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Characterization and evolution of natural aquatic biofilm communities exposed in vitro to herbicides

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ABSTRACT

River biofilms are assemblies of autotrophic and heterotrophic microorganisms that can be affected by pollutants such as those found in watersheds and wastewater treatment plants. In the laboratory, experimental biofilms were formed from river water, and their overall composition was investigated. Denaturing gradient gel electrophoresis (DGGE) and cytometry were used to assess the richness and diversity of these communities. The software Cytostack (available on request) was developed to treat and analyze the cytometric data. Measurements of chlorophyll-*a* and carotenoids were used to assess the global composition of the photoautotrophic community, whereas proteins, polysaccharides (PS) content, and esterase activities were used to assess overall changes in the mixed communities. We evaluated the effects that 3 weeks of treatment with the herbicides diuron and glyphosate (10 $\mu g\,L^{-1}$) had on these biofilms. Exposed to diuron, bacterial communities adapted, changing their composition. Glyphosate inhibited growth of one autotrophic community but caused no chlorophyll deficit. As a whole, the biofilm acted as a micro-ecosystem, able to regulate and maintain a constant level of photosynthetic pigment through the structural adaptation of the autotrophic community. These results are one more proof that microbial diversity of aquatic biofilms is influenced by chemical stresses, potentially leading to disturbances within the ecosystems.

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1. Introduction

In many aquatic ecosystems, most microbes live in matrix-enclosed biofilms and contribute substantially to energy flow and nutrient cycling (Battin et al., 2003; Costerton et al., 1987). Aquatic biofilms are complex, natural assemblages of micro-organisms that develop on submerged substrata. They typically consist of bacteria, micro-algae (mainly diatoms), protozoa and fungi embedded in a polymeric matrix. They can adapt their characteristics (composition and density of the species, extracellular matrix content) to environmental variations, and so can be used as biological indicators of these variations (Sabater et al., 2007). Because of their short-generation time, these micro-organisms respond quickly to disturbances and are thus regarded as early-warning indicators in detecting the effects of toxicants.

Chemical pollutants such as herbicides, widely used in agriculture, considerably affect aquatic biofilms because lotic aquatic ecosystems are one of the terminal receptors for these compounds. Most of these organic molecules directly affect the autotrophic communities of natural ecosystems, principally algal communities which are able to release organic compounds and thereby indirectly to disturb the heterotrophic bacterial community (Ricart et al., 2009; Sabater et al., 2007). Glyphosate and diuron are among the most frequently detected herbicides in France (SOeS, 2011). Glyphosate [N-(phosphonomethyl)glycine] is a broad-spectrum systemic and non-selective herbicide whose primary mode of action is the disruption of aromatic amino acid biosynthesis, a process that ultimately results in the reduction of protein synthesis and growth and eventually causes cellular disruption (Pérez et al., 2007). It is used to control grasses, sedges and broad-leaved weeds (Goldsborough and Brown, 1988), and under field conditions it is assumed to be rapidly and tightly adsorbed on soil particles. Diuron, N-(3, 4-dichlorophenyl)-N,N-dimethyl-urea belongs to the phenylamide family and the subclass phenylurea. This substituted urea herbicide inhibits photosynthesis by blocking electron transfer at the level of photosystem II of photosynthetic micro-organisms and plants. It is used to control a wide variety of annual and perