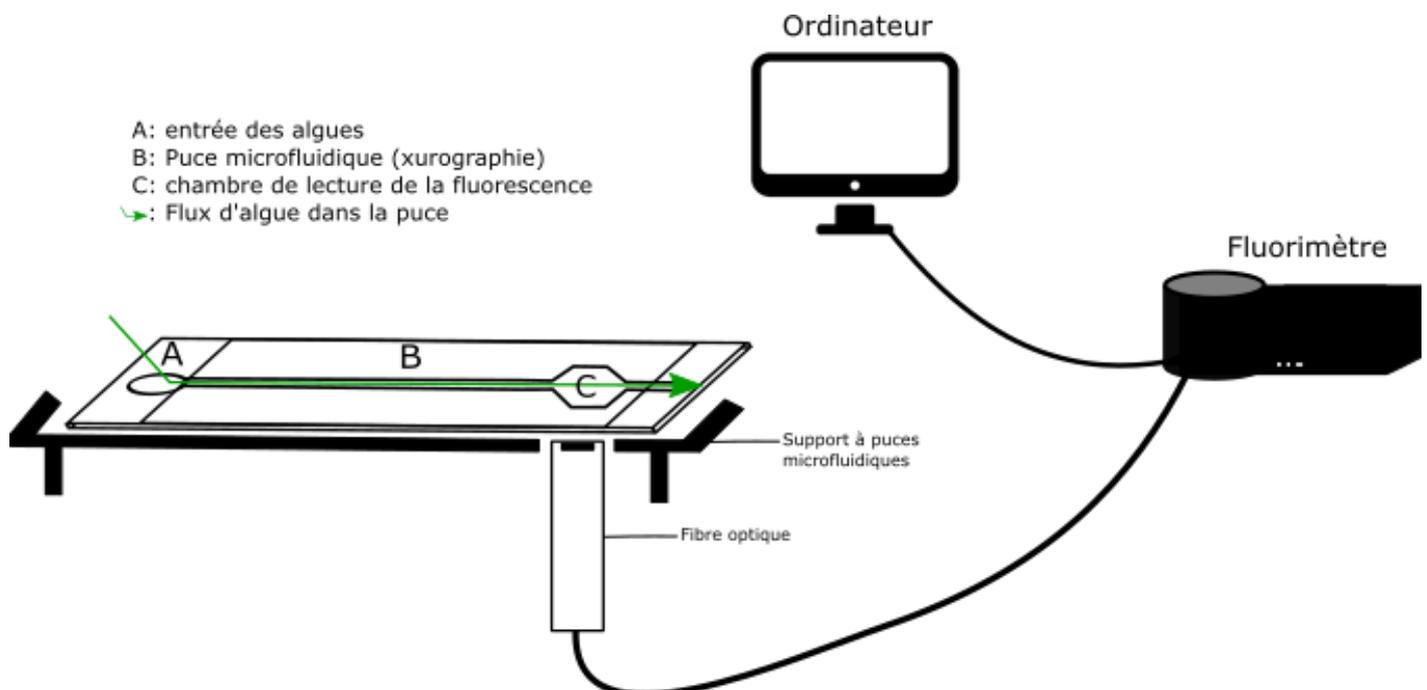


Figure 26 : Représentation schématique du dispositif utilisé pour l'évaluation de la fluorescence chlorophyllienne algale.

4.2.1.2 Matériel et méthode

4.2.1.2.1 Produits chimiques et préparation des solutions

Pour la préparation de solutions de pesticides, l'éthanol absolu ($\geq 99,5\%$) a été obtenu auprès de VWR (Radnor, États-Unis). Le Diuron ($\geq 98\%$ - Sigma-Aldrich, Saint-Louis, États-Unis) possède une solubilité relativement limitée dans l'eau (35 mg/L) contrairement à des solvants organiques tels que l'acétone ou l'éthanol. C'est la raison pour laquelle la solution stock de Diuron est préparée dans de l'éthanol pur à 10 g/L (soit 1 g de pesticide dans 100 mL d'éthanol absolu). Un volume de 100 μ L de cette solution stock est mélangé juste avant l'expérience à de l'eau ultrapure (volume de 100 mL final) pour créer des solutions « filles » à 10 mg/L utilisées pour les expériences.

4.2.1.2.2 Cultures algales

Trois souches de microalgues ont été utilisées : *Chlorella vulgaris* (*C. vulgaris*), *Pseudokirchneriella subcapitata* (*P. subcapitata*) et *Chlamydomonas reinhardtii* (*C. reinhardtii*). Ces souches d'algues ont été achetées auprès du Musée National d'Histoire Naturelle (Paris, France). *C. vulgaris* et *P. subcapitata* sont cultivées dans le milieu Lefebvre-Czarda (AFNOR, 1980). *Chlamydomonas reinhardtii* a été cultivée dans le milieu TAP (TrisAcetate Phosphate) (Annexes 1 et 2). Les souches d'algues sont entretenues de manière à disposer de cultures de microalgues « fraîches », c'est à dire dans leur phase de croissance. Pour cela, les souches sont repiquées chaque semaine dans du milieu de culture, sous condition stérile, à l'aide de verrerie et de milieu autoclavés (20 min, à 130 °C, sous 1,3 bar). Les algues sont cultivées en respectant un cycle nyctéméral 16 h/8 h lumière/obscurité sous un éclairage de 5000 lux, dans une enceinte climatique dédiée maintenue à 21 °C. La détermination de la concentration des cultures pour la préparation des solutions d'algues a été effectuée *via* comptage direct sur cellules de Thoma.

4.2.1.2.3 Création des puces microfluidiques

La technique de xurographie est utilisée pour fabriquer les puces microfluidiques. Les principales étapes de fabrication sont présentées dans la Figure 27-A. Des lames de verre (25x75x1 mm) et des lamelles de verre (24x60x0,15 mm) sont fournies par Fisher Scientific (Illkirch, France). Des rouleaux de film adhésif double face (DSPA), protégés par des films plastiques, de 100 μ m d'épaisseur ont été achetés auprès de Plusform (Tesa, Allemagne). Le design des puces microfluidiques est le suivant : un canal de 100 μ m de haut, 62 mm de long et 1 mm de large, relié à une chambre de mesure (10x6x0,1 mm). La chambre de mesure est conçue pour réaliser une lecture de fluorescence par une fibre optique reliée à un fluorimètre de laboratoire et un ordinateur enregistrant les mesures (Figure 27). Le canal et la chambre de lecture sont découpés dans un rouleau de film adhésif DSPA avec un traceur (Graphtec-CE-5000-40, Ankersmit France). Après retrait d'un des films protecteurs, l'adhésif est collé

sur une lame de verre servant de substrat. Le second film protecteur est alors retiré et une lame couvre-objet est collée afin de fermer la puce microfluidique.

4.2.1.2.4 Présentation du schéma expérimental général des expériences

Le schéma général du principe de l'expérience est présenté à la figure 27 B. Toutes les expériences sont effectuées à température ambiante (environ 21 °C). Les microalgues sont cultivées comme décrit dans la section précédente. Des volumes de 50 mL de suspension d'algues en fin de phase de croissance exponentielle (soit environ 6 jours, pour une concentration algale suffisante) sont prélevés, et centrifugés à 3000 tr/min (1600 g) dans le but de les concentrer à la valeur souhaitée. Ensuite, 160 µL de suspension concentrée de microalgues sont transférés en tube Eppendorf de 500 µL et exposés à 40 µL de solution de pesticide. Enfin, 20 µL de suspension sont ensuite collectés et déposés sur la puce microfluidique et insérés par capillarité au niveau de l'entrée de cette dernière. La puce microfluidique est alors insérée dans un support en plastique (4,1x9,5x1,8 cm) permettant de positionner une fibre optique (5 mm de diamètre) en face de la chambre de lecture de la fluorescence. La fibre optique conduit

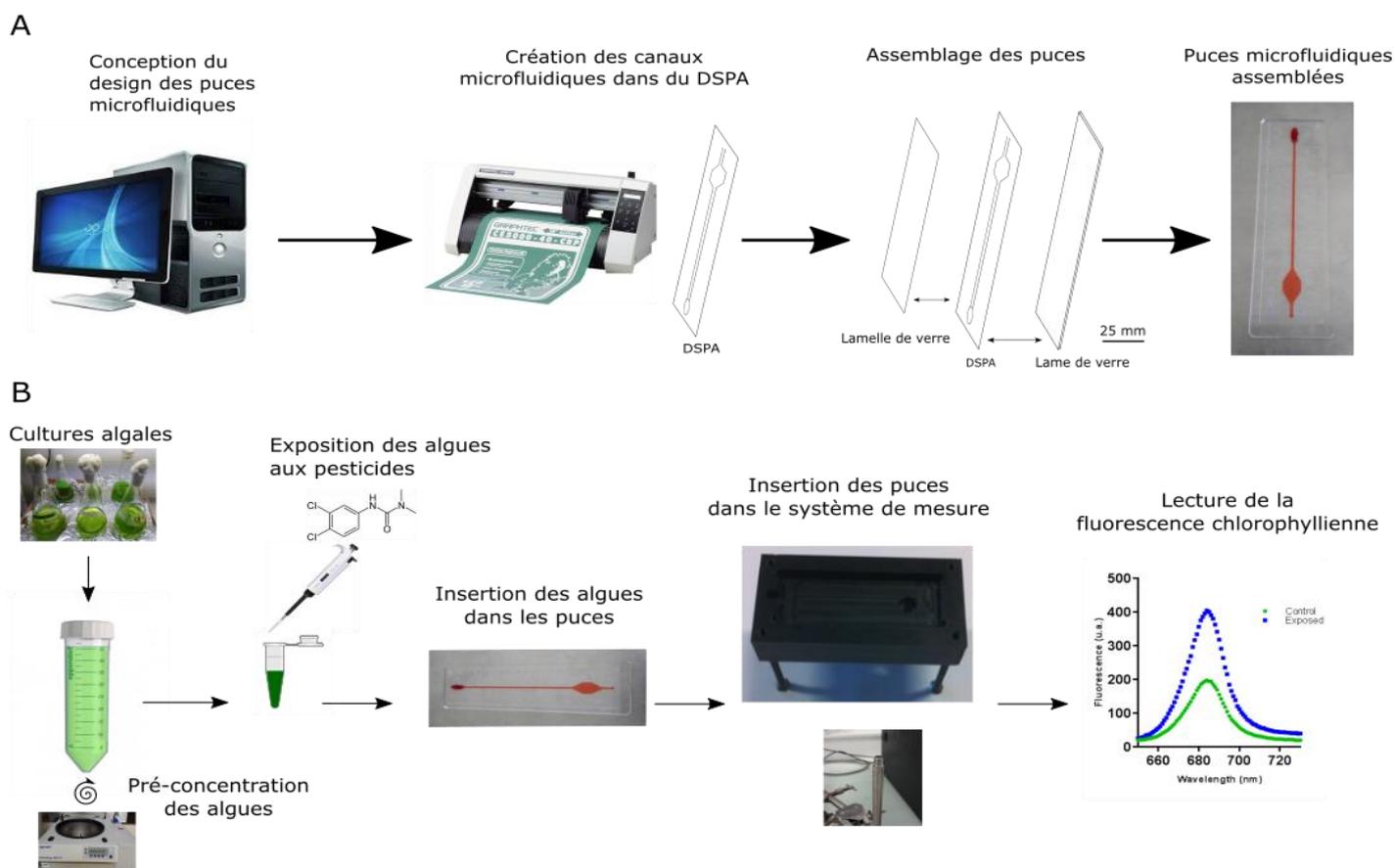


Figure 27 : Représentation schématique de notre système de détection de fluorescence algale : (A) Étapes de conception et création des puces microfluidiques pour l'étude de la perturbation des algues, basées sur la technique de xurographie. (B) Représentation schématique de la procédure de détection de la fluorescence chlorophyllienne dans les puces.

le signal de fluorescence vers un fluorimètre de laboratoire (Varian® Cary Eclipse). La fluorescence chlorophyllienne des algues témoins et exposées est déterminée à l'aide d'un couple ex./em de 460/680 nm. La perturbation de la fluorescence chlorophyllienne est calculée en utilisant l'équation suivante (2) :

$$(2) \quad F_E = (F_{ex} - F_{cont}) / F_{cont} * 100$$

Avec F_E la perturbation (augmentation ou diminution) de la fluorescence (en %), F_{ex} la fluorescence des algues exposées au pesticide (en unités arbitraires (a.u.)) et F_{cont} la fluorescence des algues contrôles (en unités arbitraires (a.u.)).

Les expériences sont effectuées en triplicatas. Un contrôle avec la plus grande concentration en éthanol utilisé dans les solutions testées (0,01 %) est préparé pour vérifier l'absence de perturbation de la fluorescence en présence d'éthanol.

Dans le cas des premières expériences préliminaires sur algues déséchées, la concentration finale des cellules (essais menés sur *P. subcapitata*) centrifugées a été de $3 \cdot 10^8$ cell./mL. Des volumes de 10 μ L de solution ont été déposés au niveau de la chambre de lecture et laissés sécher à température ambiante avant fermeture des puces. Puis, 20 μ L de Diuron à 35 mg/L (ou de l'eau pour les témoins) ont été insérés dans les puces. Les algues ont été mises en contact pour une durée de 30 minutes avant lecture de la fluorescence chlorophyllienne comme expliqué ci-dessus.

4.2.1.2.5 Optimisation des paramètres de réponse des algues aux polluants

Le travail d'optimisation des paramètres liés à la réponse des algues aux polluants a été réalisé en trois étapes successives, en validant tout d'abord la meilleure concentration, puis le temps optimal d'exposition des algues et enfin le pH, et ce à l'aide des paramètres les plus favorables à chaque fois déterminés précédemment.

- Concentration algale

La meilleure concentration algale a donc été déterminée en premier pour optimiser la réponse des microalgues. Pour cela, des aliquots de 50 mL de suspension d'algues sont centrifugés et remis en suspension dans différents volumes de milieu de culture afin d'obtenir une gamme de concentration finale (après mélange avec le Diuron) pour chaque espèce de microalgues testées. Suite à cette étape, les microalgues ont été mises en contact avec du Diuron à 100 μ g/L (eau ultra-pure pour le contrôle). Les microalgues sont laissées 40 minutes en contact avec le pesticide et sont finalement insérées dans une puce microfluidique avant la lecture de fluorescence.

- Temps d'exposition des algues

Pour cette étape, les microalgues sont utilisées à une concentration finale (après mélange avec le Diuron) correspondant à la meilleure réponse, déterminée précédemment. Pour cela, des volumes de

50 mL de suspension de microalgues sont centrifugés puis concentrés et exposés à deux concentrations différentes de Diuron (20 et 100 µg/L) dans le but de déterminer la rapidité de la réponse vis-à-vis de ce pesticide. Deux concentrations ont été choisies car celles-ci peuvent influencer la biodisponibilité du polluant pour les algues et donc le temps de réponse. Juste après la mise en contact avec le Diuron, la fluorescence est évaluée après 1,5, 5, 10, 15, 20, 30, 40 et 60 minutes d'exposition. La perturbation de la fluorescence est calculée comparativement au témoin afin de déterminer le temps d'exposition nécessaire pour obtenir une réponse stable.

- Optimisation du pH d'exposition

Pour déterminer le pH optimal de réponse des algues, des volumes de 50 mL de cultures de microalgues en phase de croissance ont été ajustés à différents pH (5, 6, 7, 8, 9) à l'aide d'acide chlorhydrique dilué et préconditionnés dans des tubes Eppendorf pendant 1 heure. Après cela, les suspensions de microalgues sont centrifugées pour les ajuster à la concentration optimale finale (après mélange avec le Diuron) déterminée auparavant. Les algues concentrées à chaque pH sont ensuite exposées au Diuron (concentration finale de 100 µg/L de la solution mère préparée au dernier moment dans de l'eau ultrapure ajustée au même pH que la solution d'algues) pendant le temps optimal également déterminé préalablement. Finalement, un échantillon de la suspension est déposé dans des puces microfluidiques, la fluorescence mesurée et la perturbation de la fluorescence calculée pour chaque condition.

- Performance du biocapteur

En utilisant tous les paramètres (concentration algale, temps d'exposition et pH) optimisés, la performance du biocapteur est évaluée. À cette fin, les trois suspensions de microalgues sont exposées à une gamme de concentration de Diuron (1, 10, 50, 100 et 1000 µg/L). Les algues ont en parallèle été exposées à la plus grande concentration en éthanol finale (0,01 %) utilisée dans cette gamme afin de vérifier qu'aucun effet observé ne provenait de ce solvant. Comme le Diuron a été utilisé par différents auteurs en tant que modèle dans la conception de nombreux biocapteurs, la sensibilité de notre système est comparée à d'autres études en termes de limites de détection et de valeurs CE₅₀. Les valeurs de CE₅₀ ont été calculées en utilisant le modèle probit de Hill (macro Regtox[®], E. Vindimian, <http://normalesup.org/~vindimian/>).

4.2.1.3 Résultats et discussion

4.2.1.3.1 Résultats préliminaires : assèchement des microalgues dans les puces.

La première idée de conception des puces microfluidiques de manière simplifiée a été de déposer au niveau de la chambre de lecture de la fluorescence une quantité d'algues et de retirer par évaporation

le milieu de culture. Le principe était de garantir la conservation des puces (par exemple à 4 °C) jusqu'à utilisation, et d'appliquer par capillarité une solution de pesticide (puis par la suite des rejets pollués). Par microscopie optique (Figure 28), nous avons pu observer que le dépôt et l'assèchement des algues (ici des *Pseudokirchneriella subcapitata*) dans la chambre entraînaient une variabilité de la répartition des celluloses algales. Un effet dit « tache de café », bien référencé dans la littérature, a été observé avec une densité surfacique des cellules supérieure dans les zones périphériques qu'au centre des chambres d'exposition/lecture (Figure 29).

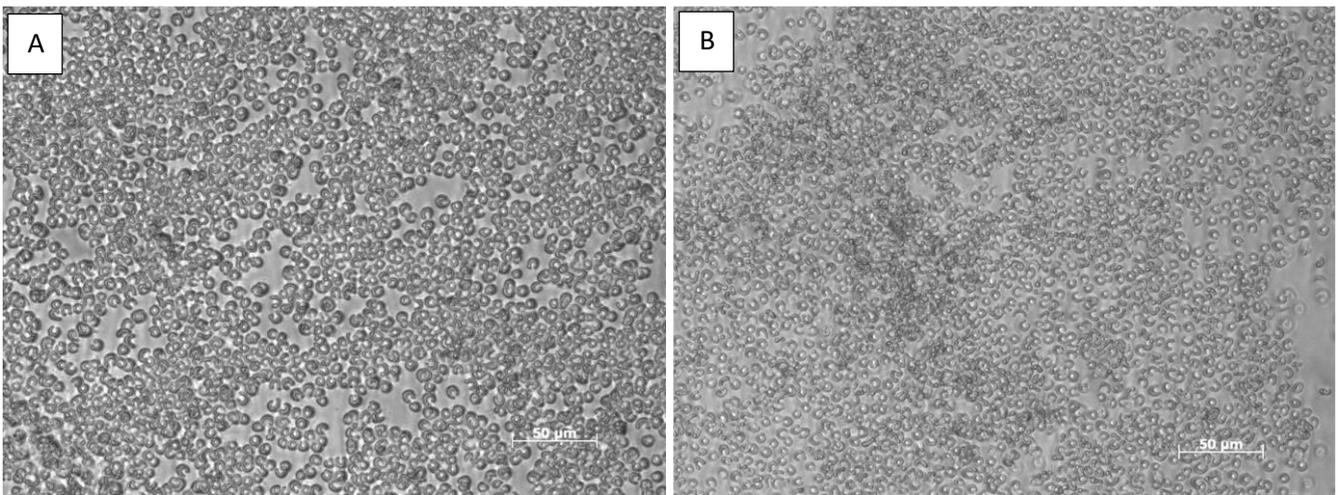


Figure 29 : Observation au microscope optique (X400) des microalgues *Pseudokirchneriella subcapitata* déposées dans la chambre d'exposition et de lecture de la fluorescence dans une puce microfluidique avant (A) et après (B) remise en contact avec une solution de Diuron.

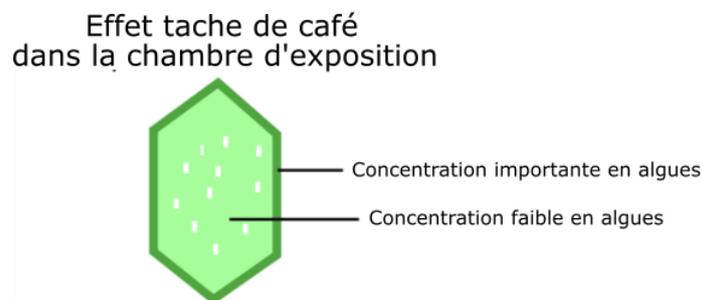


Figure 28 : Principe de l'effet tache de café appliqué aux algues déposées dans la chambre d'exposition aux pesticides.

Concernant la fluorescence des algues (*Pseudokirchneriella subcapitata*) desséchées puis réhydratées, nous avons pu mettre en évidence une réponse classique (Figure 30 A) avec un pic maximum de fluorescence ($46,45 \pm 4,23$ u.a.) à une longueur d'onde de 682,9 nm. Une certaine variabilité de la réponse a été constatée en raison de la difficulté de remplissage des puces microfluidiques au niveau de la chambre d'exposition/lecture dont une place importante était déjà occupée par les algues. Avec une concentration élevée afin d'observer une forte différence de fluorescence, l'exposition des algues au Diuron a permis d'obtenir les résultats présentés Figure 30 B. La fluorescence des algues exposées était de $50,50 \pm 3,26$ u.a., soit une augmentation de $8,70 \pm 7,04$ % comparé au témoin. Au vu de la concentration importante en Diuron utilisée et aux données disponibles

dans la littérature, cette augmentation s'avère relativement faible. L'explication la plus plausible de ce manque de réponse serait la perturbation de la physiologie des algues lors de leur dessèchement, même s'il est rapide. En effet, plusieurs auteurs dont Wiltens et al. (1978), Gray et al. (2007) ou Holzinger et

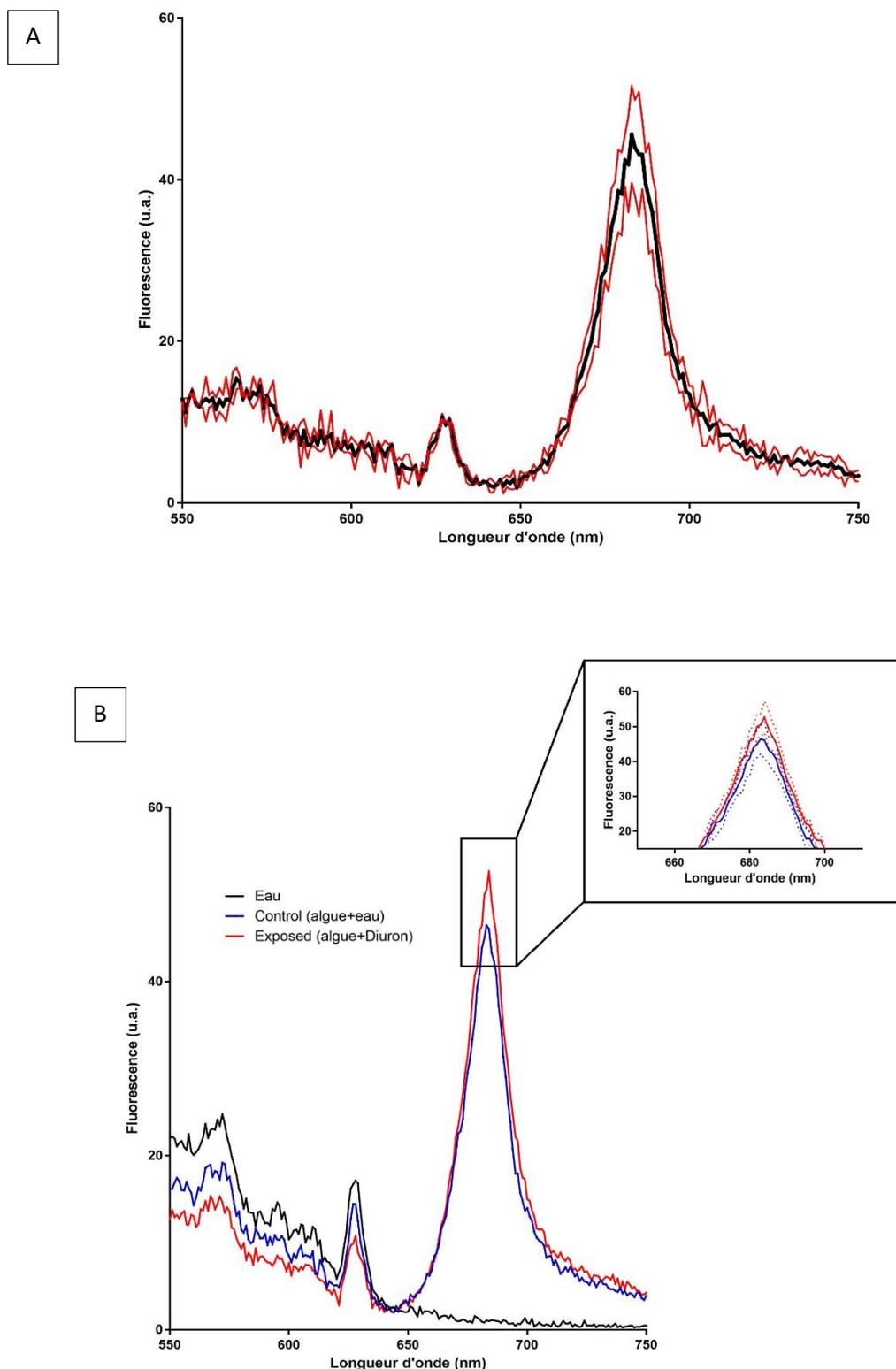


Figure 30 : (A) Profil de fluorescence chlorophyllienne des algues *P. subcapitata* remises en suspension dans de l'eau \pm écart-type (N=3), (B) Profil de réponse de fluorescence des microalgues exposées 30 minutes (rouge) ou non (bleu) au Diuron (35mg/L) \pm écart-type en en pointillés (N=3).

Karsten (2013) ont fait état de l'impact de la déshydratation et réhydratation sur la fluorescence chlorophyllienne et la réponse des algues à des pesticides tel que le Diuron. S'ils ont tous conclu que la déshydratation inhibe complètement l'activité photosynthétique et la fluorescence chlorophyllienne, ils ont également noté que selon les algues testées (aquatiques ou désertiques), la réapparition d'une activité photosynthétique (généralement faible dans le cas des algues aquatiques) peut arriver entre 30 secondes (cas des algues désertiques) et plus de 2 heures (cas des algues aquatiques) après réhydratation selon les algues étudiées. La dessiccation endommage la protéine D1 située sur le photosystème 2 des thylakoïdes et entraîne une perte d'activité photosynthétique. Le temps de réparation des cellules et de création de nouvelles protéines D1 est le facteur limitant pour la réapparition d'une activité photosynthétique. Dans notre cas, le temps d'exposition, et donc de réhydratation des algues étudiées (30 minutes), n'est vraisemblablement pas suffisant pour relancer une activité photosynthétique. Cette méthode de préparation n'est donc pas compatible pour une mise en évidence rapide de la perturbation de la photosynthèse (ici par la présence de pesticides), et souhaitée dans le cas des biocapteurs algaux pour une utilisation de terrain. Cependant, même si la méthode de mesure par algues déshydratées ne remplit pas son rôle de manière satisfaisante, tout ce travail n'a pas été fait en pure perte : il nous a permis de valider la méthode de fabrication des puces, le bon fonctionnement du remplissage capillaire avec nos échantillons, ainsi que le bon niveau de signal de fluorescence en système microfluidique, compatible avec des mesures optiques standards. Enfin, ce travail nous a permis, en procédant par une démarche récursive, de penser à l'amélioration des puces en utilisant une mesure différentielle, comme présenté dans la partie suivante (4.2.2).

4.2.1.3.2 Résultats pour les algues non déshydratées

Les expérimentations suivantes ont repris le même principe que précédemment, en utilisant des microalgues pré-exposées (ou non) au Diuron avant une insertion dans les puces microfluidiques.

La Figure 31 illustre la relation entre la gamme de concentration en microalgues et la fluorescence des algues non exposées, que nous avons observée dans les puces. Nous avons constaté une très bonne linéarité pour *P. subcapitata* et *C. vulgaris* (valeurs de R^2 respectives de 0,9829 et 0,9833). Le choix des gammes de concentration utilisées a été basé sur des essais préliminaires de réponse des algues au Diuron. La chambre de lecture de la fluorescence présentait un volume de 4,4 mm³, soit 4 µL environ. Le nombre de cellules, dans la chambre, variait de 225 000 à 1,7 million de cellules pour les *P. subcapitata* et entre 1,7 et 3,5 millions de cellules pour *C. vulgaris*.

Comme nous l'avons déjà vu dans la précédente partie de notre travail concernant les capteurs à hydrogels, la concentration en algues utilisées pour la conception des biocapteurs est un paramètre fondamental. Pour établir les conditions optimales de densité cellulaire dans la puce microfluidique, nous avons donc exposé les cultures algales de différentes concentrations à une même dose en Diuron (100 µg/L) (Figure 32). Les concentrations optimales que nous avons déterminées étaient de 2 et 7.10⁸

cell/mL pour *P. subcapitata* et *C. vulgaris* avec des réponses de $92,29 \pm 8,77$ % et $69,96 \pm 2,20$ % après exposition au Diuron.

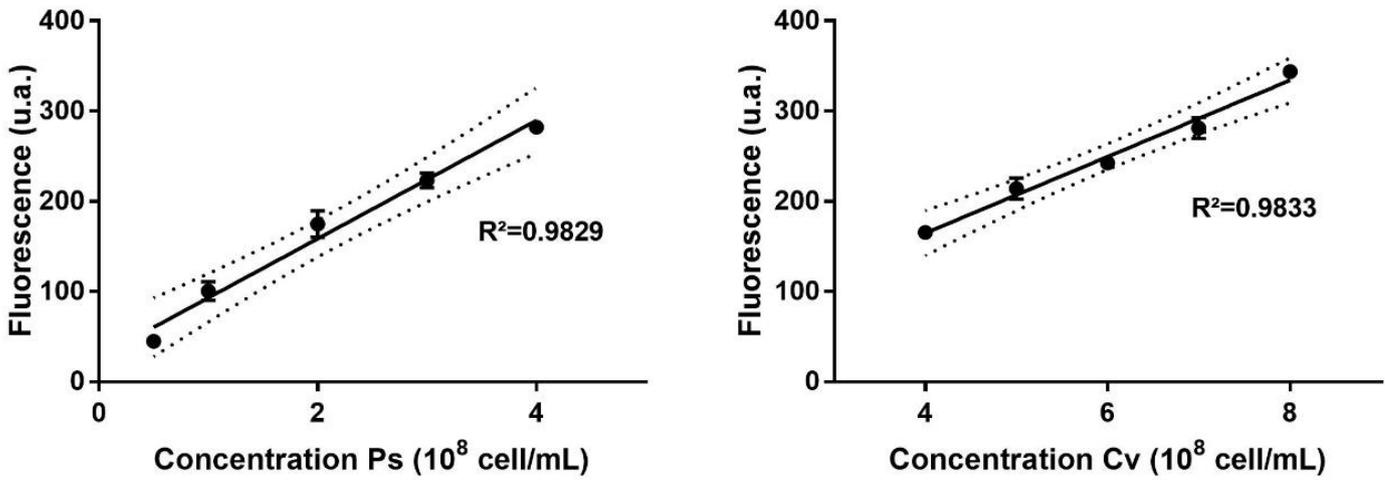


Figure 31 : Relation entre la concentration et la fluorescence chlorophyllienne des microalgues *Pseudokirchneriella subcapitata* (Ps) et *Chlorella vulgaris* (Cv) insérées par capillarités dans des puces microfluidiques (en pointillé : l'intervalle de confiance à 95 %).

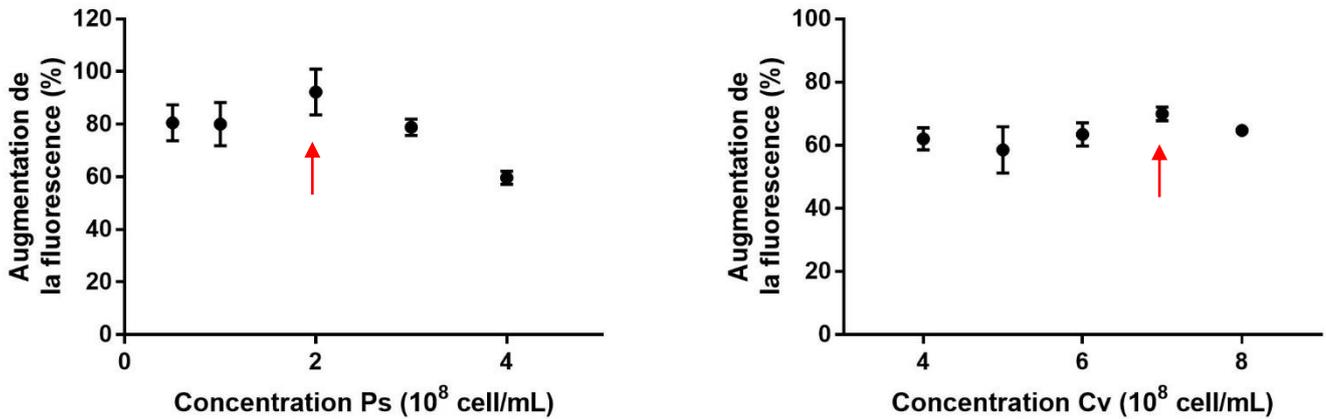


Figure 32 : Relation entre la concentration et l'augmentation de la fluorescence chlorophyllienne des microalgues *Pseudokirchneriella subcapitata* (Ps) et *Chlorella vulgaris* (Cv) exposées au Diuron (100 µg/L) insérées par capillarités dans des puces microfluidiques.

Les effets de la concentration en algues ayant déjà été abordées durant notre travail précédent sur les hydrogels, ces derniers seront de nouveaux discutés de manière plus approfondies dans la partie suivante traitant de la conception de la station de terrain (Article 5), car le même exercice d'optimisation a été effectué.

Le temps de réponse est une donnée importante dans la mise en place de biocapteurs. On cherche à obtenir une réponse rapide à la présence des contaminants toxiques, afin de pouvoir créer des outils d'alerte précoce de la pollution des écosystèmes aquatiques. Nous avons donc exposé les algues à deux concentrations en Diuron (20 et 100 µg/L) afin de déterminer d'une part le temps pour lequel la réponse

des algues est stable, et, d'autre part, si la concentration en polluant influence le temps de réponse des algues insérées dans les puces (Figure 33). Chez *P. subcapitata*, nous avons constaté une augmentation de la fluorescence en présence de Diuron durant les 20 premières minutes d'exposition quelle que soit la concentration testée, puis une stabilisation de la réponse jusqu'à la fin du temps d'exposition (1 heure). En revanche chez *C. vulgaris*, la réponse observée est nettement plus rapide, mais elle a différé légèrement entre les deux concentrations avec un temps de réponse stable quasi immédiat (1,5 minute) à 100 µg/L, contre 5 minutes à 20 µg/L.

Ces résultats confirment ceux de Podola et Melkonian (2005) qui ont constaté un phénomène de retard de l'augmentation de la fluorescence chlorophyllienne de la microalgue *Tetraselmis cordiformis* immobilisée sur filtre de cellulose et exposée à des concentrations de Diuron allant de 1 à 100 µg/L. L'augmentation de fluorescence était maximale (saturée) au bout de 20 minutes à 5 µg/L contre 10 minutes à 50 µg/L de Diuron.

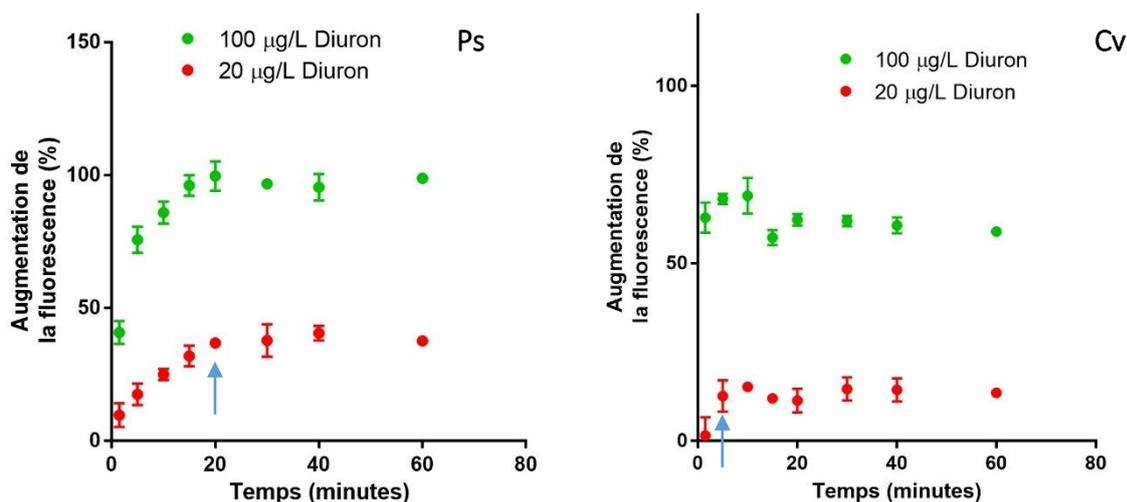


Figure 33 : Relation entre le temps d'exposition et l'augmentation de la fluorescence chlorophyllienne des microalgues *Pseudokirchneriella subcapitata* (Ps) et *Chlorella vulgaris* (Cv) exposées au Diuron (20 et 100 µg/L) insérées par capillarité dans des puces microfluidiques.

Le pH constitue aussi un paramètre essentiel de la réponse des microalgues et conditionne particulièrement la fluorescence des chlorophylles contenues dans les chloroplastes. Dans notre cas, et quelle que soit l'espèce utilisée, la réponse au Diuron (100 µg/L) a été optimale à pH 7, avec une augmentation des fluorescences respectives de $84,43 \pm 4,45$ % et $67,21 \pm 2,77$ % pour *P. subcapitata* et *C. vulgaris* (Figure 34). Ces résultats confirment les travaux de Naessens et al. (2000), Védrine et al. (2003), et Nguyen-Ngoc et Tran-Minh (2007). Le pH de réponse optimal s'explique par le fait que la concentration en H_3O^+ de la solution influence des paramètres de la photosynthèse tels que la libération des protons (H^+) permettant la création d'ATP, mais également le transfert d'électrons au sein de la chaîne photosynthétique (Conjeaud et Mathis, 1986). Par ailleurs, le pH a une influence sur la protéine

D1 du photosystème 2, donneur primaire d'électrons de la chaîne photosynthétique (Conjeaud et Mathis, 1980), et cible du Diuron et de nombreux autres polluants (voir partie démarche générale). Ce paramètre est souvent associé à la température qui influence également ces processus.

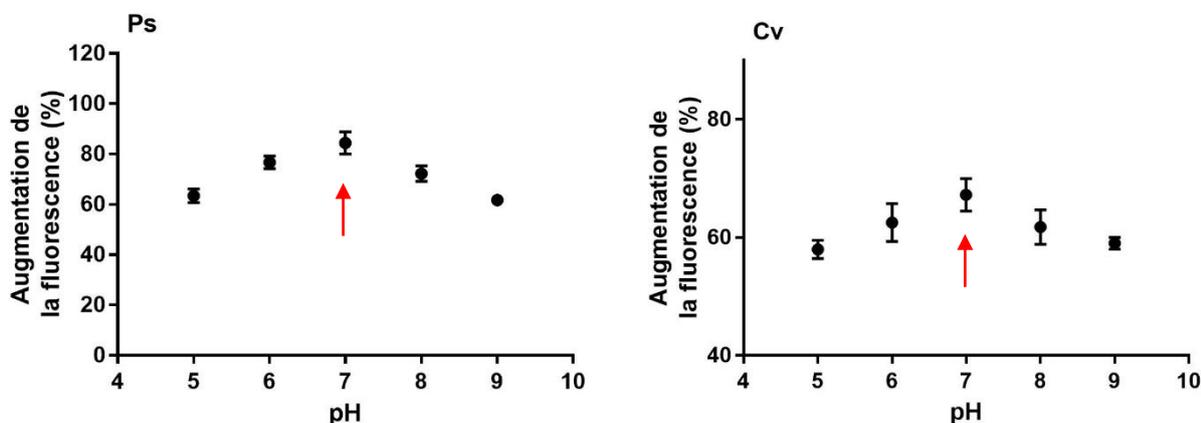


Figure 34 : Relation entre le pH et l'augmentation de la fluorescence chlorophyllienne des microalgues *Pseudokirchneriella subcapitata* (Ps) et *Chlorella vulgaris* (Cv) exposées au Diuron (100 µg/L) insérées par capillarité dans des puces microfluidiques.

Les toutes premières expérimentations sur *C. reinhardtii* ont permis d'obtenir les résultats de la Figure 35 qui illustre l'influence de la concentration algale sur l'intensité de la fluorescence. Nous constatons une bonne linéarité de la fluorescence en fonction de la concentration en algues ($R^2=0,9681$), une bonne réponse des algues au Diuron ($125,1 \pm 11,1$ % d'augmentation de la fluorescence après exposition à 100 µg/L) pour une concentration de $2 \cdot 10^7$ cell/mL, et une diminution très nette de la réponse pour des concentrations plus élevées. Toutefois, nous avons observé une importante variabilité autour de cette valeur de réponse de *C. reinhardtii* au Diuron, en répétant nos expériences (réponse allant de 50 à 125 % d'augmentation de la fluorescence, résultats non montrés). Cette variabilité a rendu

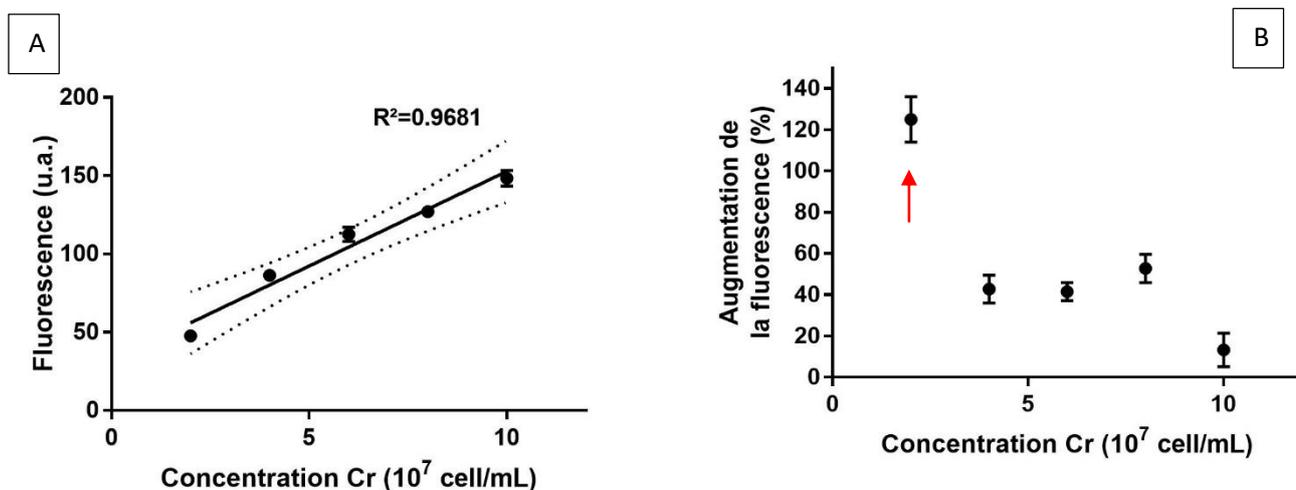


Figure 35 : Relation entre la concentration et la fluorescence chlorophyllienne (A) ou l'augmentation de la fluorescence chlorophyllienne (B) des microalgues *Chlamydomonas reinhardtii* (Cr) exposées au Diuron (100 µg/L) insérées par capillarité dans des puces microfluidiques (en pointillé : l'intervalle de confiance à 95 %).

impossible les expérimentations avec le dispositif actuel concernant le temps de réponse, le pH et la réponse de ces algues à une gamme de dilution de Diuron, et qui ont par conséquent été abandonnées.

Notre objectif final a donc été de tester la sensibilité de notre dispositif. Nous avons pour cela effectué des courbes dose-réponse avec les deux algues *P. subcapitata* et *C. vulgaris* sélectionnées et les paramètres optimums déterminés précédemment en les exposant à différentes concentrations en Diuron (1, 10, 50, 100 et 1000 $\mu\text{g/L}$). Les courbes obtenues sont présentées sur la Figure 36. Les valeurs de limites de détection et d' EC_{50} sont présentées Tableau 3. Nous avons pu observer une limite d'effet de 1 $\mu\text{g/L}$ pour *P. subcapitata* et *C. vulgaris*. Les valeurs de CE_{50} obtenues étaient de 21,38 [19,80 ; 23,02] et 46,35 [45,44 ; 47,37] $\mu\text{g/L}$. Enfin, avec une forte concentration en Diuron, d'importantes augmentations de la fluorescence (jusqu'à 148,32 % à 1 mg/L de Diuron pour *C. vulgaris*) se sont produites. Nous avons ainsi pu mettre en évidence une différence de sensibilité de nos deux algues au Diuron. Cette information s'avère utile et intéressante. Elle nous montre l'utilité de développer des biocapteurs multi-espèce. Il est en effet fort probable (et cela va être confirmé par la suite en paragraphe 4.2.2) qu'avec des rejets urbains par temps de pluie, les réponses diffèrent en fonction de l'algue et de l'échantillon.

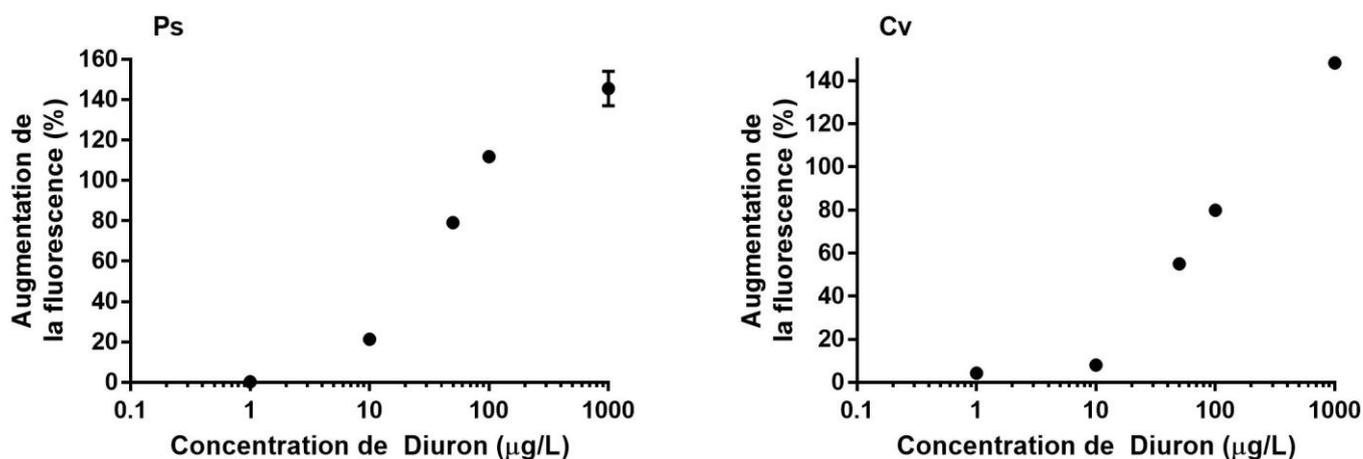


Figure 36 : Courbes dose-réponse des microalgues *Pseudokirchneriella subcapitata* (Ps) et *Chlorella vulgaris* (Cv) exposées au Diuron (1, 10, 50, 100 et 1000 $\mu\text{g/L}$) insérées par capillarité dans des puces microfluidiques.

Nos résultats de sensibilité sont comparés aux données de la littérature dans le Tableau 3. Notre biocapteur paraît avoir une sensibilité du même ordre de grandeur que la majorité des dispositifs créés par le passé, hormis dans l'étude de Lefèvre et al. (2012) où le capteur présente des valeurs de limite de détection (LDD) et de EC_{50} plus faibles que les nôtres. Cette différence peut être hypothétiquement expliquée par : (i) Une utilisation de concentration en algues bien plus faible, ce qui pourrait se traduire par une augmentation du nombre de molécules biodisponibles par algue. Cependant, ce constat est à relativiser car nous avons vu notre cas que si l'on arrivait à atteindre un optimum de perturbation de la fluorescence par le Diuron à une concentration en algue donnée, cette perturbation était moins importante à concentration plus forte ou plus faible. (ii) Une meilleure intégration de leur système, avec

des LED et photo-détecteurs organiques plus proches des systèmes microfluidiques, permettant aux microalgues de capter un plus grand flux de photons et une meilleure détection de signaux de fluorescence émis.

Cependant, il est important de préciser que nous n'avons pas considéré dans notre cas uniquement les LDD et EC₅₀ du biocapteur, mais aussi les aspects pratiques et commerciaux, en utilisant des matériaux peu chers et des techniques permettant une transposition pour des études sur le terrain. C'est l'objet des travaux réalisés par la suite et présenté dans le paragraphe 4.2.2.

Tableau 3 : Comparaison de la sensibilité de notre système avec différents capteurs algaux dédiés à la mesure de la perturbation de la photosynthèse par le Diuron.

Espèce d'algue	Méthode de mise en contact algues/transducteur	Paramètre observé	Mode d'exposition	Pesticide détecté	Limite de détection (µg/L)	EC ₅₀ (µg/L)	Auteurs
<i>C. vulgaris</i>	Immobilisation sur filtre à microfibres de quartz	Fluorescence chlorophyllienne	Flux continu	Diuron	0,025	39	Védrine et al. (2003)
<i>C. vulgaris</i>	Immobilisation par impression sérigraphique sur électrode	Variation de la production d'oxygène	Statique	Diuron	X	233,65	Shitanda et al. (2009)
<i>C. reinhardtii</i>	Microfluidique	Fluorescence chlorophyllienne	Flux continu	Diuron	X	2.56	Lefèvre et al. (2012)
<i>C. reinhardtii</i>	Système commercial Handy-PEA	Fluorescence chlorophyllienne	Statique	Diuron	X	51,26	Lefèvre et al. (2012)
<i>C. reinhardtii</i>	Microfluidique	Variation de la production d'oxygène	Statique	Diuron	46,73	X	Tsopela et al. (2014)
<i>C. reinhardtii</i>	Microfluidique	Variation de la production d'oxygène	Statique	Diuron	23,3	X	Tsopela et al. (2016)
<i>C. reinhardtii</i>	Microfluidique	Variation du pH	Flux continu	Diuron	11,65	X	Tahirbegi et al. (2017)
<i>C. reinhardtii</i>	Microfluidique	Variation de concentration d'oxygène au cours du temps	Flux continu	Diuron	11,65	X	Tahirbegi et al. (2017)
<i>C. reinhardtii</i>	Microfluidique	Fluorescence chlorophyllienne	Flux continu	Diuron	11,65	> 46,6	Tahirbegi et al. (2017)
<i>P. subcapitata</i>	Microfluidique	Fluorescence chlorophyllienne	Statique	Diuron	1	21,47	Notre étude
<i>C. vulgaris</i>	Microfluidique	Fluorescence chlorophyllienne	Statique	Diuron	1	46,35	Notre étude
<i>C. reinhardtii</i>	Microfluidique	Fluorescence chlorophyllienne	Statique	Diuron	X	X	Notre étude

Enfin, nous avons comparé, la sensibilité de notre capteur aux pesticides (ici Diuron), aux seuils de qualité de l'eau. Cette comparaison est bien sûr en partie biaisée par le fait que dans une matrice de multiples polluants viennent influencer la fluorescence chlorophyllienne (la plupart du temps de manière additive ou synergique entre eux, même si des cas d'antagonisme existent). Cependant, cette comparaison reste tout de même intéressante à discuter, car si du Diuron était présent dans un rejet/une eau contaminée, une perturbation de ces derniers serait probablement constatée. En Tableau 4, sont reportées les limites d'acceptabilité du Diuron dans l'eau potable (et par extension dans les eaux de surface) dans différents pays. En comparant nos données à ces seuils, nous avons pu vérifier que notre capteur ne permettrait pas de couvrir les seuils de qualité européens du Diuron seul (0.1 µg/L), mais largement ceux de l'Australie (30 µg/L) et du Canada (150 µg/L). Concernant les Etats-Unis, aucune

réglementation n'existe actuellement, cette molécule faisant partie des composés en cours d'étude pour une limitation. Si, depuis 2003, ce pesticide est interdit en France pour les usages agricoles (mais fréquemment détecté dans les rejets, eaux de surfaces et les sols actuellement), ce n'est pas le cas dans le reste du monde.

Tableau 4 : Seuil de qualité de l'eau pour les différentes parties du monde.

Pays/Région du monde	Union Européenne	Canada	Etats-Unis	Australie
Limite d'acceptabilité du Diuron dans l'eau ($\mu\text{g/L}$)	0,1	150	X	30
Référence	DCE	Gouvernement Canadien	US EPA	ANHMRC

4.2.1.4 Synthèse et conclusion

Cette étude de faisabilité nous a permis de contrôler la sensibilité du biocapteur vis-à-vis du Diuron, avec des limites de détection de l'ordre du microgramme par litre (soit quelques nano-molaires) pour *P. subcapitata*. Ces résultats ont été obtenus sur la base d'une optimisation des paramètres impactant la sensibilité de la réponse des algues aux pesticides (concentration algale, temps d'exposition, pH), et en cohérence avec l'état actuel de la littérature. Une différence de réponse entre les diverses espèces algales a été clairement observée : ce résultat est intéressant pour le développement de biocapteurs multi-espèces, menant à un panel de réponses variées en lien avec une exposition des algues aux RUTP *in situ*. Néanmoins, le biocapteur présente un inconvénient, à savoir la nécessité de pré-concentrer de manière importante les algues avant utilisation (afin d'obtenir un signal maximum pour permettre une détection optimum de la fluorescence et de sa perturbation) et donc le besoin d'importants volumes de cultures afin de multiplier les essais. A notre connaissance, c'est la première fois que la technique issue de la xurographie a été utilisée pour la conception de biocapteurs, plus particulièrement de biocapteurs algaux. Ce procédé ayant été validé avec des outils de laboratoire non transportables (fibre optique relié à un fluorimètre de paillasse), le paragraphe suivant est consacré à la miniaturisation du système optique pour un usage *in situ* du biocapteur, tout en gardant une solidarité entre les algues immobilisées dans les puces et le transducteur optique, et une sensibilité suffisante.

4.2.2 Application à la création d'une station portative de terrain dédiée à la détection de polluants écotoxiques

4.2.2.1 Contexte et enjeux

Nous avons validé précédemment l'utilisation des puces microfluidiques avec l'optimisation de plusieurs paramètres (temps d'exposition, pH, ...) liés aux conditions d'usage pour le développement d'un biocapteur de terrain. Les micropuces avec fibre optique ont été testées avec un pesticide modèle, le Diuron. Les résultats des courbes dose-réponse ont été satisfaisants au regard des données présentées dans la littérature scientifique, justifiant l'intérêt de l'utilisation de ces puces pour le développement d'un biocapteur. La seconde étape de nos investigations a donc consisté à concevoir et valider un dispositif permettant de réaliser des mesures écotoxicologiques *in situ*. Pour cela, un fluorimètre portatif a été développé et construit sur le concept de la puce microfluidique. Nous avons dans un premier temps créé une station portative (fluorimètre) et, dans un second temps, testé la réponse chlorophyllienne des trois microalgues en présence de Diuron. A l'aide d'un logiciel spécialement dédié, nous avons optimisé les paramètres de mesure de perturbation de la fluorescence, telles que l'intensité lumineuse excitant les microalgues, ou encore la concentration algale. Nous avons ensuite vérifié la sensibilité du biocapteur portatif en comparant la courbe dose-réponse d'exposition au Diuron avec celles présentes dans la littérature. Enfin, le dispositif a été testé avec des RUTP et des eaux usées brutes collectés durant l'année 2016, afin de valider son utilisation *in situ*.

N.B. : Ce travail a été effectué à l'aide des trois souches d'algues utilisées précédemment. Malgré l'absence de résultats probants avec l'algue *C. reinhardtii*, nous avons choisi de mener également les expérimentations suivantes sur cette espèce.

4.2.2.2 Article 5 - Xurography-based microfluidic algal biosensor and its dedicated portable measurement station for online monitoring of urban polluted samples.

Article en cours de finalisation pour soumission dans Journal of biosensors and bioelectronics

Xurography-based microfluidic algal biosensor and its dedicated portative 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 pollution discharges toxicity impact on aquatic ecosystems. The present work 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 portative fluorimeter able was developed to assess the A-chlorophyll fluorescence of microalgae inserted by capillarity in low cost and disposable xurography-based microfluidic chips. Three microalgae population 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 before to calibrate the biosensor sensitivity with Diuron, a pesticide known to be very toxic for microalgae. Finally, the biosensor was applied to 10 aqueous urban polluted samples (7 urban wet weather discharges and 3 wastewaters) in the aim to prove its reliability, reproducibility and performance to detect toxic discharges on field.

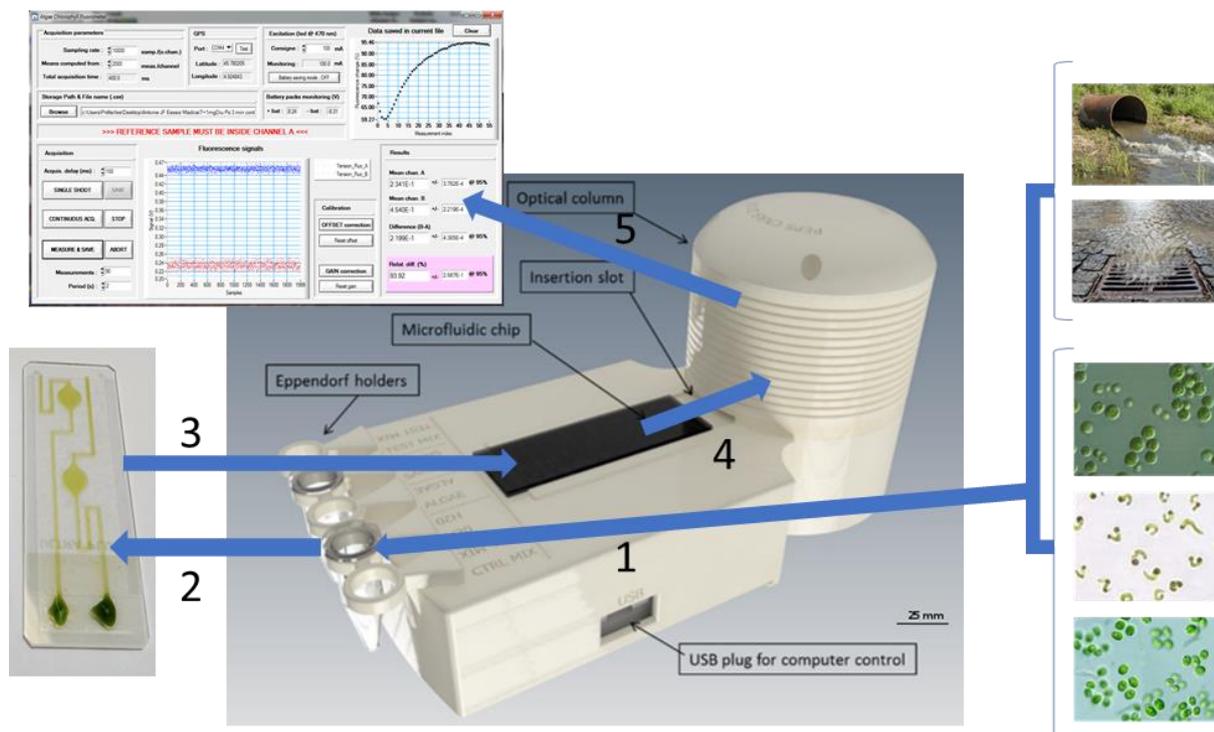
Keywords

Microfluidic biosensor, Microalgal A-chlorophyll fluorescence, Xurography, Portative fluorimeter, Pesticide, Urban polluted water.

Highlights

- We developed a portative microfluidic biosensor for *in situ* water toxicity analysis.
- Xurography technic was used to create disposable and low-cost microfluidic chips.
- Algal biosensor sensitivity was checked with Diuron pesticide.
- Biosensor was applied to real urban polluted water samples.

Graphical abstract



1. Introduction

Wide number of anthropic activities is responsible of the release of micropollutants by the production of polluted rejects. Among them, urban wet weather discharges (Zgheib et al., 2011; Gasperi et al., 2012), agricultural runoffs (Willis and McDowell, 1982), industrials, urban (Rosal et al, 2010) or hospital (Verlicchi et al., 2010) effluents can lead to multiple substantial amounts of contaminant mixture in environment: tap, underground coastal and surface waters (Masner et al, 2017).

In Europe, the Water Framework Directive (WFD, 2000/60/EC) aims at maintaining a good ecological status of water bodies exposed to such large contamination. Chemical analysis of waters bodies informs about complex chemical substances contamination. But sample collection, preparation and chemical analyses are time consuming, expensive and provide no information about biological impact (toxicity) (Axelrod et al., 2016). Moreover, common lab-scale ecotoxicological bioassays such as algal growth inhibition assays are labor intensive, time consuming, need skillful personnel and use considerable amount of reagents (Zheng et al., 2013, Gao et al., 2016, Yang et al., 2016). Therefore, they did not provide a realistic toxic impact quantification of polluted samples, because sample transportation, storage and conservation before bioassays is questionable. That is why new tools are highly expected for real time monitoring of the toxicity of water contaminated matrices (Rogers, 1995, Axelrod et al., 2016, Yang et al., 2016). For this purpose, whole-cell biosensors are very promising for the development of in situ, sensitive and fast response pollution monitoring tools (as early warning systems).

Among the microorganisms used to design whole-cell biosensors, microalgae are commonly used. Microalgae represent primary producers, at the base of the food chain. Consequently, an impact

on these organisms can affect all the higher trophic levels. It's why they provide essential information for predicting environmental impact (Zheng et al., 2012, 2013). Moreover, microalgae are very sensitive to numerous pollutants commonly detected in suburban aquatic ecosystems and do not suffer of ethical problems for their use. Among the toxicity effects observed on microalgae, photosynthesis disturbance is the widest studied, leading to 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 parameter to detect micropollutants such as Polycyclic Aromatic Hydrocarbons (Aksmann and Tukaj, 2008), pesticides (Védrine et al., 2003), heavy metals (Suresh Kumar et al., 2014) or pharmaceuticals (Liu et al., 2011). Different methods were actually developed to measure photosynthesis' disturbances (e.g. fluorescence or electrochemistry). The optically 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 point for the design of a microalgal biosensor. The sensitivity can potentially decrease down to an order of magnitude compared to non-entrapped cells (Avramescu et al., 1999, Zheng et al., 2012, Wong et al., 2013). It was also observed with hydrogels entrapment, mainly due to the diffusion barrier created between the environment and the microalgae's (Ferro et al., 2012). To overcome the problem of cell's immobilisation, microfluidic technic provides the advantage of a direct contact into micrometer-size channels between microorganisms and pollutants, without any physical barriers. Other important advantages are the low sample and reagent consumption, and low cost, flexibility in design, and 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 pollutants detection and toxicity assessment have been developed during last decade. Biosensors are mainly based on using 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 of microfluidic devices are built by "soft-lithography" using polymers, mainly polydiméthylsiloxane (PDMS), to replicate small-scale features (micro-channels) from a master mold. Despite the simplicity of the process design, it requires specific clean room facilities (Renaud et al., 2015), skillful personal. It is time consuming, and not transposable to 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), that completely cancel the advantages of microfluidic systems (e.g. miniaturization).

However, Bartholomeusz et al. (2005) developed a new process to design microfluidics 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 technic 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 any external pump, avoiding cumbersome gear deployment in situ. Recent advanced xurography-based microfluidic systems have been published such as a rapid immunosensing platform (Kim et al., 2014). Nonetheless, despite all these advantages, to the best of our knowledge, no xurography based whole-cell biosensors were described in the literature.

Consequently, we report here a new low-cost compact and stand-alone portative algal toxicity biosensor incorporating algae inserted in simple and disposable xurography-based microfluidic chips. A dedicated portative fluorimeter was developed to read immobilized algal chlorophyll fluorescence is also reported. To perform differential measurements, a single microfluidic chip embeds two separated chambers for algae entrapment, one dedicated to algae in reference medium, the other one for algae exposed to pollutants/real samples. No pump is required because chips are filled by capillarity. As recommended previously by Tsopela et al. (2016), since each alga is specifically sensitive to different pollutants, we are carried out experimental tests with three different algae strains to develop the biosensor. The fluorimeter integrates a blue (470 nm) Light Emitting Diodes (LED) as a light source, two silicon photodetectors (one for each entrapment chamber), a set of optical filters for excitation/emission light decoupling, and electronics for signal amplification, acquisition and data transfer to a laptop computer. LED provides many advantages for portative microfluidic systems, as a long lifetime, a low cost, small dimensions and a low-power consumption (Novak et al., 2007; Jokerst et al., 2012a). A dedicated software was developed to provide a user friendly interface with full control of acquisition parameters (e.g. calibration parameters, light excitation intensity, sampling frequency, number of acquisitions etc). A GPS was also linked to the fluorimeter for geolocation of the samples analyzed on the field. In a first step of 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, to prove its relevance and sensitivity as early warning system for suburban ecosystems.

2. Materials and methods

2.1 Chemical products and solution preparations

For pesticides solutions preparation, absolute ethanol ($\geq 99.5\%$) was obtained from VWR (Radnor, USA). Diuron ($\geq 98\%$) was purchased from Sigma–Aldrich (Saint-Louis, USA). Diuron stock solution was prepared in pure ethanol solution at 10 g/L (1 g of pesticide in 100 mL of absolute ethanol). Just before experiment, 100 μ L of ethanol solution of Diuron were mixed in final 100 mL of ultrapure water to create 10 mg/L Diuron work solution used for experiments.

2.2 Algal cultures

Three microalgal strains were used during this work: *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 the Lefebvre-Czarda medium (2012). *Chlamydomonas reinhardtii* were cultured in TAP medium. These strains were transplanted weekly under sterile condition, using sterilized glassware and medium (autoclaved during 20 min, at 130 °C, under 1.3 bar), in the aim to use fresh algae in their growth phase. Algae were cultivated in a nycthemeral 16 h/8 h light/dark cycle using 5000 lux lighting in special culture room maintained at 21 °C. Algal cells concentration has been determined with Thoma cell counter.

2.3 Microfluidic chips fabrication and algal insertion procedure

Xurography-based technology was used to design and construct the disposable microfluidic chips designed to allow an A-chl fluorescence lecture by a dedicated portative fluorimeter built for this purpose (see Figure 1A). The microfluidic channels and analysis chambers for algae entrapment and fluorescence measurement are cut in a 100 μ m thick double-sided pressure adhesive (DSPA) film (Plusform; Germany; Tesa, Germany) thanks to a cutting plotter (Graphtec-CE-5000-40, Ankersmit, France). Each microfluidic device provides two independent chambers (5 mm diameter, 100 μ m, and thick, 1.96 μ L) feed through microfluidic channels (100 μ m thick, 90 mm long, and 1 mm wide). The first one is the control chamber with algae in reference medium, and the second one is filled with exposed algae to pollutants. After cutting, the DSPA is scotched on a glass slide (25x75x1 mm, Fisher Scientific, Illkirch, France) used as a substrate. Then, a glass coverslip (24x60x0.15mm) is scotched to the DSPA second adhesive side to create the hood. As the length of the coverslip is shorter than the substrate ones, the remaining uncovered area is used to create the inlets where the user will have to deposit the algae suspension droplets. No external pump is required. The capillary force is used to generate the flow from each inlet to the corresponding chamber. In summary, in all experiments, algae were immobilized into the microfluidic chips by adding a drop of around 15 μ L at the inlets and let be inserted until the lecture

chamber by capillarity before fluorescence lecture. A representation of an algae filled microfluidic chip is presented in Figure 1B.

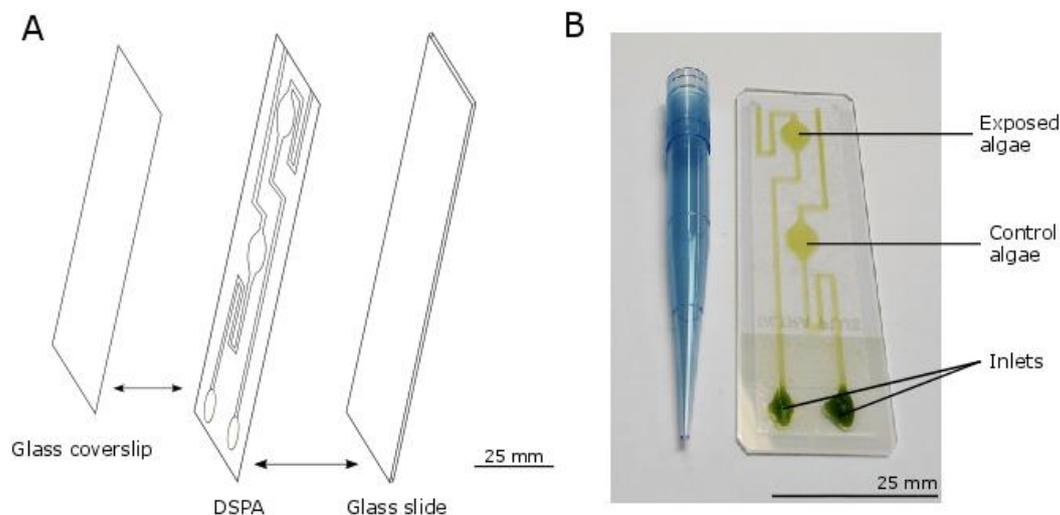


Figure 1: Xurography-based microfluidic chip creation procedure (A) and photography (B) with algae inserted by capillarity, for the algal immobilization in portative station. DSPA: Double sided pressure adhesive film.

2.4 Portative fluorimeter description

A portative fluorimeter has been developed to read the α -chlorophyll fluorescence (ex. 460-480 nm/em. 680 nm) of microalgae immobilized in microfluidic chips previously described (Figure 2). The fluorimeter was 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) allowing 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 thanks to a plastic polarizer (LPVISE2X2, Thorlabs). The light illuminates the algae inside the microfluidic chambers, and then passes through a second polarizer with crossed orientation respectively to the first one. This pair of polarizing filters enhances the rejection of the 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 thanks to 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 portative fluorimeter was composed of a LED power control and a digital acquisition system (NI USB-6002, National Instrument) to control algal exposure light intensity and collect the fluorescence signal emitted by the algae (see part 2.5). All the optical column and acquisition parts were protected by a plastic polymer case created with a 3D printer (Formlabs 1+, Formlabs, USA). This case includes Eppendorf holders for convenient field experiments. Software for fluorimeter control, data acquisition and processing has been developed (LabWindows CVI environment) and is hosted by 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.

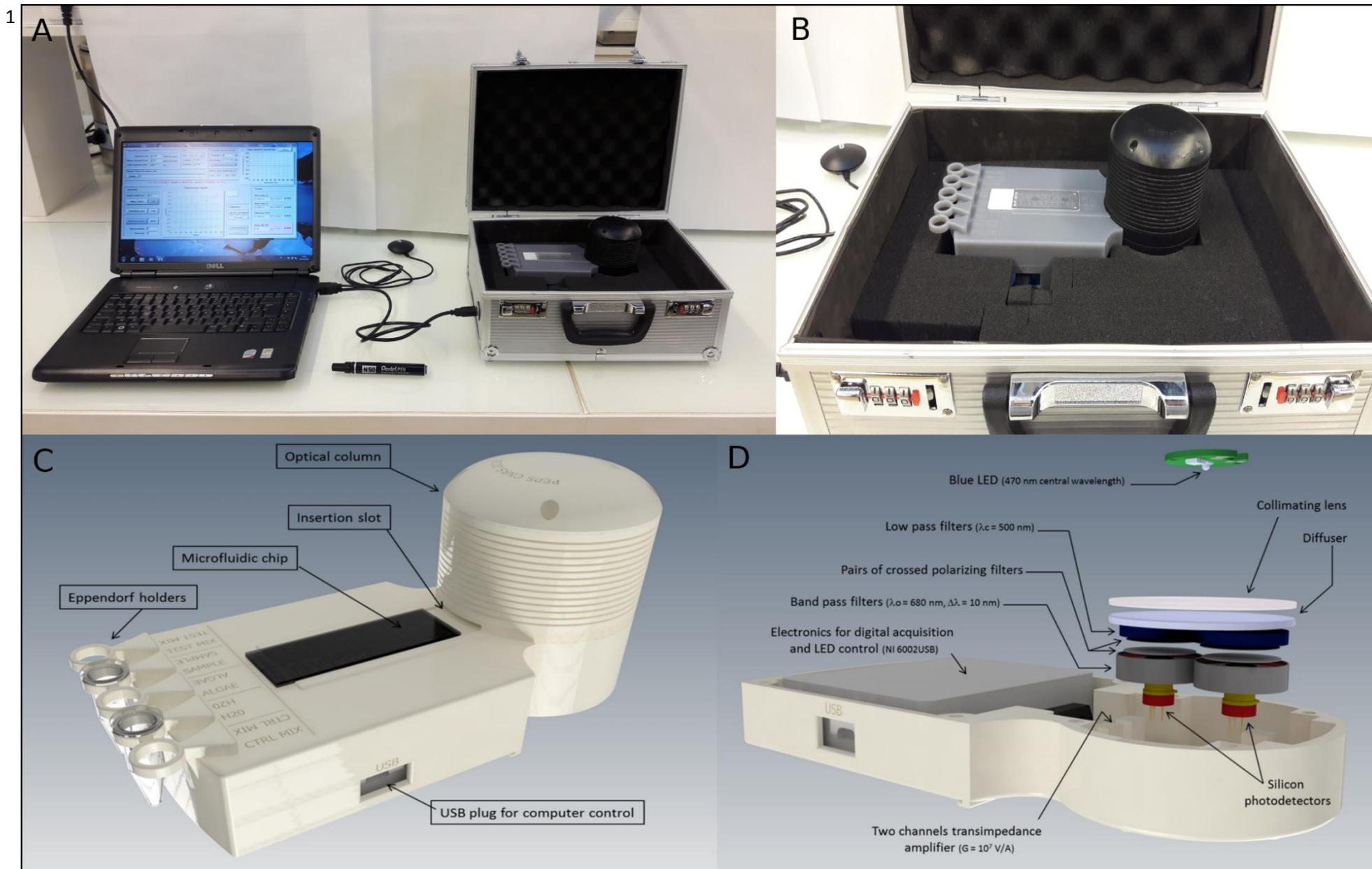


Figure 2: Photography of the whole system (Computer, GPS, and portable station) (A), portable station (B), internal (D) and external (C) representation of the portable station.

2.5 Software presentation

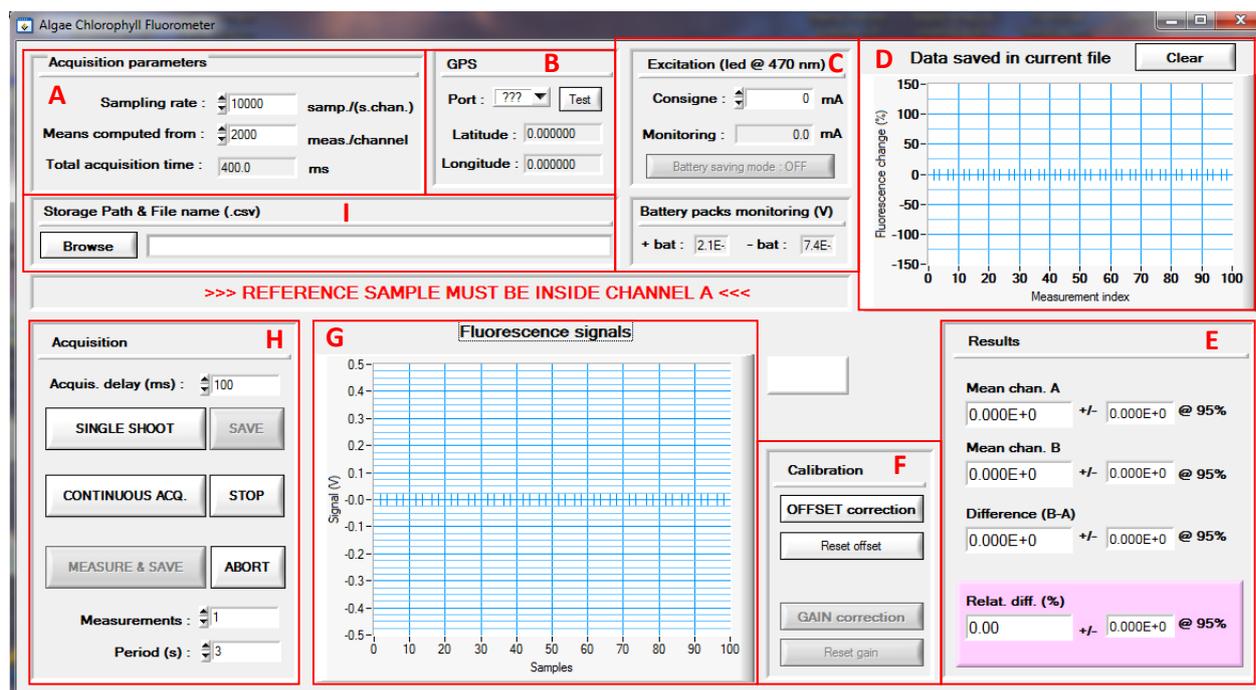


Figure 3: photography of the algal A-chl fluorimeter user interface.

The software developed to control the portable station parameters and record fluorescence signals is illustrated in Figure 3. The different parts of this one and its operating mode are presented just below. Before each algal fluorescence measurement, offset (without light excitation) and gain (with light excitation) correction of amplifiers was done for each empty microfluidic chip (Figure 3F) to insure an equal response of each one. 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 part A and H presented in the laptop screen permitted to 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 part E and G of the software (see Figure 3), and calculated relative difference of fluorescence between control and exposed algae is viewed in real time in part D and E. GPS coordinates of sample location are displayed in part B. Location and raw differential signal were saved automatically (storage path shown in part I of the software).

2.6 Algal reliability, repeatability and sensitivity parameters optimization

All experiments have been done at room temperature (around 23 °C). In our study and for all assays, algae were exposed continuously during 3 minutes to LED excitation light and fluorescence assessment was done all 2 seconds (2000 measurements each time (400 ms acquisition time) for mean and standard deviation computation), i.e. 180 fluorescence measurements for each assays. All assays have been done in triplicate during this study.

2.6.1 Algal filling in microfluidic chips characterization

Algal filling in microfluidic chips lecture chambers was observed to insure a repeatable measurement of a-chlorophyll fluorescence. Briefly, after chips filling by algal solutions (15 µL), photographs of the fluorescence lecture chambers (“control” and “exposed”) were obtained using a camera linked to a binocular microscope (Zeiss® Stemi 2000, Germany), under a 4.5 x magnification, and images were analyzed using ImageJ free software (Abràmoff et al., 2004). Following a similar principle of Jokerst et al. (2012b), mean shade of grey was used to assess for the control and exposed lecture chamber for the three algal strains an equal algal filling and so concentration into the chambers (See Figure S1 A).

2.6.2 Algal concentration

Best algal concentration was assessed to optimize the sensitivity of the response. For that, 50 mL of algal suspension in growth phase at pH adjusted to 7 were centrifuged at 3000 g and resuspended in culture medium to concentrate them and obtain a range of concentrations for each algae. After centrifugation, 160 µL of algae were put in contact with 40 µL of Diuron at a final concentration of 100 µg/L or with ultra-pure water for control. After respectively 5 min (*C. vulgaris*), 20 min (*P. subcapitata*) and 10 min (*C. reinhardtii*), a-chl fluorescence was red (with an arbitrary light excitation intensity corresponding to a LED current set to 100 mA) after microalgae insertion in a microfluidic chip, and exposed algae fluorescence enhancement was calculated using the following equation:

$$FE (\%) = \frac{F_s - F_c}{F_c} * 100$$

Were FE is the fluorescence enhancement (in %), F_s the maximum fluorescence signal acquired for algae exposed to toxicant (in Volts), F_c the maximum fluorescence signal for control algae (in Volts).

2.6.3 Light intensity

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

2.7. Performance of the biosensor

Using light excitation and algal concentration optimized parameters, biosensor performance and reliability was assessed exposing microalgae strains to a dilution range of Diuron pesticide (1, 10, 100 and 1000 µg/L). LOD (limits of detection) and EC₅₀ values were calculated using calculated using the Hill probit model (Regtox[®], E. Vindimian, <http://normalesup.org/~vindimian/>). The pesticide effects on microalgae is well referenced and dose-response curves obtained in this study have been compared to the data available from the scientific literature to validate sensitivity of our dispositive (e.g. in term of detection limits and EC₅₀ values).

2.8 Application to real urban polluted samples

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

3. Results and discussion

3.1 Construction and characterization of the properties of the portative station

The development of this algal biosensor required the construction of a special fluorimeter dedicated to the lecture of algae inserted in disposable microfluidic chips. The first step was to ensure of the correct calibration and the stability of the fluorescence signal provided without algae for different light intensity. Light intensity was controlled by the software which can deliver a current ranged between 0 and 100 mA. These data are presented in Figures S2 and S3. Figure S2 shows that despite the excitation light is filtered, a small background signal is still detected. It is also showed that a slight decrease of the signal over time is 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 investigated further. Indeed, 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 increase slightly. As the slope of the signal also increase with the led intensity, one can hypothesize that this phenomenon is due

to a small fluorescence coming from the chip (probably from the scotch tape). Nevertheless, and one again because of the differential measurement protocol, this effect is negligible.

3.2 Characterization and validation of xurography microfluidic chips for algal entrapment

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

3.3 Algal response, concentration and light intensity parameters optimization

Algal autofluorescence response along the recording time, depending of the algal concentration in the microfluidic chips, is shown in Figure 4A. Our data are in accordance to those obtained by Naessens et al. (2000) with *Chlorella vulgaris*. As regards the biosensor optimization, linearity between algal concentration of the three strains and the emitted A-chlorophyll fluorescence was firstly ensured before to ensure optimal algal concentration and light intensity. Results are presented in Figure 4B. As already discussed, algal concentrations and light intensity in contact with algae can strongly impact the sensitivity of algal biosensors (El-Ali et al., 2006, Tsopela et al., 2016). To assess the best condition for each of the two parameters, we exposed algae to the Diuron. This herbicide is known to inhibits the photosystem II (PSII) normal functioning by binding with an important affinity to the Quinone B (Q_B) binding site of the D1 protein of the PSII complex, that lead to stop 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 with electron transfer inside photosynthetic chain), excess energy is partly re-emitted by a light-chlorophyll fluorescence (Maxwell and Johnson, 2000). Finally, in presence of Diuron, energy can not be transferred into photosynthetic chain. Therefore, a more important energy dissipation by A-chlorophyll fluorescence appear compared to “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 collected helped to the software is presented in Figure S4.

In addition to the experimental validation of xurography microfluidic chips for algal entrapment, algal concentration and light intensity ranges were studied exposing algae, or not (control), at the same concentration of herbicide (100 µg/L) for each studied condition. As presented in Figure 4C and 4D, the best concentrations obtained for each algal suspension were respectively 3.10⁸, 3.10⁸ and 1.10⁸ cell/mL

for *P. subcapitata*, *C. vulgaris* and *C. reinhardtii*, leading to the maximum fluorescence enhancement. At higher concentration, a reduction of the percentage of enhancement appeared. This decrease can be explained by two assumptions: first, a reabsorption of the light emitted by neighbor cells (Védrine et al., 2003) can decrease the signal measured by photodetectors; secondly, the too high algal cell concentrations lead to a number of possible binding sites by the pesticide too high compared to the number of sites that potentially can be affected (Deblois et al., 2013; Tsopela et al., 2016), and consequently, effect be masked due to the already high fluorescence 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 80 mA condition. This result can be interpreted as a photoinhibition of the photosynthesis metabolism. When a too high quantity of photons is projected on algae, a saturation of the photosystems by the photons appeared and a decrease of the photosynthetic activity is 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 mask toxic effects, as demonstrated by Camuel et al. (2017) with *C. vulgaris* exposed to Atrazine and Diuron.



Ps

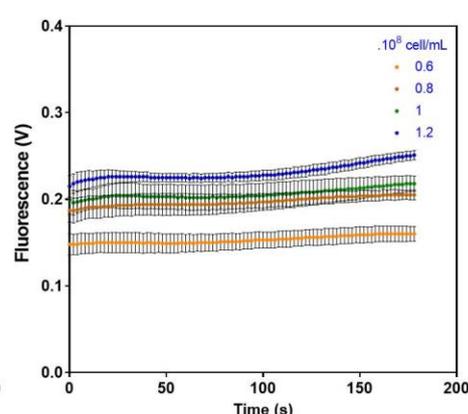
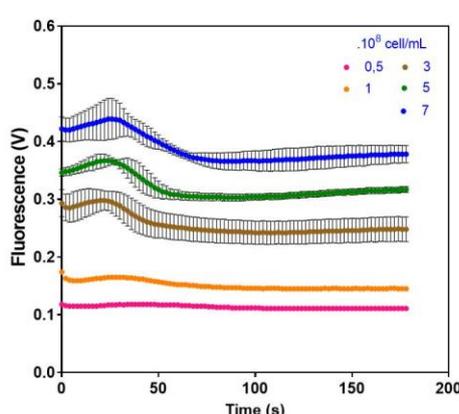
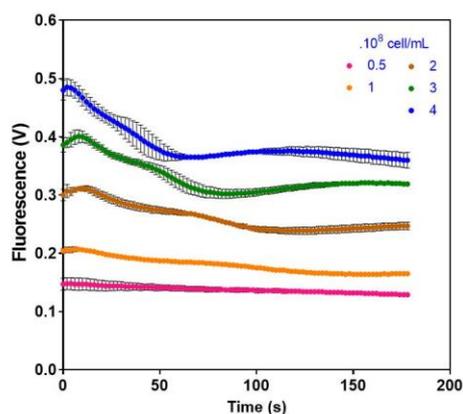


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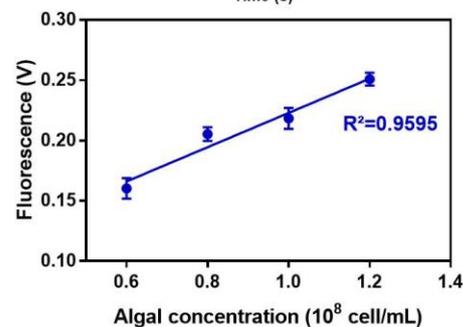
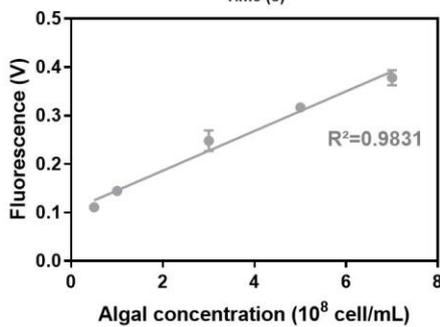
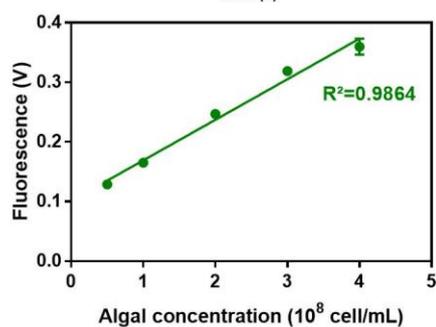


Cr

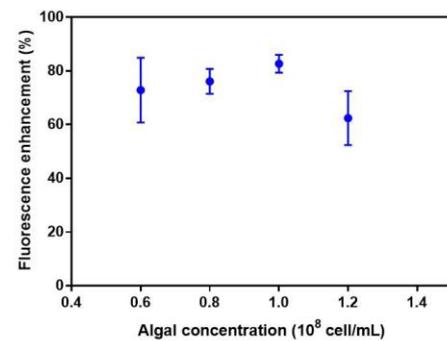
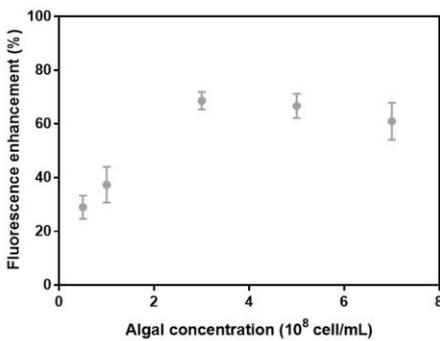
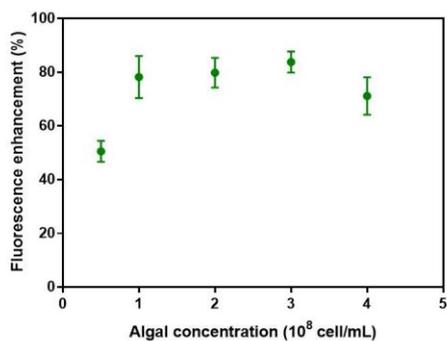
A



B



C



D

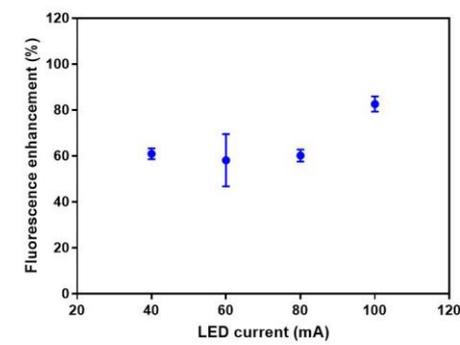
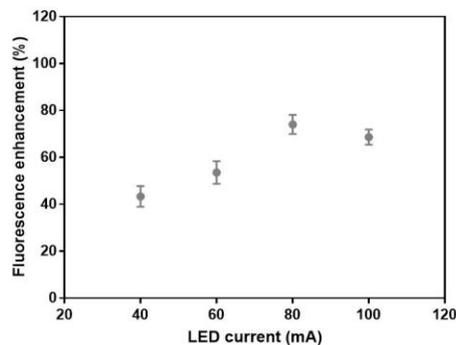
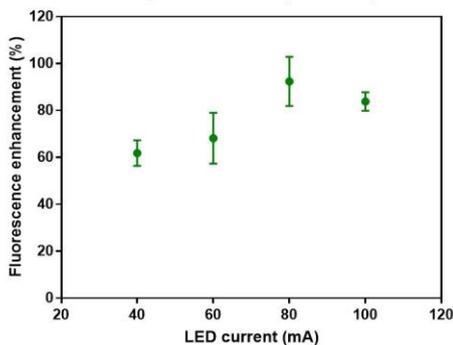


Figure 4: Algal chlorophyll fluorescence parameters optimization: Algal fluorescence response of the three algae along the time measured with the software (A), relation between chlorophyll fluorescence and the algal concentration (B), relation between algal concentration and chlorophyll fluorescence enhancement after exposure to Diuron (100 $\mu\text{g/L}$) (C), relation between light intensity and chlorophyll fluorescence enhancement after exposure to Diuron (100 $\mu\text{g/L}$) (D). *Ps* : *P. subcapitata* ; *Cv* : *C. vulgaris* ; *Cr* : *C. reinhardtii* (mean \pm S.D., N = 3).

3.4 Biosensor sensitivity evaluation

To validate the sensitivity of our biosensor comparatively to others described in the literature, Diuron dose-response curve have been determined, using for each algae the optimum parameters previously defined. Results are presented in Figure 5. All three algae appeared to be sensitive to Diuron exposure, leading to important fluorescence increase at high concentration (100 $\mu\text{g/L}$ and 1 mg/L). In this configuration, *C. reinhardtii* was the most sensitive algae comparing limits of detection (LOD) and EC_{50} data for Diuron (Table S3). It is important to specify that variability observed in the dose-response curves with biosensors is due to different sources of errors: technical operating errors, xurography-based

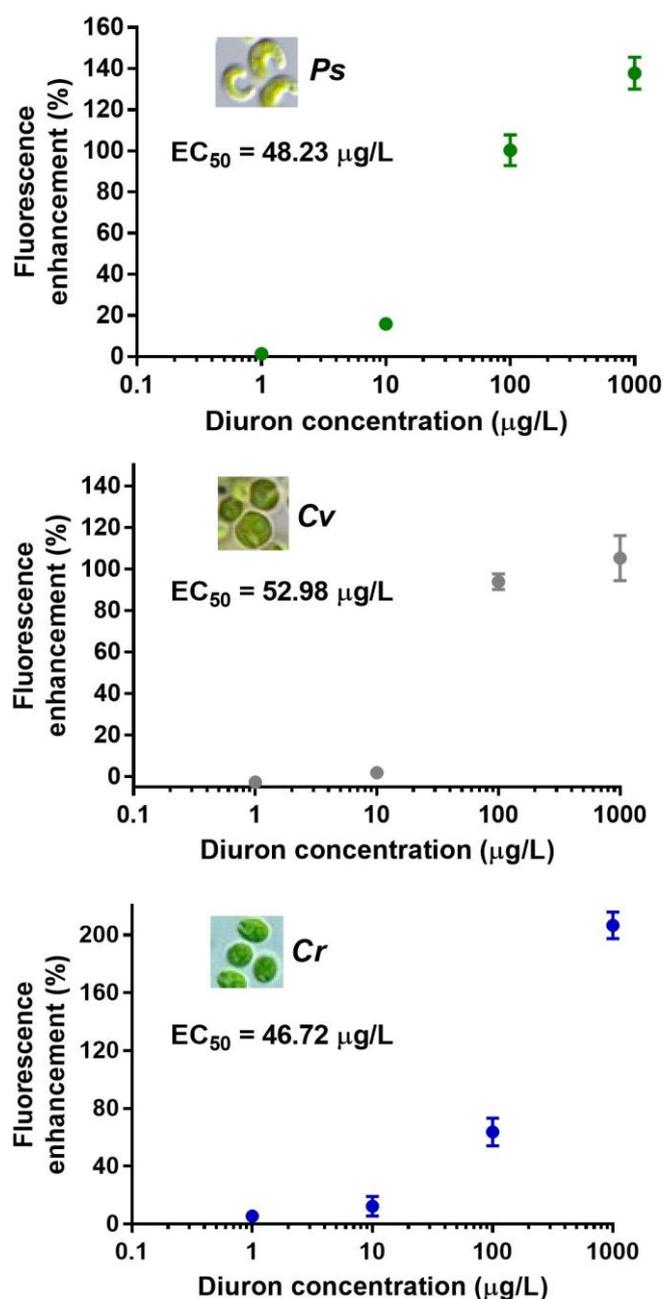


Figure 5: Biosensor dose-response curves for algae exposed to the Diuron. *Ps* : *P. subcapitata* ; *Cv* : *C. vulgaris* ; *Cr* : *C. reinhardtii* (mean \pm S.D., N = 3).

microfluidic chip construction precision and algal insertion potential problem inside the chip, negligible (as observed before), and position of the chip into the fluorimeter. Despite all these potentially sources of errors, the variability was relatively limited and led to a sensitive biosensor.

Our results were confronted to 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 (Shitenda 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 than our device. Nonetheless, Lefevre et al. (2012) manufactured PDMS-based microfluidic biosensor containing and integrated system of excitation/detection composed of organic light-emitting diode (OLED) and photodetectors (OPD) placed directly underneath and overhead the microfluidic channels. Then, they obtained very low EC₅₀ compared to this study. Plausible explanation is a lower distance between the algae and the optical components (due to flexibility of OLED and OPD) and the better sensitivity of OPD compared to commercial photodetectors. As already evocated in part 3.3, another explanation reside in a lower algal concentration used by Lefevre et al. (2012), leading to a number of possible binding sites by the pesticide more important compared to the present configuration, and so to lower EC₅₀ values.

3.5 Application to real environmental samples

Our biosensor was applied to 10 real environmental samples (UWWD, receiving water, municipal and hospital wastewaters) collected in different sites of the Rhône Valley (France). Algal strains responses are illustrated in Figure 6. Toxic effects were observed for a large part of the samples, depending of the algae exposed, with percentage of fluorescence enhancement until $29.22 \% \pm 2.83$ for *P. subcapitata* exposed to the hospital wastewater A. Compared to the previous experiments with pure pesticide molecule where only fluorescence increase was determined, enhancement or decrease of fluorescence were observed depending of the sample. Fluorescence decrease after pollutant exposure was widely described in literature. Several pollutant families impact other sites of the photosynthetic chain than the Quinone B (Suresh Kumar et al., 2014), as Photosystem I (paraquat) (Fai et al., 2007) or ATPase proteins. These interactions with photosynthetic systems, can so lead to a decrease of algal a-chlorophyll fluorescence, as for example observed with algae (*P. subcapitata* and *C. vulgaris*) exposed to pollutants detected in our environmental samples such as pharmaceuticals (Ciprofloxacin and Sulfamethoxazole) (Liu et al., 2011) or Glyphosate (Naessens et al., 2000). Consequently, toxic effects observed in our case result of algal exposure to very complex mixtures of micropollutants with additives, synergic or antagonism effects of algal photosynthetic apparatus and so chlorophyll fluorescence disturbance. It's why biosensors are particularly relevant for an early detection toxic impact of urban

samples but would not replace physico-chemical laboratory analyses of substances that are likely sources of toxicity.

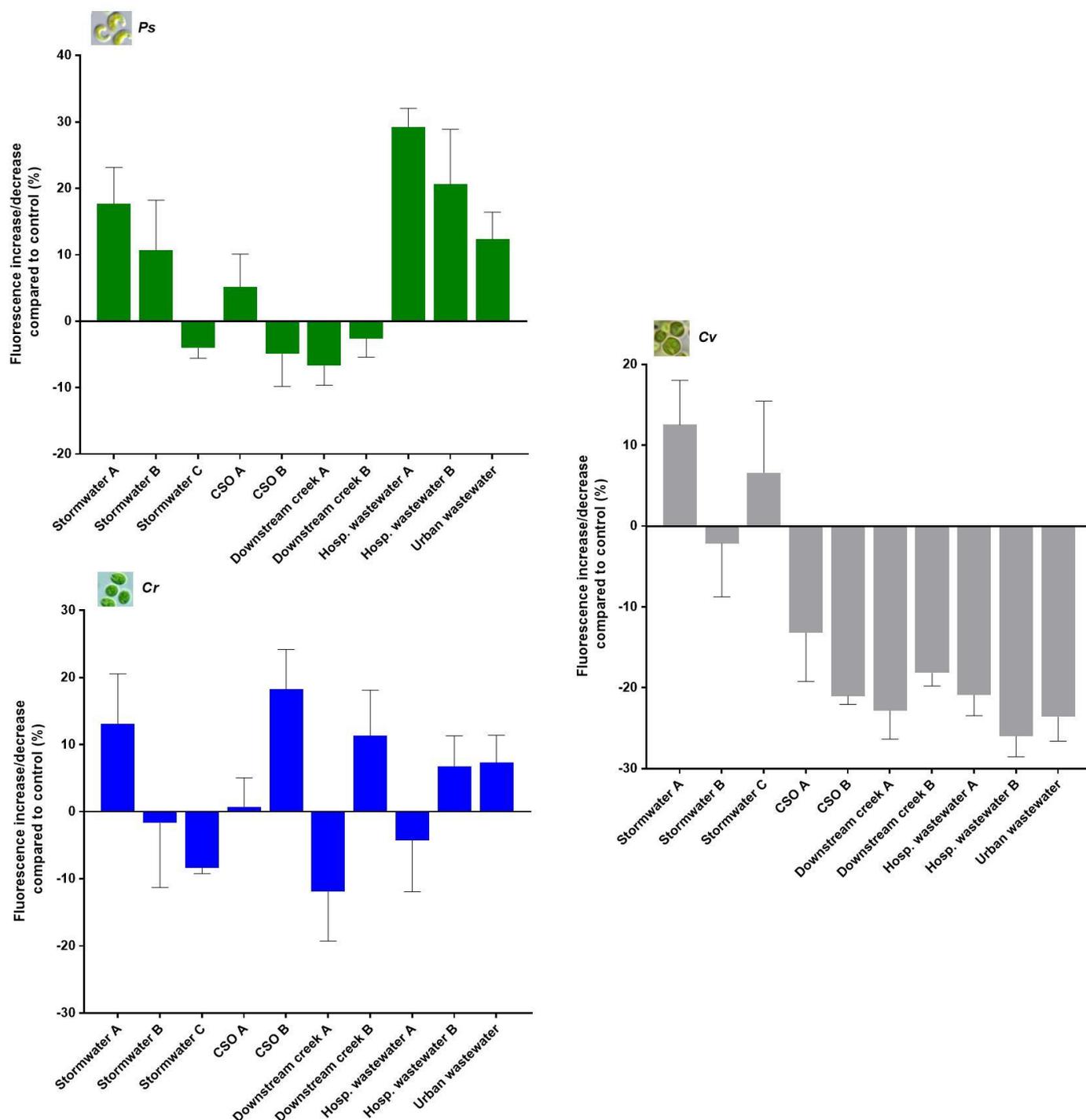


Figure 6: Biosensor response for *P. subcapitata* (Ps), *C. vulgaris* (Cv) and *C. reinhardtii* (Cr) algae exposed to the 10 environmental samples (mean \pm S.D., N = 3).

The investigation also evidenced the relevance of using three different algal strains. Indeed, algal response varied considerably, depending of the sample. For instance, hospital wastewater B sample led to a 20.60 % \pm 8.27 chlorophyll fluorescence increase of *P. subcapitata*, whereas it generated 26.00

% \pm 2.53 of decrease of *C. reinhardtii* fluorescence compared to control. The difference between the three algae strain's 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 or pharmaceuticals (Takamura et al., 1989; Magnusson et al., 2010; Brezovsek et al., 2014).

Finally, to our knowledge, only two other photosynthesis disturbance algal biosensors were currently applied to environmental samples (Buckova et al., 2017; Durrieu et al., 2016). In their 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 stormwater samples toxicity. Algal sensors are sufficiently sensitive to detect the toxicity of polluted effluents. However, a limit remains and should be pointed out in these two studies. These one detected significant disturbances after 12 h and 24 h exposure. With the biosensor proposed in this study, 20 minutes' maximum were necessary to detect a toxicity effect.

Meanwhile, these algal biosensors are not relevant to identify the source of toxicity, and then required in-depth chemical analyses in laboratory. Algal biosensors constitute only "early warning systems" for toxic conditions detection but with no analytical capability. Nevertheless, these biosensors present some advantages compared to conventional chemical analyzes: (i) The ecotoxicological effects observed with biosensors are taking into account all pollutants present in discharges, when physico-chemical analyses can only find what they are researching and can so forget hundreds of ecotoxic molecules; (ii) The bioavailability of all the micropollutants for the algae is considered (interactions between chemicals and particles sorption/desorption phenomenon) (Becouze-Lareure et al., 2016; Fahl et al., 1995).

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 tap freshwaters. This device, able to be used *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 constraints technique, the xurography, and transparent and inert materials (glass slides). Simple two-entries (control and exposed) chips were constructed, using capillarity phenomenon to insert microalgae inside. Three microalgae strains were used (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*) because of their extensive literature information and their difference of sensitivity for environmental pollutants. Fluorimeter and microfluidic chips were characterized 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, the Diuron, as a model. We demonstrated that

variation in these two parameters could change considerably the algal response to pollutants. The sensitivity of our device was successfully demonstrated doing dose-response curves with different Diuron concentrations and comparing data with literature. Different sensitivities were observed depending of the algae, with a best one for *C. reinhardtii* (lowest LOD and EC₅₀). Finally, the station was applied to real environmental samples, such as urban stormwaters, effluents or contaminated rivers, and toxic effect were detected after short exposure times (≤ 20 min). The necessity to developed multi-species sensors was demonstrated by the fact that significant differences of response to urban samples were observed depending of the algae species. Nevertheless, in our case, a high algal concentration could be a source of decrease of sensitivity with low concentration of pollutants in samples. Futures investigations are necessary to study lower algal concentration exposure. Further experiments are also required to develop multi-channel chips and detection system to analyze replicates in parallel and decrease analysis time.

Acknowledgments

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Supplementary data

Table S1: Results of the physico-chemical analyses performed on the 7 urban wet-weather discharges and contaminated creek samples, following the methods described in Gosset et al. (*in press*).

Parameter	Stormwater A	Stormwater B	Stormwater C	CSO A	CSO B	Downstream creek A	Downstream creek B
Date	13/04/2016	02/06/2016	02/06/2016	09/05/2016	19/05/2016	09/05/2016	19/05/2016
Location	Chassieu	IUT- La Doua (Vileurbanne)	Miribel	Grezieux-la-Varenne	Grezieux-la-Varenne	Grezieux-la-Varenne	Grezieux-la-Varenne
pH	7.66	7.28	7.56	7.93	7.1	7.72	8.31
Conductivity (µS/cm)	156.3	66.7	213	285	255.5	176.7	309
TSS (mg/L)	207.28	13.5	46.52	164.55	467.3	131	26.6
DOC (mg/L)	5.01	2.21	2.91	11.7	15.8	4.3	15
Na ⁺ (mg/L)	7.72	0.45	0.62	16.77	16.02	9.91	18.67
NH ₄ ⁺ (mg/L)	N.D.	N.D.	N.D.	13.69	7.33	N.D.	0.11
K ⁺ (mg/L)	1.24	1.57	3.26	4.91	4.56	3.3	5.1
Mg ²⁺ (mg/L)	1.38	0.22	0.59	1.9	2.02	2.38	4.89
Ca ²⁺ (mg/L)	25.44	9.47	26.76	24.72	27.12	24.18	41.05
Cl ⁻ (mg/L)	10.37	0.49	0.86	20.82	19.9	13.34	28.27
NO ₂ ⁻ (mg/L)	N.D.	N.D.	N.D.	0.34	0.67	N.D.	N.D.
NO ₃ ²⁻ (mg/L)	9.07	N.D.	2.44	5.6	4.16	3.81	10.73
PO ₄ ³⁻ (mg/L)	N.D.	N.D.	0.43	2.84	5.39	N.D.	N.D.
SO ₄ ²⁻ (mg/L)	8.77	1.02	1.24	15.77	14.5	11.78	24.07
<u>Pesticides (µg/L)</u>							
Glyphosate	0.88	0.743	0.605	13.7	1.2	1.518	1.19
AMPA	N.D.	1.813	0.683	1.775	0.595	0.545	N.D.
Fosetyl-aluminium	N.D.	N.D.	0.808	N.D.	0.768	N.D.	1.358
Diuron	N.D.	N.D.	N.D.	N.D.	2.46	N.D.	N.D.
2.4.6-Trichlorophenol	N.D.	N.D.	N.D.	N.D.	0.125	N.D.	N.D.
<u>Alkylphenols (µg/L)</u>							
Nonylphenol	N.D.	N.D.	N.D.	0.149	0.191	N.D.	N.D.
<u>Heavy metals (µg/L)</u>							
Cadmium	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Chromium	6.38	0.18	0.28	0.4	0.26	0.3	0.3
Copper	10.82	6.55	2.09	7.06	10.61	6.36	9.17
Lead	N.D.	N.D.	N.D.	N.D.	0.45	N.D.	N.D.
Iron	N.D.	13.38	18.67	N.D.	N.D.	N.D.	N.D.
Nickel	2.2	1.88	0.84	0.683	0.5	1.06	0.72
<u>Others (µg/L)</u>							
Tributyl phosphate	N.D.	N.D.	N.D.	0.102	0.416	N.D.	N.D.

CSO: Combined sewer overflow. N.D.: Not Detected. All of the organic compounds detected in the 7 samples are detailed in Gosset et al. (*in press*).

Table S2: Results of the physico-chemical analyses performed on the 3 urban and hospital effluents following the methods described by Perrodin et al. (2016).

Parameter	Unit	Urban wastewater	Hospital wastewater A	Hospital wastewater B	
<u>Routine physico-chemical parameters</u>					
pH	-	8.4	8.1	8	
Conductivity	µS/cm	1273	2433	2879	
BOD ₅	mg/L	188	325	294	
COD (Chemical Oxygen Demand)	mg/L	477	692	611	
DOC (Dissolved Organic Carbon)	mg/L	40	NM	55.9	
TOC (Total Organic Carbon)	mg/L	74.3	NM	143	
TSS (Total Suspended Solids)	mg/L	260	212	200	
NH ₄ ⁺	mg/L	61.6	46.8	42.6	
NO ₂ ⁻	mg/L	0.016	0.0711	0.0264	
NO ₃ ⁻	mg/L	0.17	<LQ	0.38	
PO ₄ ³⁻	mg/L	4.16	6.48	4.82	
TKN	mg/L	81.9	61.4	59.2	
Total phosphorous	mg/L	9.23	11.6	8.45	
Sulphides	mg/L	<LQ	NM	NM	
Sulphates	mg/L	17.5	NM	NM	
AOX (Adsorbable Organically Halides)	mg/L	0.04	0.91	0.12	
<u>Metals</u>					
Arsenic	µg/L	<LD (<LQ)	<LQ (<LD)	<LQ (<LQ)	
Cadmium	µg/L	<LD (<LQ)	<LQ (<LD)	<LQ (<LQ)	
Chrome	µg/L	<LD (4)	0.5 (3)	0.5 (2)	
Copper	µg/L	<LD (58)	18 (67)	5 (70)	
Gadolinium	µg/L	<LD (<LQ)	1 (2)	1 (2)	
Lead	µg/L	<LD (5)	<LQ (4)	<LQ (3)	
Mercury	µg/L	<LD (<LQ)	<LQ (<LQ)	<LQ (<LQ)	
Nickel	µg/L	<LD (<LQ)	<LQ (<LQ)	<LQ (<LQ)	
Zinc	µg/L	15 (150)	19 (88)	10 (87)	
<u>Organic pollutants (dissolved fraction)</u>					
<i>Family</i>	<i>Name</i>				
Analgesics	Paracetamol	ng/L	180500	831000	1005500
Antibiotics	Ciprofloxacin	ng/L	<LQ	31300	17650
Antibiotics	N4-acetyl-sulfaméthoxazole (SMX impurity A)	ng/L	857.5	16550	7365
Antibiotics	4-Amino-N-(3-methyl-5-isoxazolyl)benzenesulfonamide (SMX impurity F)	ng/L	<LQ	2570	4440
Antibiotics	Sulfamethoxazole B-D-Glucuronide (SMX glucuronide)	ng/L	6.67	14700	8945
Antibiotics	Vancomycin	ng/L	<LQ	301.5	1150
Anticonvulsants	Carbamazepine	ng/L	505	574	542
Anticonvulsants	Sulfamethoxazole	ng/L	399	16550	4345
Anti-inflammatory drugs	1.3-Dihydro-2H-indol-2-one (DCF impurity E)	ng/L	39250	11150	51900
Anti-inflammatory drugs	4-hydroxy diclofénac (DCF 4HO)	ng/L	2675	2585	332.5
Anti-inflammatory drugs	5-hydroxy diclofénac (DCF 5HO)	ng/L	31350	1970	943.5
Anti-inflammatory drugs	Diclofenac	ng/L	1276	339	93.65
Anti-inflammatory drugs	Ibuprofen	ng/L	12250	11000	12500
Anti-inflammatory drugs	Ketoprofen	ng/L	2900	23950	9645
Beta-Blockers	Atenolol	ng/L	952.5	5110	6060
Beta-Blockers	Propranolol	ng/L	307	236.5	371
Others	Salicylic acid	ng/L	2690	44750	18750
Personal care products	Cetyl betain	µg/L	2.035	<LQ	<LQ
Personal care products	Incromine SD	µg/L	6.81	<LQ	<LQ
Personal care products	Stepanquat GA 90	µg/L	130.5	<LQ	<LQ
Surfactants	Anionic surfactant	mg/L	0.63	0.3	<LQ
Surfactants	Benzyltrimethyldecylammonium chloride (BDDAC)	µg/L	18.3	<LQ	<LQ
Surfactants	Benzyltrimethyltetradecylammonium chloride (BDTAC)	µg/L	9.585	<LQ	<LQ

Surfactants	Cationic surfactant	mg/L	<LQ	0.9	0.1
Surfactants	Comperlan 100	µg/L	0.00833	<LQ	<LQ
Surfactants	Sodium decylbenzenesulphonate C10	µg/L	1195	<LQ	<LQ
Surfactants	Sodium decylbenzenesulphonate C11	µg/L	825.5	<LQ	<LQ
Surfactants	Sodium decylbenzenesulphonate C12	µg/L	883	<LQ	<LQ
Surfactants	Sodium decylbenzenesulphonate C13	µg/L	876	<LQ	<LQ
Surfactants	Sodium dodecyl sulfate	µg/L	0.0833	<LQ	<LQ
Surfactants	Triton X100	µg/L	0.0167	<LQ	<LQ
Synthesis intermediates and solvents	Sodium 2-ethylhexyl sulfate	µg/L	0.000833	<LQ	<LQ
Synthesis intermediates and solvents	Texapon N701 S	µg/L	61	<LQ	<LQ
<u>Organic pollutants (whole sample)</u>					
	<i>Family</i>	<i>Name</i>			
Surfactants		4-tert-octylphenol	µg/L	0.005	<LQ
Surfactants		Non-ionic surfactant	mg/L	1.3	0.1
Surfactants		Nonylphénol diethoxylate (NP2OE)	µg/L	0.18	<LQ
Surfactants		Nonylphenol monoethoxylate (NP1OE)	µg/L	0.15	<LQ
Synthesis intermediates and solvents		Chloroform	µg/L	0.089	1.5
Synthesis intermediates and solvents		Toluene	µg/L	0.25	<LQ
Synthesis intermediates and solvents		Xylenes	µg/L	<LQ	0.64

LD : Limit of Detection ; LQ : Limit of Quantification ; NM : Not Measured ;

Regarding metal analyses, data correspond to dissolved fraction and whole sample (in brackets).

List of the metals and organic compounds sought in the effluents : [2-[(2,6-Dichlorophenyl)amino]phenyl]methanol (DCF impurity C) ; 1-(2,6-Dichlorophenyl)-1,3-dihydro-2H-indol-2-one (DCF lactam) ; 1,1,1-trichloroethane ; 1,1,2,2-tetrachloroethane ; 1,1,2-trichloroethane ; 1,1,2-trichlorotrifluoroethane ; 1,1-dichloroethane ; 1,1-dichloroethylene ; 1,2,3-trimethylbenzene ; 1,2,4-trimethylbenzene ; 1,2-dibromoethane ; 1,2-dichlorobenzene ; 1,2-dichloroethane ; 1,2-dichloroethylene (cis) ; 1,2-dichloroethylene (trans) ; 1,2-dichloropropane ; 1,3,5-trimethylbenzene ; 1,3-dichlorobenzene ; 1,3-Dihydro-2H-indol-2-one (DCF impurity E) ; 1,4-dichlorobenzene ; 2,3-dichloropropene ; 2-[(2,6-Dichlorophenyl)amino]benzaldehyde (DCF impurity B) ; 2-[(2-chlorophenyl)amino] benzaldehyde (DCF CPAB) ; 2-chlorotoluene ; 3-chloropropene ; 3-chlorotoluene ; 4-Amino-N-(3-methyl-5-isoxazolyl)benzenesulfonamide (SMX impurity F) ; 4-chlorotoluene ; 4-hydroxy diclofénac (DCF 4HO) ; 4-isopropyltoluene (cymene) ; 4-Nitro-sulfaméthoxazole (SMX-NO₂) ; 4-n-nonylphenol ; 4-tert-octylphenol ; 5-hydroxy diclofénac (DCF 5HO) ; Anionic surfactant ; Arsenic ; Atenolol ; Aztreonam ; Benzene ; Benzotriazole ; Benzylidimethyldodecylammonium chloride (BDDAC) ; Benzylidimethyltetradecylammonium chloride (BDTAC) ; Bromochloromethane ; Bromoforme ; Cadmium ; Carbamazepine ; Carbon tetrachloride ; Cationic surfactant ; Cetyl betain ; Chloroform ; Chrome ; Ciprofloxacin ; Cis-1,3-dichloropropylene ; Comperlan 100 ; Copper ; Dibromochloromethane ; Dichlorobromomethane ; Dichloromethane ; Diclofenac ; Diclofénac acyl-B-D-glucuronide (DCF glucuronide) ; Diclofénac carboxylic acid (DCF COOH) ; Econazole ; Ethinylestradiol ; Ethylbenzene ; Gadolinium ; Hexachloroethane ; Ibuprofen ; Incromine SD ; Isopropylbenzene (cumene) ; Ketoprofen ; Lauryl pyridinium chloride ; Lead ; Mercury ; Meropenem ; Methyl chloride ; Methyl tert-butyl ether (MTBE) ; Monochlorobenzene ; N4-acetyl-sulfaméthoxazole (SMX impurity A) ; n-butylbenzene ; Nickel ; Non-ionic surfactant ; Nonylphénol diethoxylate (NP2OE) ; Nonylphenol monoethoxylate (NP1OE) ; Paracetamol ; Propranolol ; Salicylic acid ; Sodium 2-ethylhexyl sulfate ; Sodium decylbenzenesulphonate C10 ; Sodium decylbenzenesulphonate C11 ; Sodium decylbenzenesulphonate C12 ; Sodium decylbenzenesulphonate C13 ; Sodium dodecyl sulfate ; Stepanquat GA 90 ; Styrene ; Sulfamethoxazole ; Sulfaméthoxazole B-D-Glucuronide (SMX glucuronide) ; Tert-butylbenzene ; Tetrachloroethylene ; Texapon N701 S ; Toluene ; Trans-1,3-dichloropropylene ; Trichloroethylene ; Trichlorofluoromethane ; Triton X100 ; Vancomycin ; Vinyl chloride ; Xylenes ; Zinc

Table S3: Comparative sensitivity of the biosensor developed in this project to literature studies (X: No data)

Algal specie	“Immobilization” method	Observed parameter	Exposition procedure	Detected herbicide	Detection limit (µg/L)	EC ₅₀ (µg/L)	Authors
<i>C. vulgaris</i>	Silica hydrogel	Chlorophyll fluorescence	Continuous flow	Diuron	1	X	Nguyen-Ngoc, H. and Tran-Minh (2007)
<i>C. vulgaris</i>	Serigraphic impression on electrode	Oxygen production variation	Static	Diuron	X	233,65	Shitanda et al. (2009)
<i>C. reinhardtii</i>	Microfluidic	Chlorophyll fluorescence	Continuous flow	Diuron	X	2.56	Lefèvre et al. (2012)
<i>C. reinhardtii</i>	Commercial system Handy-PEA	Chlorophyll fluorescence	Static	Diuron	X	51,26	Lefèvre et al. (2012)
<i>C. reinhardtii</i>	Microfluidic	Oxygen production variation	Static	Diuron	46,73	X	Tsopela et al. (2014)
<i>C. reinhardtii</i>	Microfluidic	Oxygen production variation	Static	Diuron	23,3	X	Tsopela et al. (2016)
<i>C. reinhardtii</i>	Microfluidic	pH variation	Continuous flow	Diuron	11,65	X	Tahirbegi et al. (2017)
<i>C. reinhardtii</i>	Microfluidic	Oxygen production variation	Continuous flow	Diuron	11,65	X	Tahirbegi et al. (2017)
<i>C. reinhardtii</i>	Microfluidic	Chlorophyll fluorescence	Continuous flow	Diuron	11,65	> 46,6	Tahirbegi et al. (2017)
<i>P. subcapitata</i>	Microfluidic	Chlorophyll fluorescence	Static	Diuron	1	48.23	Our study
<i>C. vulgaris</i>	Microfluidic	Chlorophyll fluorescence	Static	Diuron	10	52.98	Our study
<i>C. reinhardtii</i>	Microfluidic	Chlorophyll fluorescence	Static	Diuron	1	46.72	Our study

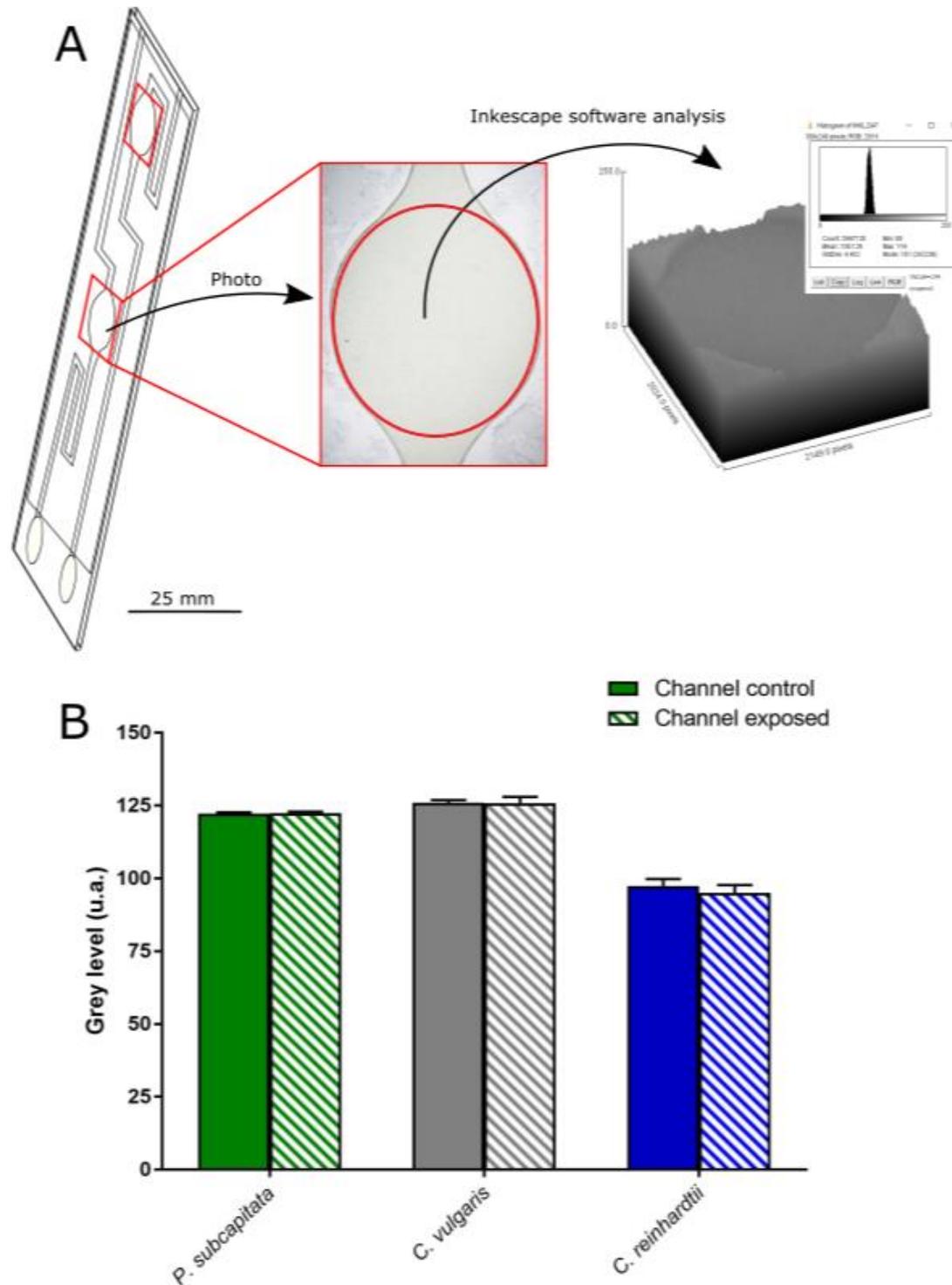


Figure S1: Evaluation of algal concentration filling into microfluidic chips for control and exposed channels (A). Mean grey level into the control and exposed lecture chambers filled with the three microalgae (mean \pm S.D., N = 3).

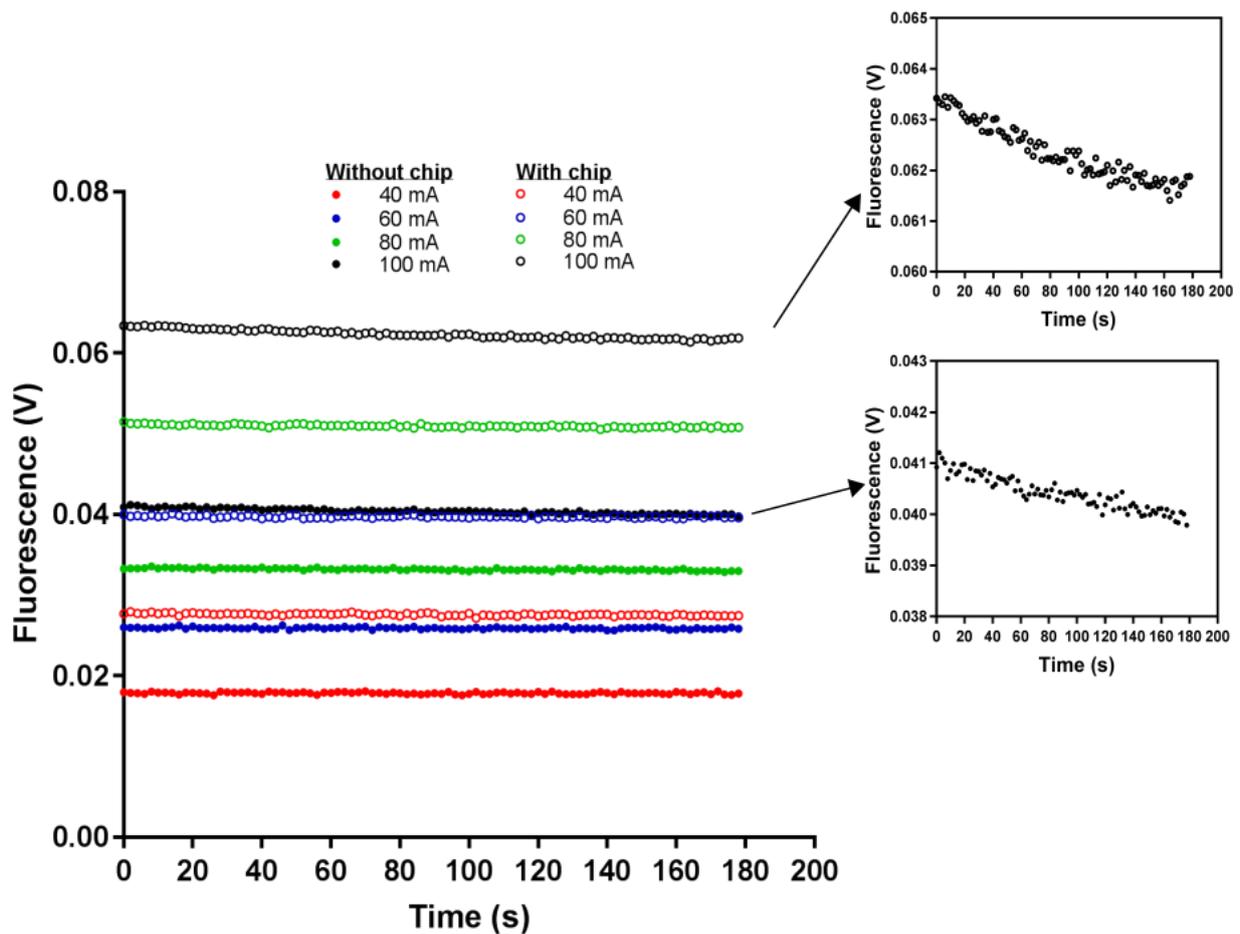


Figure S2: Fluorescence signal emission detected by photodetectors along the time without and with inserted microfluidic chip in the portable fluorimeter depending of LED current excitation.

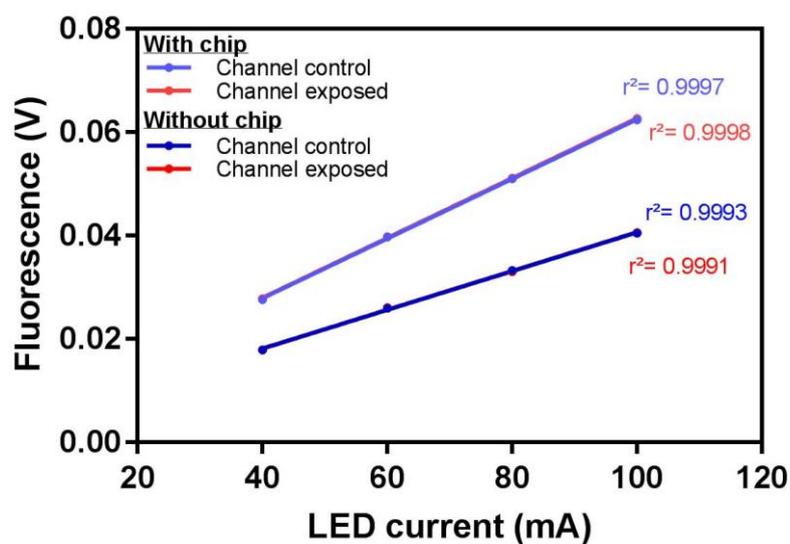


Figure S3: Relation between LED light excitation and fluorescence detection for the portable fluorimeter without and with inserted microfluidic chip (N=3).

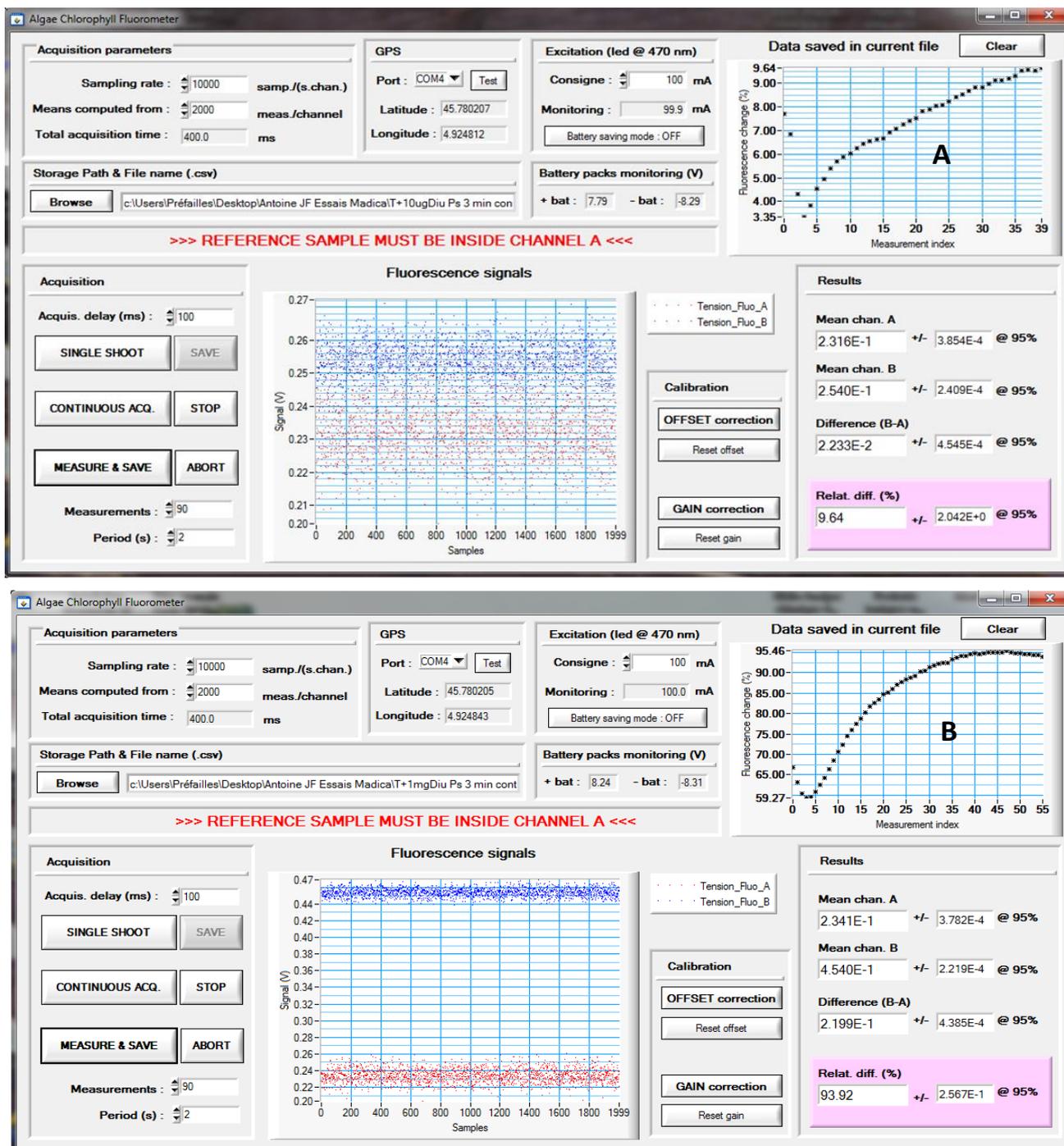


Figure S4: Example of fluorescence data acquisition with the dedicated software for *P. subcapitata* ($3 \cdot 10^8$ cell/mL, 100 mA LED current intensity) exposed to 10 $\mu\text{g/L}$ and 1 mg/L of Diuron.

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4.2.2.3 Synthèse et discussion

Nos travaux ont permis le développement et la validation d'un biocapteur original, basé sur la mesure de la fluorescence algale dans une cellule microfluidique. Ce biocapteur dédié à des mesures *in situ* ne nécessite pas d'alimentation électrique externe (fonctionnement sur batteries et sans pompe). Les résultats de cette étude ayant conduit à l'aboutissement de cette station portative résultent de : la création de puces microfluidiques par xurographie répétibles, l'optimisation des paramètres de mesures de la perturbation de la fluorescence chlorophyllienne des algues insérées dans les puces et le fluorimètre, la vérification de la sensibilité du capteur à l'aide d'une molécule modèle (le Diuron) et enfin l'application à des RUTP et eaux usées de station d'épuration. D'un coût de fabrication et d'usage très faible, nous avons pu créer un biocapteur qui présente une bonne sensibilité, comparativement aux biocapteurs décrits dans la littérature, avec des limites de détection du Diuron de l'ordre de 1 µg/L pour deux des 3 microalgues étudiées. Nous avons également mis en évidence l'intérêt d'utiliser plusieurs microalgues avec des réponses très différentes selon les algues et les rejets.

Toutefois, plusieurs voies d'amélioration de ce prototype sont envisagées afin de garantir sa fiabilité : augmenter la sensibilité du capteur (parvenir à baisser les concentrations en algues utilisées) et faciliter son utilisation sur le terrain (réussir à créer des puces, un fluorimètre et un logiciel permettant d'effectuer des mesures de plusieurs paramètres et sur plusieurs répliques à la fois).

5 Conclusion générale et perspectives

5.1 Conclusion générale

La pollution des rejets urbains par temps de pluie représente une pollution complexe et dont la toxicité est difficilement prévisible de par sa composition variable dans le temps (événements pluvieux) et l'espace (bassins versants). L'étude de l'impact de ces rejets est par conséquent difficile et il est nécessaire de développer des outils adaptés afin d'analyser leur toxicité. Afin de contribuer à la recherche d'une solution, ce travail s'est focalisé sur le développement et l'application de tests écotoxicologiques de laboratoire et de biocapteurs dédiés à la mesure *in situ*, en se basant principalement sur le compartiment des microalgues, à la base des chaînes trophiques.

Dans un premier temps, l'étude bibliographique menée sur les rejets urbains par temps de pluie nous a permis de mettre en avant plusieurs points :

- Tout d'abord, nous avons obtenu et publié la liste de l'ensemble des substances polluantes recherchées, à ce jour, dans les RUTP par la communauté scientifique, à savoir 207 molécules. 165 PNEC ont ainsi été établies, dont 52 que nous avons déterminées nous-même. Pour 47 de ces molécules, les données écotoxicologiques se sont avérées insuffisantes pour établir une PNEC. Le danger écotoxicologique des molécules retrouvées dans les RUTP a été caractérisé *via* le calcul de quotient de danger, prenant en compte la concentration des polluants dans les RUTP ainsi que leur écotoxicité. Finalement, 97 quotients de danger ont pu être calculés et les molécules les plus dangereuses ont ainsi pu être référencées (principalement des métaux lourds, médicaments et pesticides).

- Suite à cette première étude démontrant de manière chiffrée le danger écotoxicologique lié aux micropolluants présents dans les RUTP, nous nous sommes intéressés aux différentes méthodes actuellement utilisées pour détecter l'impact des RUTP sur la faune/flore des écosystèmes aquatiques receveurs, leurs avantages et limites. Ainsi, nous avons pu constater le faible nombre d'études disponibles, comparé à d'autres matrices telles que les eaux usées de station d'épuration. Leur étude est en effet complexe en raison du caractère épisodique et difficilement prédictible des événements pluvieux. De plus, un très faible nombre d'essais *in situ* a été développé, ce qui peut être expliqué par des expérimentations difficiles à concevoir pour les raisons évoquées à l'instant. L'utilisation de biocapteurs (par exemple algaux) peut donc être une bonne alternative à ces essais, comme outils d'alerte *in situ*.

Nous avons dans un second temps travaillé en laboratoire au développement d'une batterie de 4 biomarqueurs (perméabilité membranaire, fluorescence chlorophyllienne, activités enzymatiques estérases et phosphatases alcalines) sur une microalgue unicellulaire d'eau douce (*Chlorella vulgaris*). Ces biomarqueurs ont été appliqués à la campagne d'échantillonnage de 2015 de RUTP prélevés sur plusieurs sites de la région Lyonnaise, avec des typologies de bassins versants de ces sites très variés.

L'écotoxicité des rejets a ainsi pu être mise en évidence sur les microalgues après de courtes expositions (2 h et 24 h, les événements pluvieux dépassant rarement cette durée), et ce quel que soit le biomarqueur utilisé. Les analyses chimiques ont confirmé la présence de concentrations significatives de micropolluants tels que des pesticides (glyphosate ou encore Fosetyl-aluminium), des métaux lourds (Cu^{2+} ou Zn^{2+}) ou encore d'autres composés organiques tels que le nonylphénol pouvant expliquer l'écotoxicité mesurée. Toutefois, il est important de noter une difficulté liée à l'étude des activités enzymatiques, et la présence de bactéries dans les RUTP pouvant interférer avec les substrats utilisés pour les essais.

En comparaison, une batterie de bioessais monospécifiques a été testée sur les mêmes échantillons prélevés. Les bioessais mis en oeuvre suivent des lignes directrices européennes, et sont couramment utilisés en écotoxicologie, notamment pour étudier les RUTP et les eaux usées. Cette batterie de bioessais est composée des essais d'inhibition de la mobilité de *Daphnia magna*, et d'inhibition de la croissance de *Pseudokirchneriella subcapitata*, *Chlorella vulgaris* et *Heterocypis incongruens*. Ces organismes ont été sélectionnés car ils représentent différents niveaux trophiques. L'ensemble des essais réalisés s'est traduit par une absence de toxicité totale vis-à-vis des échantillons testés, pouvant être expliquée par leur manque de sensibilité, ou la présence de nutriments dans les rejets. Finalement, ce travail expérimental a permis de valider l'utilisation de ces biomarqueurs algaux pour l'étude des RUTP.

Nous avons également mené une analyse préliminaire sur le développement et l'utilisation de nouveaux biomarqueurs du stress oxydant pour l'étude des RUTP chez *Chlorella vulgaris*. Les premières expérimentations ont donné des résultats intéressants, et plus particulièrement ceux sur la peroxydation membranaire des microalgues (augmentation importante de la peroxydation pour deux des 7 échantillons testés).

Dans un troisième temps, nous avons travaillé à l'application de ces biomarqueurs algaux, afin de développer des biocapteurs algaux, à l'aide de trois espèces algales (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* et *Chlamydomonas reinhardtii*). Nous nous sommes essentiellement concentrés sur la fluorescence chlorophyllienne, le paramètre étudié le plus simple à adapter en biocapteur (aucun substrat ou produit chimique nécessaire, peu d'interférences dues aux RUTP ou autres rejets) et qui a montré des réponses significatives pour un nombre important d'échantillons testés.

Deux types de biocapteurs à fluorescence ont ainsi été développés avec pour objectif principal de réussir une mise en contact optimale des algues avec les polluants étudiés : un biocapteur à hydrogels (alginate/silice) et un biocapteur à puces microfluidiques. Chacun des types de biocapteurs développés au cours de cette étude possède ses avantages et inconvénients. En ce qui concerne les biocapteurs à hydrogels, ils autorisent un suivi continu dans le temps de la pollution des RUTP, avec des algues immobilisées dans un matériau de bonne qualité optique. Cependant, l'emprisonnement des microalgues dans des hydrogels d'alginate/silice conduit à la création d'une barrière physique défavorable au transfert des polluants et particules vers les microalgues, mais aussi l'adsorption de certaines substances

à la surface de ces gels. Cela peut entraîner une baisse de sensibilité de ces derniers, comme nous l'avons observé avec les trois polluants testés (Diuron, Atrazine et Isoproturon). En ce qui concerne les biocapteurs basés sur la microfluidique, cette technique a l'avantage d'éviter toute barrière physique entre les microalgues et les polluants. Quelle que soit la technique de mise en contact des microalgues testée dans notre étude avec les transducteurs, les deux biocapteurs présentent à l'heure actuelle deux principales limites. La première est que les particules en suspension dans les rejets testés doivent être éliminées pour éviter tout risque de colmatage des canaux fluidiques micrométriques dans lesquels les mélanges algues/rejets sont insérés. Une étape de décantation est donc nécessaire. De la même manière, afin de concevoir un système simple, autonome (ne nécessitant pas de branchements électriques externes, pompes) et robuste, la mesure en continu nous est rapidement apparue comme trop complexe pour être envisagée. Par conséquent, l'outil développé correspond à un « bioessai de terrain » permettant d'effectuer des campagnes de mesures sur différents sites de prélèvements sélectionnés (« approche semi-continue »). Comme évoqué par Guedri (2010), le déploiement sur le terrain des biocapteurs se heurte à des limites telles que l'automatisation ou la conservation/régénération des bio-récepteurs. Au cours de nos travaux, nous avons réussi à contourner ces difficultés. Grâce à la technique de préparation des puces microfluidiques par xurographie, nous avons été en mesure de développer un outil peu cher, portable, de réponse rapide et sensible (avec des limites de détection allant jusqu'à 1 µg/L pour des essais effectués avec une molécule modèle, le Diuron). Ce dispositif est utilisable sur le terrain afin d'effectuer des mesures *in situ* sur divers effluents liquides tels que les RUTP, les eaux usées ou les eaux de surfaces. Il s'agit là d'un premier bioessai de terrain, très utile pour les gestionnaires des eaux pluviales en termes d'outils de prévention de risques de pollution.

5.2 Perspectives

Suite aux résultats que nous avons obtenus, les perspectives qui découlent de nos expérimentations sont nombreuses. Les principales voies envisagées sont présentées ci-dessous.

5.2.1 Bioessais et biomarqueurs de laboratoire

Notre étude s'est axée sur 3 espèces de microalgues unicellulaires d'eau douce. L'utilisation de nouvelles espèces bioindicatrices de microalgues présente l'intérêt de pouvoir réaliser un « screening » plus large de l'impact des RUTP sur le compartiment microalgues. De plus, d'autres bioindicateurs tels que les levures (*saccharomyces cerevisiae*), faciles à cultiver et représentant un autre compartiment écosystémique (les décomposeurs), et qui possèdent des enzymes similaires aux microalgues (estérases, enzymes du stress oxydant) sont susceptibles d'être utilisés dans ce type de bioessai.

En ce qui concerne les bioessais écotoxicologiques sur microalgues, il serait intéressant de continuer à développer des biomarqueurs du stress oxydant. Les résultats présentés par Ferro (2013)

ainsi que ceux issus de nos travaux présentés dans ce mémoire confirment en effet que les biomarqueurs du stress oxydant sont de bons marqueurs précoces de toxicité de ces rejets sur les microalgues.

En termes de bioessais monospécifiques, les résultats obtenus durant notre travail n'ont montré aucun effet toxique des RUTP sur les espèces étudiées, qui semblent insuffisamment sensibles pour l'étude de ce type d'échantillons. Il est donc nécessaire de poursuivre la mise au point de ces bioessais afin d'essayer d'améliorer leur sensibilité. Par exemple, Angerville et al. (2013) ont récemment montré, à l'aide d'une batterie de bioessais appliquée à des échantillons de déversoirs d'orage, que l'essai d'inhibition de reproduction du microcrustacé *Ceriodaphnia dubia* donnait des résultats plus sensibles que les autres organismes aquatiques. Il serait donc pertinent de creuser cette piste (Annexe 5, Gosset et al., *in press*).

5.2.2 Choix des bio-récepteurs pour le développement de biocapteurs

En termes de biocapteurs, plusieurs voies de recherche s'ouvrent suite à cette étude :

Il faut rappeler que les eaux côtières sont, au même titre que les milieux dulçaquicoles, touchées de manière importante par les RUTP et les eaux usées. Ainsi, concernant le choix des bio-récepteurs, il pourrait être opportun de réaliser des investigations sur les microalgues marines telles que certaines chlorophycées (e.g. *Nephroselmis pyriformis* ou *Navicula* sp.) ou diatomées (e.g. *Skeletonema costatum* ou *Dunaliella tertiolecta*) (Macedo et al., 2008 ; Magnusson et al., 2008 ; Durrieu et al., 2011). Des études antérieures (en bioessais ou biocapteurs) ont présenté des résultats prometteurs, notamment vis-à-vis d'herbicides impactant la photosynthèse tels que le Diuron ou le Bentazon.

Il pourrait également être utile de travailler à d'autres échelles trophiques, en utilisant des organismes tels que des levures, des micro-crustacés ou des poissons. Des biomarqueurs similaires aux microalgues, telle que l'activité estérase, existent chez les levures et pourraient également être adaptés sous forme de biocapteurs. S'agissant des micro-crustacés, Perrulini et al. (2014) ont pour la première fois co-encapsulé des daphnies (*D. magna*) avec des microalgues (*P. subcapitata*) utilisant un procédé similaire à celui que nous avons employé (double-encapsulation). Ces organismes pourraient donc être déployés *in situ* pour la surveillance des eaux. Enfin, en ce qui concerne les poissons, des systèmes tels que des truitomètres (sorte d'aquarium contenant des truites très sensibles à la pollution) sont exploités, par exemple au sein de centres de gestion de l'eau potable, afin de surveiller la qualité de cette dernière. Cependant, avec la réglementation et la démarche éthique actuellement développée par l'Europe et l'Amérique du Nord, tendant à une diminution de l'utilisation des espèces animales, plusieurs équipes de recherches développent des biocapteurs de toxicité à partir de lignées cellulaires de poissons, comme alternative à la manipulation d'organismes entiers (cultures cellulaires de gonades de truite arc-en-ciel (*Oncorhynchus mykiss*) RTG-2) (O Hara et al., 2017). Les premiers résultats sont encourageants pour la suite et ouvrent la porte à la conception de nombreux nouveaux outils écotoxicologiques.

5.2.3 Mise en contact microalgues/transducteur pour le développement de biocapteurs

Une perspective importante pour continuer à optimiser les biocapteurs est de poursuivre les investigations sur la mise en contact bio-récepteurs/transducteurs.

En particulier, l'optimisation de l'encapsulation des microalgues est nécessaire, en travaillant sur la porosité des hydrogels et la sélectivité de ces derniers aux polluants, pour créer des biocapteurs permettant une détection spécifique de certaines familles de molécules. De même, si l'on a montré que l'on pouvait augmenter la sensibilité des biocapteurs à hydrogels, un travail important reste à faire afin de transférer ces capteurs sur le terrain, en limitant la variabilité de réponse observée. Enfin, il faudrait encore affiner la sensibilité et la reproductibilité des résultats.

Les biocapteurs à microfluidique ont indéniablement montré leur utilité et leur sensibilité à l'étude de la toxicité des RUTP sur les microalgues. Cependant, il existe plusieurs voies d'optimisation. La conception des systèmes microfluidiques pourrait être enrichie afin de faire progresser la sensibilité des biocapteurs. Comme déjà évoqué lors de la partie précédente, de nouvelles techniques de microfluidiques se développent pour concentrer les éléments (par ex. ioniques) en utilisant de faibles volumes. Il serait *a priori* possible d'envisager de préconcentrer les molécules présentes dans les rejets faiblement pollués comme les RUTP de manière à pouvoir détecter leur présence (méthode déjà mise en place par Yuan et al. (2015) avec de la fluorescéine, ou Lafleur et Salin (2009) avec des métaux lourds), même si la représentativité de l'échantillon peut en pâtir. Une autre voie possible serait l'utilisation de la technologie de Lab-on-a-Disc (LOD) ou Lab-on-a-CD comme cela a déjà été réalisé par une centrifugation de cellules sanguines (Zhang et al., 2008). Les LOD consistent en une intégration des systèmes microfluidiques au sein de disques reliés à un rotor (voir exemple Figure 37) permettant, grâce à la force centrifuge exercée par ce dernier, des phénomènes de pompages, mélange et l'analyse des fluides présents au sein des canaux microfluidiques. L'idée ici serait que les microalgues et polluants soient insérés dans des LOD par différentes voies se rejoignant avant d'être mélangés et centrifugés vers une chambre de lecture dédiée qui puisse permettre une analyse de la perturbation de la fluorescence. L'utilisation de LOD comporte de nombreux avantages. Elle permet d'éviter le recours à des pompes ou seringues pour faire avancer et mixer/centrifuger les liquides insérés, et d'éviter les interconnexions fluidiques complexes au sein des microcanaux. Cette technique permet également de multiplier le nombre de répliques analysés et d'étudier plusieurs microalgues en parallèle. Enfin grâce aux technologies de pointe maintenant bien développées (micro-électrodes, photo-détecteurs et diodes électroluminescentes organiques pouvant être souples et plats), il est possible d'intégrer des transducteurs au niveau des chambres de lecture pouvant permettre l'acquisition de nombreuses données en parallèle (comme par exemple : pH, température, turbidité, conductivité) essentielles pour connaître la physico-chimie des rejets analysés par le biocapteur, sans devoir effectuer les mesures en laboratoire.

La seule limite dont il faut tenir compte est le coût de fabrication de tels disques qui pourrait freiner tout transfert vers des acteurs publics ou privés, hors recherche scientifique.

5.2.4 Choix des biomarqueurs pour le développement de biocapteurs : vers des

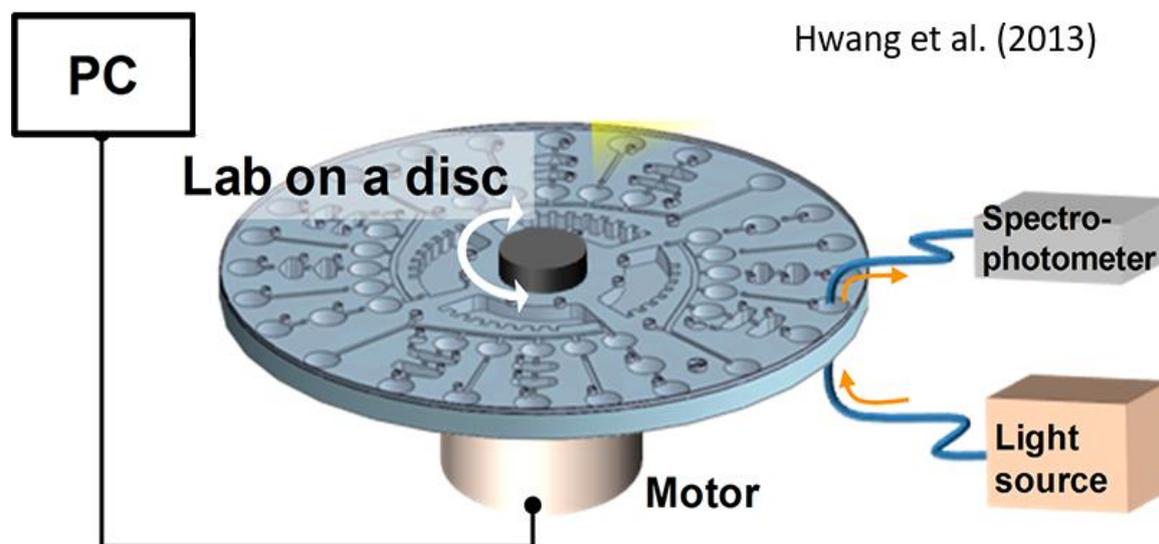


Figure 37 : Principe d'un Lab-On-a-Disc (Modifié d'après Hwang et al., 2013).

biocapteurs multiparamétriques ?

Nous avons centré notre étude sur la perturbation de la fluorescence chlorophyllienne, marqueur pertinent et facile à mesurer pour créer des outils sensibles. Toutefois, d'autres biomarqueurs, telles que les activités estérases ou phosphatases alcalines, ont également prouvé leur sensibilité et seraient susceptibles d'être mis en œuvre pour le développement de biocapteurs. Une approche multi-marqueurs est en effet pertinente pour détecter des effets toxiques *in situ* de manière rapide et sensible, même avec ces matrices diluées. Chouteau et al. (2004, 2005) ont développé des biocapteurs algaux électrochimiques (conductimétriques) utilisant des microélectrodes et permettant de suivre simultanément ces deux biomarqueurs enzymatiques. D'autres auteurs comme Tsopele et al. (2016) se sont déjà intéressés à l'intégration de biocapteurs algaux électrochimiques dans des puces microfluidiques.

Il pourrait par conséquent être utile de combiner dans une approche microfluidique à la fois l'étude optique (étude de la photosynthèse) et électrochimique (étude des biomarqueurs enzymatiques), en créant des puces adaptées à cette approche et reliées à la fois à un fluorimètre (du type de celui réalisé dans notre étude) et un système amplificateur (Lock In) portatifs. Cela pourrait également parfaitement s'effectuer dans le cadre de la création de LOD précédemment évoqués

5.2.5 Mesures *in situ* et praticité des biocapteurs pour le terrain

Malgré l'avancée qu'elle constitue, la fabrication de la station portative (fluorimètre) développée au cours de ce travail est une première étape de développement d'un biocapteur à microalgues. Actuellement limitée, la miniaturisation de l'équipement, et en particulier du fluorimètre, pourrait être envisagée en travaillant sur le choix des composants (comme les filtres, photodétecteurs, ou le système d'acquisition digital). De même, une multiplication des voies en parallèle (comme effectué par Lefevre et al., 2012) de notre capteur pourrait autoriser à prendre en compte directement des répliques, sans devoir effectuer des répétitions de mesures à la suite.

Les mesures de toxicité réalisées dans ce travail ont été faites sur des RUTP et eaux usées que nous avons prélevés et conservés en laboratoire. Il reste à réaliser de véritables campagnes de mesures *in situ* afin de valider définitivement notre capteur.

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ANNEXES

ANNEXE 1

Composition du milieu de culture AFNOR pour microalgues *Chlorella vulgaris* et

Pseudokirchneriella subcapitata

Préparation des solutions mères pour la création du milieu de culture AFNOR :

Composition des solutions mères		Quantité à diluer	Volume final de la solution
Solution-mère 1			
Nitrate de calcium	Ca(NO ₃) ₂ , 4H ₂ O	4g	100mL
Solution-mère 2			
Nitrate de potassium	KNO ₃	10g	100mL
Solution-mère 3			
Sulfate de magnésium	MgSO ₄ , 7H ₂ O	3g	100mL
Solution-mère 4			
Monohydrogénophosphate de potassium anhydre	K ₂ HPO ₄	4g	100mL
Solution-mère 5			
Sulfate de cuivre	CuSO ₄ , 5H ₂ O	0,06g	1L
Heptamolybdate d'ammonium	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0,06g	
Sulfate de zinc	ZnSO ₄ , 7H ₂ O	0,06g	
Chlorure de cobalt	CoCl ₂ , 6H ₂ O	0,06g	
Nitrate de manganèse	Mn(NO ₃) ₂ , 4H ₂ O	0,06g	
Acide citrique	C ₆ H ₈ O ₇ , 7H ₂ O	0,06g	
Acide borique	H ₃ BO ₃	0,06g	
Solution-mère 6			
Citrate de fer ammoniacal (ou citrate de fer)	C ₆ H ₁₁ FeNO ₇	0,625g	1L
Sulfate de fer	FeSO ₄ , 7H ₂ O	0,625g	
Chlorure de fer	FeCl ₃ , 6H ₂ O	0,625g	

Toutes les solutions mères préparées sont autoclavées à (121 ± 1) °C pendant 20 minutes.

Préparation du milieu de culture et d'essais des microalgues :

- 1 - Diluer 1 mL des solutions 1, 2, 3 et 4, ainsi que 500 µL des solutions 5 et 6 dans 1 litre d'eau ultra pure final.
- 2 – Autoclaver à 121 (± 1) °C pendant 20 minutes. Vérifier que le pH est de 7,1 ± 0,1. Ajuster le pH à l'aide d'acide chlorhydrique ou d'hydroxyde de sodium ou potassium si nécessaire.

ANNEXE 2

Composition du milieu de culture TAP pour microalgues *Chlamydomonas reinhardtii*

Préparation des solutions mères pour la création du milieu de culture et d'essai TAP :

Composition des solutions mères		Quantité à diluer	Volume final de la solution
Solution Tris-acide acétique glacial			
Acide acétique glacial	C ₂ H ₄ O ₂	10mL	100mL
Tris base		24,2g	
Tampon phosphate			
Hydrogénophosphate de potassium	K ₂ HPO ₄	10,8g	100mL
Phosphate de potassium monobasique	KH ₂ PO ₄	5,6g	
Solution Hutner's trace metals			
Ajouter 5 g de Na ₂ EDTA dans 400mL d'eau ultrapure puis ajuster le pH entre 6,5 et 6,8 avec du KOH 2N. Ensuite ajouter un à un les ingrédients ci-dessous en les dissolvant 1 à 1 au fur et à mesure.			
Sulfate de fer heptahydraté	FeSO ₄ 7H ₂ O	0,5g	0,5L
Sulfate de zinc heptahydraté	ZnSO ₄ 7H ₂ O	2,2g	
Acide borique	H ₃ BO ₃	1,14g	
Chlorure de manganèse tétrahydraté	MnCl ₂ 4H ₂ O	0,51g	
Sulfate de cuivre pentahydraté	CuSO ₄ 5H ₂ O	0,16g	
Sodium molybdate dihydraté	Na ₂ MoO ₄ 2H ₂ O	0,098g	
Chlorure de cobalt hexahydraté	CoCl ₂ 6H ₂ O	0,16g	
Solution A			
Chlorure d'ammonium	NH ₄ Cl	20g	0,5L
Sulfate de magnésium heptahydraté	MgSO ₄ 7H ₂ O	5g	
Chlorure de calcium dihydraté	CaCl ₂ 2H ₂ O	2,5g	

Toutes les solutions mères préparées sont autoclavées à (121 ± 1) °C pendant 20 minutes.

Préparation du milieu de culture et d'essais des microalgues :

1 - Diluer :

- 10 mL des solutions Tris-acide acétique glacial et A,
- 5 mL tampon phosphate,
- 1 mL de solution Hutner,
- 0,250 mL d'acide acétique glacial (>99 % (C₂H₄O₂)),

dans 1 litre d'eau ultra pure final.

2 – Autoclaver à 121 (± 1) °C pendant 20 minutes. Vérifier que le pH est de 7,1 ± 0,1. Ajuster le pH à l'aide d'acide chlorhydrique ou d'hydroxyde de sodium ou potassium si nécessaire.

ANNEXE 3

Liste des produits utilisés pour les différentes expériences

Utilisation	Produit	Fournisseur
Milieux de culture	Voir compositions milieux AFNOR et TAP en Annexes 1 et 2	Voir Annexes 1 et 2
Réactif pour la mesure de la perméabilité membranaire	Sytox Green® (5mM)	Thermo Fisher Scientific
Réactif pour la mesure de la phosphatase alcaline	Méthylumbelliférylphosphate (MUP)	Sigma Aldrich
Réactif pour la mesure de l'estérase	Fluorescéine Diacétate (FDA)	Sigma Aldrich
Tampon et réactifs pour la mesure de peroxydation lipidique	Acide trichloroacétique (TCA)	Sigma Aldrich
	Acide Thiobarbiturique (TBA)	Sigma Aldrich
	Acide chlorhydrique	VWR
	EDTA	Sigma Aldrich
	MgCl ₂	Sigma Aldrich
	Polyvinylpyrrolidone	Sigma Aldrich
	Tris-HCl	Sigma Aldrich
Polluants	RUTP	X
	Effluents (Urbain/Hospitaliers)	X
	Diuron (≥98 %)	Sigma Aldrich
	Atrazine PESTANAL®	Riedel-de-Haën
	Isoproturon PESTANAL®	Riedel-de-Haën
	Ethanol aboslu (pour solutions mère de pesticide) (≥99.5 %)	VWR
Réactifs pour l'immobilisation par procédé sol-gel	Alginate de sodium	AppliChem GmbH
	Chlorure de calcium (≥99.9 %)	Merck
	Silicate de sodium (NaOH 10 % SiO ₂ 27 %)	Sigma Aldrich
	Acide chlorhydrique	VWR
	Hydrogénophosphate dipotassique	Merck
	Dihydrogénophosphate de potassium	CHIMIE-PLUS
	Silice colloïdale (Ludox®) (40 wt.%)	Sigma Aldrich
Création de puces microfluidiques	Lamelle	Fisher Scientific
	Lame	Fisher Scientific
	Adhésif double face	Plusform

ANNEXE 4



Feasibility of using a translucent inorganic hydrogel to build a biosensor using immobilized algal cells

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Abstract Anthropogenic activities generate contaminants, as pesticides and other pollutants, in the aquatic environment which present a real threat to ecosystems and human health. Thus, monitoring tools become essential for water managers to detect these chemicals before the occurrence of adverse effects. In this aim, algal cell biosensors, based on photosystem II activity measurement, have been designed for several years in previous studies. In this work, we study a new immobilization technique of algal cells in the aim of improving the performance of these biosensors. Immobilization was here achieved by encapsulation in a hybrid alginate/silica translucent hydrogel. The feasibility of this process was here assessed, and the biosensor designed was tested on the detection of chemicals in urban rainwaters.

Keywords Algae · Pesticides · Photosystem II · Biosensors · Encapsulation

Introduction

The sealing of the surfaces of urban environments reduces the infiltration of rainwater to underground aquifers and increases runoff in heavily anthropized areas. The leaching of urban

soils results in the transfer of a cocktail of chemical compounds comprising a large proportion of pesticides to the aquatic environment (Aalderink et al. 1990; Bertrand-Krajewski et al. 2008; Aryal et al. 2010). These cocktails stem from the maintenance of gardens and green spaces. Thus, a large number of molecules, such as atrazine and diuron whose use has nonetheless been forbidden for several years, are regularly detected in aquatic environments (Dubois and Lacouture 2011). These compounds have harmful effects on the functioning of ecosystems as they degrade habitats (Angerville 2009; Armstrong et al. 1980). The presence of carcinogenic and neurotoxic substances, as well as endocrine disruptors, has often been demonstrated in animals, and their potential impacts on human health also lead to great concern (Aubertot 2005). This observation has led the French and European authorities to strengthen legislation in order to organize actions aimed at reversing this process, notably through the adoption at the end of 2009 of the “Pesticide Package”, composed of four texts: two directives and two regulations in force since June 2011 (Directive 2009/128/CE). Their objective is to reduce risk by requiring member countries to set up action plans that involve reinforced monitoring of the quality of aquatic environments. In this context, biosensors capable of continuous measurement in situ are indispensable tools for decision-makers. In previous works, algal biosensors based on metabolism measurements have been developed by Chouteau et al. (2004, 2005) for heavy metal and pesticide detection in freshwater.

A biosensor can be considered as a combination of a bioreceptor, the biological component, and a transducer, the detection method. The total effect of a biosensor is to transform a biological event into an electrical signal. The first link of a biosensor is the bioreceptor, which has a particularly selective site that identifies the analyte. The bioreceptor ensures molecular recognition and may transform the analyte in

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some way detectable by the transducer (Tran-Minh 1993; Ciucu et al. 2001; Dzyadevych et al. 2003).

Hereafter, we present a unicellular algal biosensor based on photosystem II activity measurement to evaluate levels of photosynthesis. The algae were chosen for their high sensitivity to pollutants and the position they occupy at the base of trophic chains. They are essential in aquatic ecosystems since they provide oxygen and organic substances to other life forms. Chemical action of pollutants can affect their photosynthetic activity, resulting in oxygen depletion and a decrease in aquatic biodiversity. Therefore, algae can serve as biological monitors of water quality and as biological indicators in the assessment of environmental impact of pollutants. Thus, the tool developed is of considerable ecological pertinence. Photosystem II was reported to be able to detect herbicides in the environment (Giardi et al. 2001). About 30 % of herbicides are targeting the vegetal photosystem II (PSII) (Moreland 1980; Draber et al. 1991). They include derivatives of phenylurea, triazine, and phenolic herbicides. These substances inhibit photosynthetic electron flow by blocking the PSII quinone binding site and thus modify chlorophyll fluorescence. So, PSII activity has so far been used because of its higher sensitivity (Samson and Popovic 1988; Fai et al. 2007).

Since the sensitivity of biosensors is directly linked to the method of bioreceptor immobilization, we present in this paper a method of tool improvement by encapsulating the algae in a hybrid alginate/silica material. The design of biosensors based on direct silica encapsulation of algal cells is intrinsically limited by the restricted viability and high level of cellular stress developed during and after direct entrapment in the silica host. Recent work has demonstrated the possibility of cell division inside inorganic matrices by means of a two-step encapsulation procedure based on sol-gel chemistry (Perullini et al. 2005). This strategy expands the range of possible applications as cells can not only be entrapped within silica hydrogels, but plant and algal cells are also able to grow inside, even for periods of months (Perullini et al. 2007; Sicard et al. 2011). In addition, the mesoporous texture of the silica hydrogel provides a shield preventing the release of entrapped cells, as well as the contamination of the inner culture by exogenous strains. Moreover, in a previous work, translucent alginate-silica matrices have been investigated for the entrapment of three algal strains in order to develop a prototype of biosensor (Ferro et al. 2012). In the mentioned work, after a biocompatibility assay of the inorganic host material with the biological guests (*Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Pseudokirchneriella subcapitata*), the detection and quantification of pure herbicides in artificial samples were evaluated, as a proof of concept for the development of a biosensor. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) and atrazine, as examples of herbicides that are known to inhibit the algal PSII, were evaluated, and a

limit of detection of 0.1 μM of atrazine was established for the most sensitive algal species (*C. reinhardtii*).

The goal of the present work is to evaluate the performance of the developed biosensor on rainwater from urban catchments contaminated by pesticides. Hereafter, we present the analysis of samples of rainwater from urban catchments contaminated by pesticides compared with bioassays on non-immobilized algae. The results obtained lead to considering the application of the biosensor developed to detect pesticides in the environment.

Material and methods

Three algal strains were used: *C. vulgaris* (CV), *P. subcapitata* (PS), and *C. reinhardtii* (CR). They were purchased from the Culture Collection of Algae and Protozoa (Cumbria, UK).

C. vulgaris and *P. subcapitata* were grown in Lefebvre-Czarda medium (AFNOR 1980), whereas *C. reinhardtii* was grown in Tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965), and these were transplanted weekly under sterile conditions (autoclaving 20 min, 130 °C, 1.3 bars). Algae were maintained in a nycthemeral cycle of 16 h of illumination at 5000 lx and 8 h of darkness.

Photosystem II activity was measured by chlorophyll fluorescence emission which was done using a fluorometer for an excitation wavelength of 460 nm and an emission wavelength of 680 nm. The transducer is an optical fibre placed in contact with the immobilized algae.

Algal cell immobilization was carried out in two steps. The algae were encapsulated beforehand in a calcium alginate matrix which in turn was immobilized in an inorganic silica matrix, thus minimizing the contact of algae with soluble silica precursors. A mixture of 50 μL of cells suspended with 50 μL of Tris-HCl buffer (10 mM, pH 7.5) and 100 μL 2 % Na(I) alginate (10^4 cells/mL) was introduced in a multiwell plate and further immersed in 0.1 M CaCl_2 for 10 min in order to achieve alginate crosslinking. After that, a 1:3 mixture (pH=6.5) of sodium silicate (Riedel-de Haën; NaOH 10 %, SiO_2 27 %) and pre-formed silica particles (LUDOX HS-40, 40 % in water; Aldrich) was poured on the top, leading to a mesoporous monolithic structure after sol-gel transition. The silica hydrogel thickness of 1.9 mm optimizes the optical quality and mass transport.

Material biocompatibility was assessed in a previous work (Ferro et al. 2012) by comparing the growth of the encapsulated algae with that of free algae, under the same temperature and light conditions. The results obtained showed that the growth of CV was not affected by encapsulation, whereas that of the two other species was slightly inhibited (6 % for PS and 13 % for CR). We considered that these inhibition rates remained acceptable to continue the tests with the three species of algae.

The optical quality of the hybrid alginate/silica material was compared to that obtained from free algae in the presence of sodium alginate and encapsulated in calcium alginate. To do this, chlorophyll fluorescence emission measurements were compared under the different conditions described. The results revealed a slight loss of fluorescence intensity in the presence of sodium alginate that was not increased by gelification in the calcium alginate or by inclusion in the hybrid material. These losses were considered acceptable for the experiments to continue.

Determination of photosynthetic activity was evaluated using the method described by (Ban-Dar et al. 1989) based on measuring chlorophyll fluorescence emission which is inversely proportional to the level of photosynthesis. Thus, photosynthetic activity can be determined by measuring fluorescence before and after contamination by dichloromethyl urea (DCMU) at 4 mg/L, as it leads to the total blocking of photosynthesis and thus maximum fluorescence. Fluorescence enhancement after DCMU treatment is expressed as percentage of fluorescence after DCMU exposure reported to the fluorescence before exposure. The degradation of photosynthetic activity was tested at first with atrazine, as a pesticide model, and after with rainwater. Photosynthetic inhibition was expressed as residual activity:

$$A_{\text{res}}(\%) = \frac{\Delta F_{\text{sample}}}{\Delta F_{\text{control}}} * 100$$

with $\Delta F = F_u - F_0$ (F_u is the fluorescence after DCMU (at 4mg/L) exposure and F_0 the fluorescence before DCMU exposure.

The rainwater samples were taken from two retention basins (Chassieu and Ecully) in the suburb of Lyon in which water is stored before re-infiltration into the groundwater.

Results and discussion

Atrazine, whose effect is well known on photosynthesis, is used in this study as a pesticide model to test the impact of the encapsulation on the sensor's efficiency.

Ferro et al. (2012) have shown in a previous study that DCMU at 4 mg/L (concentration which leads to the total blocking of photosynthesis) can produce the enhancement of chlorophyll fluorescence on free algae, in the presence of sodium alginate, encapsulated in calcium alginate or immobilized in two steps in an alginate/silica gel. This study was carried out on three algal species (*CV*, *PS*, and *CR*) and showed that the increase of fluorescence was not significantly different between the free algae and the algae immobilized in one or two steps for the *PS* and *CR* strains. However, the increase of fluorescence was much lower for the *CV* strain once the cells had been immobilized. It is difficult to explain this phenomenon: it is not possible to know at this stage if

algae are damaged by immobilization or if encapsulation reduces the DCMU effect.

In this paper, a study of photosynthetic activity after exposure to different concentrations of atrazine was carried out in the aim to give more information about the behaviour of algae after encapsulation in one or two steps. The results in Table 1 express the residual photosynthetic activity, A_{res} (%), as described in the "Material and methods" section, after a 40 min exposure at different atrazine concentrations ranging from 10^{-3} to $10 \mu\text{M}$. Experiments were conducted on the three algal species with three replicates for each measurement.

Results show that photosynthetic activity of free algae decreases, in a dose-dependent manner, with the concentration of atrazine in the same way for the three species. This decrease is also observed for algae encapsulated in one or two steps; however, the decrease stops in alginate above $1 \mu\text{M}$.

Thus, the limit of detection (LOD) was not observed on free algae; it seems lower than $10^{-3} \mu\text{M}$. A_{res} increases with the number of immobilization steps (higher in calcium alginate than on free algae, higher in alginate/SiO₂ than in alginate). This means that the encapsulation material provides a barrier to diffusion of atrazine towards algal cells. This can explain why atrazine at $10^{-3} \mu\text{M}$ has no effect on *CV* and *CR* immobilized in two steps; in this case, the LOD is between 10^{-3} and $10^{-2} \mu\text{M}$. These results show, however, the feasibility of detecting atrazine with algae immobilized in one or two steps. On the other hand, the optical biosensors designed with a double encapsulation of algal cells could present the advantage to protect algae from external damages. Photosynthetic activity seems to be preserved after the two immobilization steps for the three strains studied here.

Table 1 Residual photosynthetic activity (A_{res} (%)) of control: free algae (Control), in a mixture with sodium alginate (Na-Alg), encapsulated in calcium alginate (Ca-Alg), and in a double encapsulation alginate/SiO₂ (Ca-Alg-SiO₂) after exposure to different atrazine concentrations (10^{-3} to $10 \mu\text{M}$)

Atrazine (μM)		0.001	0.01	0.1	1	10
<i>CV</i>	Control	90±3.2	40±0.4	10±2.1	2±0.1	0±0
	Na-Alg	95±1.2	70±4.2	50±1.8	28±2.3	32±3
	Ca-Alg	102±2	80±1.6	60±5.6	32±2.4	28±2.5
	Ca-Alg-SiO ₂	103±2	92±1.6	45±0.5	35±4	12±2
<i>PS</i>	Control	80±1.6	70±2.9	42±2	18±1.3	0±0
	Na-Alg	83±1.9	80±1.4	75±2.3	21±0.5	10±0.8
	Ca-Alg	78±5.2	85±1.5	59±2.5	25±1.9	18±4.2
	Ca-Alg-SiO ₂	95±3.7	92±4.3	61±2.6	29±2.4	20±4.5
<i>CR</i>	Control	95±3	80±2.6	47±6.2	3±0.6	0±0
	Na-Alg	92±1.9	85±3.2	65±2.7	27±4.2	30±4.7
	Ca-Alg	98±1.5	78±2.8	68±4.7	25±3.9	21±4
	Ca-Alg-SiO ₂	105±8	90±6.4	72±3.7	38±4.2	15±8

Table 2 Organic composition of samples collected after different rainfalls in the Chassieu and Ecully basins. The lowest and highest concentrations measured are presented

Chemical (ng/L)	Chassieu	Ecully
Atrazine	1.7–2.5	<–2
Diuron	20–43	12.2–238
Hexachlorocyclohexane	<–7.2	<
Isoproturon	1.7–2.7	<
Simazine	<	2.8–2.9
4-Nonylphenol	26–555	10–23
Parateroctylphenol	11–33	<
Fluoranthene	4–5.9	<
Naphtalene	108–182	117
Acenaphthene	9.4–19	10
Fluorene	14–18	15
Phenanthrene	16–53	<
Pyrene	3.6–30.8	<

< means a level lower than the limit of detection

Assays after rainwater exposure

In the aim to assess the performance of encapsulated algae for detection of chemicals in the environment, photosynthetic activity was measured in the three strains during the different steps of encapsulation after a 24-h exposure of rainwater collected during several rainfalls inside two basins.

Water collected in Chassieu comes from an industrial area and those from Ecully come from agricultural land. Organic compounds were measured in the samples collected; results are presented in Table 2, and they show that there are more pesticides in the Ecully basin (diuron particularly) and more hydrocarbons in the Chassieu basin.

Figures 1 and 2 show the results of A_{res} after exposure to a sample of rainwater pooled after several events. The results show an impact of leachates on the photosynthetic activity of

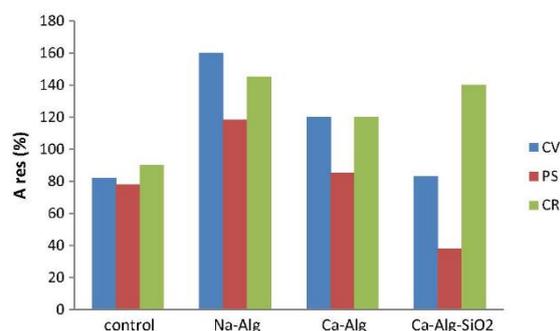


Fig. 1 Residual photosynthetic activity (A_{res} (%)) of control: free algae (*control*), in a mixture with sodium alginate (*Na-Alg*), encapsulated in calcium alginate (*Ca-Alg*), and in a double encapsulation (*Ca-Alg-SiO₂*) after a 24-h exposure to rainwater collected in the Chassieu basin

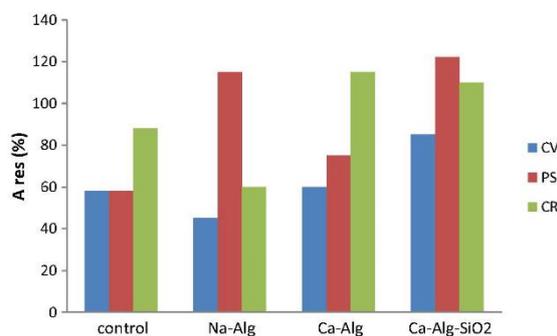


Fig. 2 Residual photosynthetic activity (A_{res} (%)) of control: free algae (*control*), in a mixture with sodium alginate (*Na-Alg*), encapsulated in calcium alginate (*Ca-Alg*), and in a double encapsulation (*Ca-Alg-SiO₂*) after a 24-h exposure to rainwater collected in the Ecully basin

free algae for the three species (residual photosynthetic activity <100 %) after exposure to rainwaters collected in the two basins.

If we compare A_{res} of algae encapsulated in alginate with that of free algae, we can see that A_{res} is >100 % for *CV* and *CR* and near 100 % for *PS* after exposure to Chassieu water, whereas the results are different with Ecully water: in this case, A_{res} is <100 % excepted for *PS* in the presence of alginate and *CR* after encapsulation. After encapsulation in two steps, A_{res} is <100 % for *CV*, which means that photosynthetic activity is inhibited. In this last case, photosynthetic activity is inhibited for *PS* with Chassieu water and enhanced with Ecully water. At last, A_{res} is <100 % with the two types of water.

These results show a difference of behaviour not easy to understand between Chassieu and Ecully waters. It is not very surprising because these waters have different organic compositions. The enhancement of photosynthetic activity in the presence of alginate could perhaps be explained by a synergic effect between the alginate and some molecules present in the leachates. Rainwater contains a complex cocktail of molecules and measurements could reflect several effects with synergism or antagonisms.

Conclusion

In spite of the different uncertainties and doubts identified in this work, the feasibility of using a translucent inorganic hydro-gel to build an optical biosensor using immobilized algal cells appears to have been validated. According to our results and in agreement with previous studies, the immobilization of green algae in a sol-gel silica matrix by means of the two-step procedure presents high biocompatibility. This encapsulation method is easy to carry out and a matrix with good optical and mechanical performances is obtained. In addition, the precursors of silica and polymer particles used in this

synthesis are inexpensive reagents, which is interesting from the standpoint of possible future applications. The transparency and structure of the algal layer permit direct contact with optical fibres to produce a fluorescence emission that can be detected by a fluorometer. This whole-cell biosensor functioning without reagent is of great interest economically for monitoring different chemical levels in the environment. Studies on the choice of the algal species best adapted as a function of the molecules to be detected remain to be performed.

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ANNEXE 5

Gosset et al. (In press in *Journal of Environmental Science and Health, Part A -Toxic/Hazardous Substances and Environmental Engineering*)

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Assessment of long term ecotoxicity of urban stormwaters using a multigenerational bioassay on *Ceriodaphnia dubia*: A preliminary study

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ABSTRACT

Standardized ecotoxicity bioassays show some limits to assess properly long-term residual toxicity of complex mixture of pollutants often present at low concentration, such as stormwaters. Among invertebrate organisms used for ecotoxicity testing, the microcrustacean *Ceriodaphnia dubia* (*C. dubia*) is considered as one of the most sensitive, especially regarding reproduction impairment as a toxicity endpoint. Consequently, this work explores the interest to perform a multigenerational assay based on the study of the reproduction of *C. dubia* to assess long-term ecotoxicity of complex mixture, using stormwater samples. With this in mind, a battery of standardized bioassays (*Daphnia magna* mobility, *Pseudokirchneriella subcapitata* population growth, *Heterocypris incongruens* growth and one generation *C. dubia* reproduction inhibition assays) was performed in parallel to a three generation *C. dubia* reproduction inhibition assay on 2 stormwater samples. Results highlighted that while all standardized bioassays failed to reveal residual toxicity in the stormwater samples, the *C. dubia* multigenerational assay exhibited an higher sensitivity than the previous ones. No adverse effect was observed for the first exposed generation, but an increase in mortality and a reproduction disturbance was obtained in the second and third exposed generation depending of the sample. Further experiments are now needed to optimize the exposure protocol of this multigenerational assay.

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micropollutant

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Introduction

In Europe, the Urban Wastewater Directive (91/271/EEC) requires the management of all urban wastewaters, i.e. effluents and stormwaters, even though available treatments (mainly retention and/or infiltration basins) are relatively limited in efficiency notably due to the large volumes to be treated. Furthermore, European Water Framework Directive (EWFD, 2000/60/EC) requires to maintain the good ecological status of water bodies receiving such effluents. To achieve this goal, it remains of value to implement effective monitoring methods such as bioassays likely to reveal the residual ecotoxicity of chemically complex mixtures of contaminants at low concentrations such as stormwater.

Many standardized bioassays are currently used to assess the ecotoxicity of urban stormwaters. 24-h *Daphnia magna* (*D. magna*) immobilization, 72-h *Pseudokirchneriella subcapitata* (*P. subcapitata*) growth, 6-d *Heterocypris incongruens* (*H. incongruens*) and 7-d *Lemna minor* (*L. minor*) growth assays are the most common ones. However, it has been shown that such standardized bioassays often fail to properly assess stormwater toxicity, mainly due to their low contamination level, and/or to the presence of nutrients interfering with the measured endpoint, in particular growth.^{11,21} Angerville et al.¹¹ showed that *C. dubia* reproduction bioassay was the most

sensitive to assess the toxicity of this kind of matrixes. Moreover, previous studies using cladocerans (*D. magna*) highlighted that many environmental pollutants (pesticides, heavy metals, pharmaceuticals, phthalates, etc.) led to reproduction impairment with different modes of toxic action: estrogenic and anti-estrogenic, androgenic and anti-androgenic activities through activation/blocking of specific receptors, modulation of steroid metabolism (others than estrogens and androgens), disturbance of ecdysis or juvenile hormone mimicry.^{3–5} Endocrine disrupting activities can lead to adverse effects on exposed organisms, but also on their progeny, creating adverse effects on subsequent generations.^{6,7} Nowadays, there is undoubtedly an important concern about the long term ecotoxicity assessment of single chemicals and of multicontaminated matrixes and the scientific community is actively working to create harmonization (OECD guidelines) of multigenerational bioassays on cladocerans (*D. magna*) to detect long-term impairments, as done recently by Barata et al.⁶

Multigenerational (3–5 generations) reproduction inhibition tests have already been developed on *C. dubia*. Rose et al.^{8,9} used such an approach for testing the long-term reproduction toxicity of low concentration of 3,4-dichloroaniline (3,4 DCA), a by-product of the herbicide diuron. These authors observed that 3,4-DCA at environmentally realistic concentrations led to

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a stimulation of the reproduction (overproduction of neonates) starting at the second generation exposed (total of 4 generations studied), resulting in tolerance (physiological acclimation or genetically based resistance) to 3,4 DCA at concentration up to $2.5 \mu\text{g L}^{-1}$. On the contrary, Lamichhane et al.^[10,11] observed that two pharmaceuticals (carbamazepine and sertraline) led to a decrease in offspring production in three successive *C. dubia* generations exposed, respectively, to 196.7 and $4.8 \mu\text{g L}^{-1}$.

The main objective of this study was to implement an ecotoxicological battery of bioassays, comprising *D. magna* acute bioassay and *P. subcapitata*, *H. incongruens* and *C. dubia* chronic bioassays, as well as a multigenerational bioassay carried out on *C. dubia*, to assess the toxicity of two stormwater effluents. For the later, *C. dubia* reproduction assay was derived from the standardized bioassay (NF ISO 20665^[12]) and applied in three successive generations in order to assess with a good reliability a possible long-term toxicity of urban stormwater samples collected.

Materials and methods

Sampling sites

Two samples of stormwaters were collected. The first one (CHA) stemming from a sub-urban zone of Lyon City (France), corresponded to the outfall of a retention–infiltration basin receiving stormwater from a highly urbanized industrial area of 185 ha, with an imperviousness rate of 75%.^[13] The second one (MIR) was collected in the overflow of three interconnected retention ponds, in the North-East of Lyon City corresponding to a small watershed of 11 ha draining a sub-urban area under high agricultural pressure. The two samples were collected between March and April 2015 and the main characteristics of the corresponding storm events are summarized in Table 1.

Grab stormwater samples were collected manually during the storm events, using 2 L high-density polyethylene flasks previously washed three times with the discharge. Samples were transported immediately to the laboratory and was filtered ($0.45 \mu\text{m}$ pore size) or not, depending on the assay. Then, 120 mL aliquots (filtered and nonfiltered) were stored at -20°C until testing.

Physico-chemical analyses of samples

Several physico-chemical parameters (pH, Conductivity, Dissolved Organic Carbon (DOC), Total suspended Solids (TSS), etc.) were measured according to European standards as described by Angerville et al.^[1] Using $0.45 \mu\text{m}$ filtered samples, a total of 105 organic compounds were analyzed by an accredited laboratory (CARSO laboratory in Venissieux)

according to European standards (34 pesticides, 15 Polycyclic Aromatic Hydrocarbons (PAHs), 22 Volatile Organic Compounds (VOCs), 16 Benzene derivatives, 12 Phenol derivatives and 3 others). A list of all organic compounds and analytical methods employed are summarized in Table 1S. Additionally, eight major metals (Pb, Cr, Ni, Cd, Cu, Fe, Ca and Zn) were analyzed by ICP-MS (Hitachi spectrophotometer).

Algal (*Pseudokirchneriella subcapitata*) culture and growth inhibition test

The algal strain was purchased from the French National Museum of Natural History (Paris, France). *P. subcapitata* were cultured in Lefebvre-Czarda medium.^[14] Algae cultures were transplanted weekly under sterile condition, using sterilized glassware and medium (autoclaved during 20 min, at 130°C , under 1.3 bar), with the aim of using fresh algae in their growth phase. Algae were maintained at 21°C in a culture room under a 16 h/8 h light/dark cycle (5000 lux illumination). Growth inhibition assays were realized following the OECD guidelines,^[15] and EC_{20} 72 h values were calculated using the Hill probit model (Regtox[®], E. Vindimian, <http://normalesup.org/~vindimian/>).

Ostracode (*Heterocypris incongruens*) lethality and growth inhibition test

A 6-day subchronic toxicity test was conducted following the NF EN ISO 14371 standard.^[16] Organisms were exposed to 50% (standard value) or to 95% v/v of stormwater with minor modifications. Briefly, test was carried out using the OSTRACODTOXKIT F[®]. Cysts were kept for 48 h in standard freshwater at 25°C and under a 3000–4000 lux illumination to hatch. Ten 4 h-posthatching neonates (6 replicates of 10 organisms) were sampled and their survival and body length were measured after 6-day exposure using the image analysis Motic[®] software.

Cladoceran stock culture

Water flea *D. magna* and *C. dubia* (IRSTEA, France) were bred (weekly transplantation, continuous parthenogenetic reproduction) in two liter glass jars, under 500 ± 200 lux illumination and at a temperature of $25 \pm 2^\circ\text{C}$ for *C. dubia* and at $20 \pm 2^\circ\text{C}$ for *D. magna*, under a 16 h light/8h dark photoperiod. *D. magna* was cultured in Volvic[®] water. *C. dubia* was cultured in Evian[®] water supplemented by $75 \mu\text{g L}^{-1}$ thiamine chlorhydrate, $1 \mu\text{g L}^{-1}$ cyanocobalamine and $0.75 \mu\text{g L}^{-1}$ biotine. According to the NF EN ISO 20665 standard,^[12] organisms were daily fed on *P. subcapitata* algae culture (40×10^6 cells/mL) and on fish food (2.5g L^{-1} JBL NovoRed[®] solution).

Daphnia magna mobility inhibition test

The mobility inhibition bioassay (24 and 48 h acute toxicity test) was conducted following the NF EN ISO 6341 standard.^[17] EC_{20} values were calculated using the Hill probit model (Regtox[®]).

Table 1. Characteristics of the rain events corresponding to stormwater samples.

Sample name	Rain event duration (hh:min)	Rain height (mm)	Rain intensity (mm.h^{-1})	Dry period before rain event (dd:hh:min)
CHA	20:06	17.2	0.86	03:03:48
MIR	04:30	5.4	1.20	01:22:30

***Ceriodaphnia dubia* multigenerational reproduction inhibition test**

C. dubia reproduction test was performed on three successive generations following a modified protocol derived from the standardized one generation-reproduction bioassay NF ISO 20665.^[12] Due to the rather small volume of stormwater samples available, bioassay was developed and conducted in 6-well plates. In each well, one organism was exposed during 10 days in 10 mL (instead of using 50 mL in the ISO standard) of either 0.45 μm filtered stormwater sample at a concentration of 90% (v/v) or to the control medium (see Section "Cladoceran stock culture"). Main physico-chemical characteristics (pH and conductivity) of control medium and of tested stormwaters were in the same range what enabled to expose organisms up to 90% of stormwater. Exposure medium consisted of 9 mL of sample (stormwater or control water) mixed with 1 mL of *C. dubia* food suspension. Exposure medium was renewed every two days with thawed stormwater in order to insure constant exposure conditions. Twelve replicate organisms were used per tested condition, each replicate consisting of one neonate (age < 24 h) obtained from the stock culture of *C. dubia* and considered as the first generation (F0). Every two days when exposure medium was renewed, neonates were counted and removed. Organisms were then fed and parent mortality measured. Neonates from the second brood (of the twelve replicates) were mixed and randomly used to produce the next generation (F1) exposed again to the wastewater samples or to the control medium. Experiment was repeated for three successive generations (Fig. 1). Two parameters were followed at each generation (i.e. after 10 days of exposure): first, the population growth expressed as the total number of neonates per mother (sum of neonates produced along the successive broods), and second, the reproduction success expressed as the number of neonates per alive mother at each generation, enabling to take into account a possible effect of mother mortality on reproduction

success. Regarding the NF ISO 20665 standard, a reproduction inhibition and a mortality lower than 30 and 20%, respectively, were considered as nonsignificant.

Statistical analysis

Data from *H. incongruens* and *C. dubia* assays were analyzed using GraphPad Prism version 7 for Windows (GraphPad software, San Diego, CA, USA). Distribution normality and homoscedasticity were tested for each parameter using Shapiro-Wilk and Brown-Forsythe tests. When these conditions were respected, comparisons between groups were carried out using the ANOVA test followed by post-hoc tests, and if not, by using the nonparametric Kruskal-Wallis test followed by post-hoc tests.

Results

Chemical analyses

Table 2 illustrates physico-chemical analyses of the stormwaters. Metal concentrations were lower than the Predicted No Effect Concentration (PNEC) values established by INERIS (National Institute for the Industrial Environment and Risks, France), except for copper and zinc. Regarding organic micropollutants, some pesticides were detected in the $\mu\text{g L}^{-1}$ range: for example the fungicide fosetyl-aluminium in CHA sample ($2.6 \mu\text{g L}^{-1}$), the herbicide glyphosate ($69.25 \mu\text{g L}^{-1}$, higher than the PNEC value) and one of its degradation by-product AMPA (acide 2-amino-3-(5-méthyl-3-hydroxy-1,2-oxazol-4-yl)propanoïque) in MIR sample ($20.15 \mu\text{g L}^{-1}$). In CHA sample, two other organic pollutants, benzyl chloride and tributyl phosphate, were detected but concentration remained below their PNEC values. Other compounds, such as the PAH fluorene, were found in both samples. All other compounds among the 105 analyzed were under their limit of quantification.

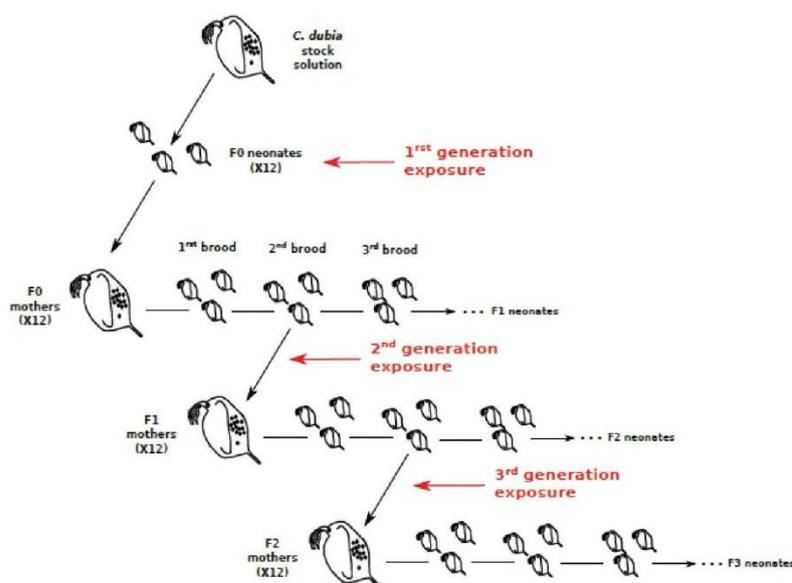


Figure 1. Exposure protocol of three successive generations in *Ceriodaphnia dubia*.

Table 2. Chemical analyzes of urban stormwater samples.

Parameter	Unit	CHA sample	MIR sample	Freshwater aquatic PNEC ^[36] (From INERIS)
pH	—	8.0	7.2	/
Conductivity	$\mu\text{S}/\text{cm}$	103.6	105.1	/
TSS	mg L^{-1}	82	56.26	/
DOC	mg L^{-1}	7.1	6.53	/
NO_3^-	mg L^{-1}	1.6	7.0	/
NH_4^+	mg L^{-1}	1.1	0.3	/
SO_4^{2-}	mg L^{-1}	0.3	3.4	/
Cl^-	mg L^{-1}	0.7	1.6	/
PO_4^{3-}	mg L^{-1}	0.01	0.3	/
K^+	mg L^{-1}	1.27	3.3	/
Metals				
Pb	$\mu\text{g L}^{-1}$	0.10	<0.05*	5
Cr	$\mu\text{g L}^{-1}$	0.63	0.31	4.7
Ni	$\mu\text{g L}^{-1}$	1.23	0.59	1.7
Cd	$\mu\text{g L}^{-1}$	0.09	<0.002	0.19
Cu	$\mu\text{g L}^{-1}$	12.48	4.38	1.6
Fe	mg L^{-1}	<0.005	<0.006	/
Ca	mg L^{-1}	20,42	25,51	/
Zn	mg L^{-1}	0.08	0.02	0.0078
Organic compounds[#]				
Benzyle chloride	$\mu\text{g L}^{-1}$	< 0.1	0.5	1
Fluorene	$\mu\text{g L}^{-1}$	3.45	5.93	0.25
AMPA	$\mu\text{g L}^{-1}$	0.98	20.15	80
Glyphosate	$\mu\text{g L}^{-1}$	< 0.5	69.25	28
Fosetyl-aluminium	$\mu\text{g L}^{-1}$	2.6	< 0.5	590
Tributyl phosphate	$\mu\text{g L}^{-1}$	0.12	< 0.05	82

PNEC = predicted no effect concentration; TSS = total suspended solids; DOC = dissolved organic carbon = AMPA = 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propionic acid.

/: no data available.

*: < limit of quantification.

[#]: organic chemicals listed here are those detected at a level \geq limit of quantification among 105 compounds analyzed.

D. magna, P. subcapitata and H. incongruens bioassays

As illustrated in Table 3, EC_{20} value was above 90% whatever the stormwater sample, suggesting a lack of acute toxicity towards *D. magna* (24 and 48 h of exposure), as well as towards *P. subcapitata* (72 h exposure).

Results of the ostracod growth assay are presented in Figure 2. After exposure to a 50% stormwater concentration, ostracod growth was not significantly affected neither by CHA nor by MIR sample. With 95% of sample in the exposure medium, both samples led to a slight growth stimulation ($P < 0.05$). No mortality was observed at the end of the assay.

C. dubia multigenerational bioassay

Validation of the method

The NF ISO 20665 standard requires some validation criteria for the reproduction *C. dubia* bioassay in control exposure conditions: less than 20% mortality of parents, at least three broods at the end of exposure for 60% of the parents, less than 20% of males in parent generation and at least

15 neonates produced per parent. As presented in Table 4, mortality of parents at each generation remained null in the control. Furthermore, no male was detected whatever the generation. It must be stressed that probably due to the reduction of the exposure volume (10 mL vs 50 mL), the number of broods and neonates produced per mother did not respect the standard. However, values remained closed

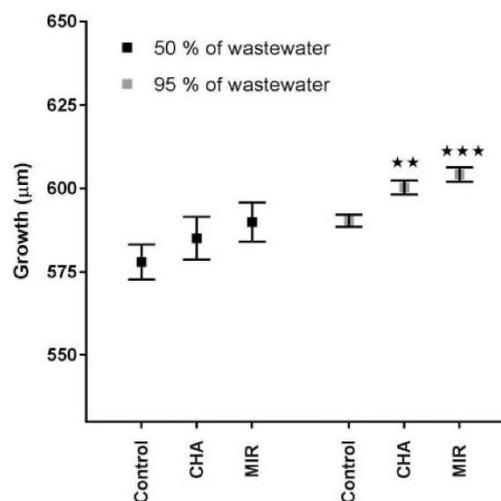


Figure 2. *Heterocypris incongruens* growth inhibition assay. Six replicates of 10 organisms were performed per assay. Histograms represent the mean growth of control neonates or neonates exposed to stormwater. Bars show standard errors ($n = 6$). Asterisks indicate a significant difference from the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 3. *D. magna* immobilization and *P. subcapitata* growth inhibition tests: EC_{20} values after 24 and 48 h exposure of *D. magna* ($n = 4$) and 72 h exposure of *P. subcapitata* ($n = 3$).

	CHA sample	MIR sample
<i>Daphnia magna</i> bioassay		
EC_{20-24h}	> 90%	> 90%
EC_{20-48h}	> 90%	> 90%
<i>Pseudokirchneriella subcapitata</i> bioassay		
EC_{20-72h}	> 90%	> 90%

Table 4. Percentage of mortality in *Ceriodaphnia dubia* parents (F0, F1 and F2) along the exposure to wastewater samples and to control medium. Limit of significance is 20% (from AFNOR standard, 2009).

	Control	CHA	MIR
F0	0	8.33	8.33
F1	0	54.54	8.33
F2	0	0	80

to the requested criteria (2.33; 3.33 and 3 broods per mother; 11.1; 10.7 and 12.5 neonates per mother for F1, F2 and F3 generations, respectively).

First generation effect on ceriodaphnia dubia

Studying the first generation of *C. dubia* corresponds to the standard chronic reproduction bioassay, despite minor modifications applied to the protocol as stated before. In control and in exposed organisms, mortality of the parents was under or equal to 8.3%, and consequently was considered as not significant. Responses of *C. dubia* to the different stormwater samples are given in Figure 3 and Table 4. No chronic toxicity (population growth and reproduction) was observed at the first exposed generation (F1).

Second and third generations

CHA stormwater sample led to a strong inhibition of *C. dubia* population growth (60.81% of control) and of reproduction (53.14% of control) at the second generation F2 ($P = 0.0151$ and 0.00188 , respectively), and to a high parent mortality rate (54.54% increase compared to control). On the contrary, a significant stimulation of both growth and reproduction was obtained at the third generation (F3) of daphnids exposed to CHA stormwater (Fig. 3).

An opposite trend was observed after exposure to MIR stormwater sample, i.e. a significant stimulation of reproduction

(38.71%) at generation F2 ($P = 0.0347$) and a high and significant inhibition at the third generation (F3) regarding both population growth (64.67% $P < 0.0001$) and reproduction (52%; no statistics possible because of the high parent mortality rate (80%) resulting in only 2 available replicates).

Discussion

Chemical analyses (realized once and considered representative of the 10 day-exposure since medium was renewed every other day) show that for the two sampled stormwaters, several pollutants were detected, the chemical pattern depending on the characteristics of the corresponding watershed. Concerning organic micropollutants, their presence can be easily explained by the different human activities along the watersheds studied. Here, high concentration of glyphosate, regarding its PNEC value, and of AMPA (respectively 69.25 and $20.15 \mu\text{g L}^{-1}$) found in MIR sample may be related to a high agricultural pressure in the corresponding watershed. For the CHA sample, the presence of tributyl phosphate and of benzyle chloride could arise from plastics and wood industry settled in the Chassieu industrial area. Moreover, heavy metals (here Cu, Cd, Cr, Ni or Zn), and PAHs (here fluorene) are commonly found in urban stormwater discharges,^[18,19] due to road traffic, vehicle brake emissions and atmospheric depositions.

The absence of acute toxicity in *D. magna*. (mobility) observed in both samples tested is in accordance with previous results of Waara et al.,^[20] Angerville et al.,^[1] or Bi et al.,^[21] obtained after exposure to urban wet weather discharges, confirming the tendency that acute toxicity bioassays are not enough sensitive for stormwater toxicity evaluation.

When conducting standard chronic assays with algae and ostracods, no toxicity was observed either. A slight growth

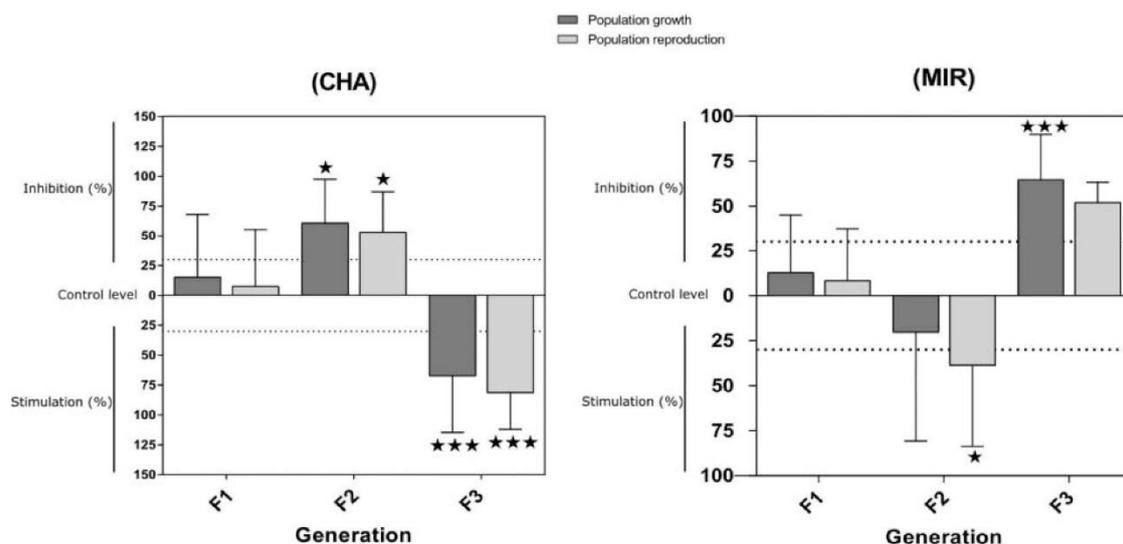


Figure 3. Multigenerational *Ceriodaphnia dubia* bioassay. The stormwater concentration tested is 90%. Effect on population growth is expressed as the mean of variation percentage compared to control of 12 replicates \pm standard deviation. Effect on population reproduction is expressed as the mean of variation percentage compared to the control for the n surviving mothers \pm standard deviation. Dotted lines represent the significance limit of 30% established in NF ISO 20665 standard. Asterisks indicate a statistical significant difference from the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

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stimulation of *H. incongruens* was even recorded when organisms were exposed to 95% of MIR or CHA sample. Although not commonly described in the case of stormwaters, such an effect was observed by Wigh et al.^[22] with wastewater effluents stemming from a treatment plant. Several authors as Sterner,^[23] Elser et al.,^[24] or Civitello et al.^[25] have suggested that enrichment due to nutrients such as phosphorus and nitrogen can stimulate the growth of producers and consumers and their reproduction. Here, nutrients could have contributed to stimulate the micro-algal growth and consequently, to increase the amount of available food for primary consumers (e.g. $7.0 \text{ mg L}^{-1} \text{ NO}_3^-$ in MIR sample dissolved phase).

The main objective of this work was to overcome the issues encountered in the ecotoxicity assessment of complex matrixes such as stormwater with standardized methods. Therefore, a *C. dubia* multigenerational assay was performed. The choice of this organism and of reproduction as an end point was driven by previous results obtained in our laboratory by Angerville et al.^[1] They observed that *C. dubia* reproduction bioassay was the most sensitive one among a rather broad ecotoxicity test battery applied to urban combined sewer overflows (acute bioassays on: *Vibrio Fischeri*, *D. magna*, *Danio rerio*; chronic bioassays on: *P. subcapitata*, *D. magna*, *Brachionus calyciflorus*, *C. dubia*).

The novelty of the assay performed here is the threefold repetition (i.e. on 3 successive generations) of the one generation NF ISO 20665 assay in a miniaturized version (10 vs 50 mL exposure volume). This was done in an attempt to evaluate long term toxic events. Sufficient fulfilment of most of the validity criteria supported by the standardized method was achieved excepted for the number of brood that was slightly lower than three in average. In 2003, Pandard.^[26] attempted to perform the *C. dubia* reproduction assay in 25 mL instead of 50 mL and described the same trend. This point certainly deserves further improvements or a reevaluation of a minimum valid brood number in the perspective of a refined assay.

As performed here, one can first stress that the *C. dubia* reproduction bioassay carried out with stormwater samples did not highlight any significant toxic effect (mortality or reproduction impairment) at the first generation. This result is in accordance with the studies of Kayhanian et al.,^[27] McQueen et al.,^[28] and Angerville et al.,^[1] showing with the standardized assay that for a majority of urban runoffs samples, no effect was detected after one single *C. dubia* generation exposure. As hypothesized, when second and third generations were exposed, acute and chronic toxicity appeared after both MIR and CHA sample exposure. Two kinetics of toxicity response of *C. dubia* were obtained: the one observed after exposure to MIR sample, is a stimulation of population reproduction at the second generation followed by an acute toxicity and reproduction impairment at the third generation. The second one observed after exposure to CHA sample, is a possible resistance/acclimation of organisms at the third generation, expressed as a stimulation of reproduction in F2 mothers, after an increase in mortality and reproduction inhibition measured at the second generation. Caution must be taken when considering the physico-chemistry of the samples to explain such results because of the possible interaction between chemicals and the fact that chemical analysis is limited to some compounds. Nevertheless, regarding MIR sample we can

hypothesize that the results could be partially related to the presence of glyphosate and its degradation product AMPA, glyphosate concentration being much higher than its PNEC value. This herbicide disturbs aromatase activity involved in the synthesis of estrogens and thus has been identified as an endocrine disruptor.^[29] Cuhra et al.^[30] observed a reduction of the fecundity and an increased abortion rate (determined counting the number of aborted eggs along the time) after exposure to 1.35 mg L^{-1} of glyphosate and a reduction of offspring length at lower concentrations ($50 \text{ } \mu\text{g L}^{-1}$) in *D. magna*.

Trends observed after CHA sample exposure are more complex to interpret, but heavy metals could be responsible for the observed effects. Chemical analyses (Table 2) showed rather high concentrations of heavy metals regarding their PNEC values (in particular Cu and Zn, at respectively, 12.48 and $80 \text{ } \mu\text{g L}^{-1}$). Cooper et al.^[31] observed that a mixture of the three metals Cu, Pb, and Zn could lead to a significant reproduction impairment on 7-days exposed *C. dubia* at concentrations of 1.3 (Cu), 1.1 (Pb) and 13.0 (Zn) $\mu\text{g L}^{-1}$, and to a high mortality at 10.6 (Cu), 9.0 (Pb) and 101.1 (Zn) $\mu\text{g L}^{-1}$, respectively. In CHA sample, Cu, Pb and Zn concentrations although being slightly lower than in Cooper et al.^[31] could have led to the delayed (F2) reproductive defect showed in *C. dubia*. On the contrary, some studies observed a tolerance/acclimation of cladocerans to copper ($1\text{--}35 \text{ } \mu\text{g L}^{-1}$) and zinc ($50 \text{ } \mu\text{g L}^{-1}$) at environmental realistic concentration as found in CHA samples.^[32,33] Considering the high mortality rate observed in F1 parents (56%), it could be possible that a resistance/acclimation of the neonates borne during the stormwater exposure appeared and led to a higher production of neonates at the last (F3) generation.

We can also note that a significant concentration (considering its PNEC value) of fluorene was measured in the two samples (respectively 3.45 and $5.95 \text{ } \mu\text{g L}^{-1}$ in CHA and MIR samples). If the acute and chronic (e.g. reproduction) ecotoxicity of fluorene is known (e.g. *C. dubia* reproduction bioassay CE_{10} at $25 \text{ } \mu\text{g L}^{-1}$),^[34] its mechanism of action remains unclear and its toxicity is difficult to predict because depending for example on light exposure (possible UV photo-activation of PAHs).^[35]

The role of epigenetic events to explain obtained results cannot be excluded since it was demonstrated by Vandegehuchte et al.^[36,37] in another cladoceran organism (*D. magna*) that sub-lethal concentrations of some heavy metals (such as zinc) or organic compounds (such as the 5-azacytidine drug) could modify the methylation pattern and subsequent transcription of several genes without stability among several generations.

Conclusion

A battery of acute and chronic bioassays was implemented for the ecotoxicity assessment of stormwaters. No toxic effect was observed neither with any of the acute and chronic standardized bioassays applied (*D. magna* mobility, *P. subcapitata* and *H. incongruens* growth), nor with the *C. dubia* reproduction bioassay performed on one generation (NF ISO 20665 slightly amended). We modified the *C. dubia* reproduction bioassay to perform the test over three successive generations in order to evaluate if this could improve the battery sensitivity. This study is the first attempt to apply a multigenerational reproduction

bioassay on *C. dubia*, for the toxicity detection of complex urban matrixes such as urban stormwaters. This work contributes to an issue of growing interest: the pollutant long-term effect assessment through a multigenerational approach, as recently proposed as part of a new OECD guideline targeting *D. magna* reproduction.^[6] Present results show that the *C. dubia* three generation reproduction bioassay appears to be a relevant approach, since exposure of the second and third generations enables to reveal a disturbance of some fitness parameters (reproduction and mortality). In order to confirm the interest and thus the added value of such a multigenerational reproduction assay it is now necessary to complete the development of the assay (e.g. by studying changes in the size, weight, and/ or other physiological defects of *C. dubia* parents and neonates).

Conflict of interest

The authors declare that they have no competing interests.

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Supplementary Material

Table 1S. List of the organic compounds, analytical methods and standards used for stormwater physico-chemical analyses in this study.

Compound	Analytical method used	Standard
VOC : Volatil Organic Compounds		
<i>Organohalogenatedsolvents</i>		
1,1,2,2-tetrachloroethane	HS/GC/MS	NF EN ISO 10301
1,1,1-trichloroethane	HS/GC/MS	NF EN ISO 10301
1,1,2-trichloroethane	HS/GC/MS	NF EN ISO 10301
1,1-dichloroethane	HS/GC/MS	NF EN ISO 10301
1,1-dichloroethylene	HS/GC/MS	NF EN ISO 10301
1,2-dichloroethane	HS/GC/MS	NF EN ISO 10301
Cis 1,2-dichloroéthylène	HS/GC/MS	NF EN ISO 10301
Trans 1,2-dichloroéthylène	HS/GC/MS	NF EN ISO 10301
1-chloronaphtalene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
2-chloronaphtalene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Vinyl chlorid	HS/GC/MS	NF EN ISO 10301
Dichloromethane	HS/GC/MS	NF EN ISO 10301
Hexachlorobutadiene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Pentachloroethane	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Tétrachloroéthylène	HS/GC/MS	NF EN ISO 10301
Carbon tetrachloride	HS/GC/MS	NF EN ISO 10301
Trichloroethylene	HS/GC/MS	NF EN ISO 10301
Sum of tri and tetrachlorethylene	HS/GC/MS	NF EN ISO 10301
Sum of 1,2-dichloroethylene	HS/GC/MS	NF EN ISO 10301
Others		
Dichlorobenzidine	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Benzidine	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Benzyl chlorid	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
benzylidene chlorid	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
4-chloroacetanilide	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
PAH : Polycyclic Aromatic Hydrocarbons		
Fluoranthene	HPLC/FLUO	NFT90-115
Benzo (b) fluoranthene	HPLC/FLUO	NFT90-115
Benzo (k) fluoranthene	HPLC/FLUO	NFT90-115
Benzo (a) pyrene	HPLC/FLUO	NFT90-115
Benzo (ghi) perylene	HPLC/FLUO	NFT90-115
Indeno (1,2,3 cd) pyrene	HPLC/FLUO	NFT90-115
Anthracene	HPLC/FLUO	NFT90-115
Acénaphthene	HPLC/FLUO	NFT90-115
Chrysene	HPLC/FLUO	NFT90-115
Dibenzo (a,h) anthracene	HPLC/FLUO	NFT90-115
Fluorene	HPLC/FLUO	NFT90-115
Naphtalene	HPLC/FLUO	NFT90-115
Pyrene	HPLC/FLUO	NFT90-115
Phenanthrene	HPLC/FLUO	NFT90-115
Benzo (a) anthracene	HPLC/FLUO	NFT90-115
Pesticides		
<i>Nitrogen Pesticides</i>		
Atrazine	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Simazine	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
<i>Organochlorine Pesticides</i>		
Lindane (γ-HCH)	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
α-HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Aldrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Dieldrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695

(Continued on next page)

Table 1S. (Continued).

Endrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDT	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDT	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Isodrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
β -HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
δ -HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Endosulfan	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Heptachlor	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDD	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDD	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDE	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDE	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
(cis+trans) Chlordane	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Sum of HCH isomers (except α -HCH)	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
α -HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Chlordecone	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Organophosphorus pesticides		
Chlorfenvinifos	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Ethyl-Chlorpyrifos	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Pesticides (others)		
AMPA	HPLC/MS/MS after direct injection	Internal method
Glyphosate	HPLC/MS/MS after direct injection	Internal method
Fosetyl-aluminium	HPLC/MS/MS after direct injection	Internal method
Alachlor	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Trifluralin	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Oxadiazon	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Biphenyl	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Substituted urea		
Diuron	HPLC/DAD after liquid-liquid extraction	Internal method
Isoproturon	HPLC/DAD after liquid-liquid extraction	Internal method
Chlorotoluron	HPLC/DAD after liquid-liquid extraction	Internal method
Linuron	HPLC/DAD after liquid-liquid extraction	Internal method
Benzene derivatives		
Chlorobenzenes		
1,2,4,5-tetrachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Hexachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Pentachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Monochlorobenzene	HS/GC/MS	NF EN ISO 11423-1
Bromobenzene	HS/GC/MS	NF EN ISO 11423-1
2-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
3-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
4-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
1,2-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,3-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,4-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,2,4-trichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,3,5-trichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
Sum of trichlorobenzenes	HS/GC/MS	NF EN ISO 11423-1
Sum of dichlorobenzenes	HS/GC/MS	NF EN ISO 11423-1
Sum of monochlorotoluenes	HS/GC/MS	NF EN ISO 11423-1
Chloronitrobenzenes		
1-chloro, 4-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
1-chloro, 2-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
1-chloro, 3-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Phenol derivatives		
Chlorophenols		
2-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
3-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
4-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4-dichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4,5-trichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4,6-trichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
Pentachlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
Alkylphenols		
4-nonylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-n octylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-tert octylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Sum of octylphenols	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Nonylphenol (CAS 25154-52-3)	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Sum of nonylphenols	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-tert butylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Other compounds		
4,4'-biphenol	GC/MS/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Tributyl phosphate	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Hexachlorocyclopentadiene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468

DAD (Diode-Array Detection), FLUO (Fluorescence), GC (Gas Chromatography), HPLC (High Performance Liquid Chromatography), HS (HeadSpace), MS (Mass Spectrometry).

FOLIO ADMINISTRATIF

THESE DE L'UNIVERSITE DE LYON OPEREE AU SEIN DE L'INSA LYON

NOM : GOSSET

DATE de SOUTENANCE : 06/04/2018

Prénoms : Antoine, Clément, Alain, Serge

TITRE : Evaluation de l'écotoxicité des Rejets Urbains par Temps de Pluie : Développement d'une batterie de bioessais et application à la conception de biocapteurs.

NATURE : Doctorat

Numéro d'ordre : 2018LYSEI027

Ecole doctorale : Chimie, Procédé, Environnement

Spécialité : Environnement

RESUME : Les Rejets Urbains par Temps de Pluie (RUTP) représentent une pollution très complexe et variable de par la diversité des événements pluvieux et des bassins versants lessivés. Les RUTP sont, dans la majorité des cas, rejetés dans des milieux récepteurs aquatiques péri-urbains tels que les lacs, rivières ou eaux souterraines, sans traitement d'épuration. La pollution déversée, qui peut être très diluée, est le plus souvent liée à des événements relativement courts et difficiles à prévoir. L'impact écotoxique des RUTP peut donc s'avérer difficile à évaluer, en particulier par des mesures directes *in situ*. Parmi les organismes utilisés en écotoxicologie, les microalgues sont extrêmement intéressantes. En effet, elles constituent la base des réseaux trophiques, sont sensibles à une large gamme de polluants et sont très sensibles à la présence de substances exogènes. C'est la raison pour laquelle nous avons développé dans un premier temps une batterie de biomarqueurs cellulaires sur microalgue (perturbation de la physiologie (comme la photosynthèse) de *Chlorella vulgaris*), afin de montrer leur intérêt pour détecter rapidement et sensiblement l'impact toxique d'échantillons de RUTP collectés sur la région Lyonnaise. La réponse de ces biomarqueurs a été comparée en laboratoire à une batterie de bioessais écotoxicologiques monospécifiques classiques sur microalgues et microcrustacés (e.g. essais de croissance, reproduction). Dans un second temps, nous avons travaillé à l'adaptation de ces biomarqueurs afin de créer des outils de détection *in situ*. Des biocapteurs à cellules entières, basés sur une mesure de la perturbation de la photosynthèse (fluorescence chlorophyllienne) de microalgues, ont été développés. Pour leur création, deux techniques de mise en contact bio-récepteur/transducteur ont été testées : (i) la double encapsulation des microalgues dans des hydrogels alginate/silice utilisant un procédé sol-gel, et (ii) l'inclusion des microalgues dans des puces microfluidiques fabriquées par xurographie. Une station portative autonome de terrain a été élaborée et testée avec efficacité pour effectuer des mesures *in situ* de la toxicité des RUTP, et des milieux aquatiques urbains contaminés.

MOTS-CLÉS : Rejet Urbain par Temps de Pluie, Ecotoxicologie, Microcrustacé, Microalgue, Biomarqueurs, Bioessai, Biocapteur, Fluorescence chlorophyllienne, Hydrogel, Microfluidique, Xurographie.

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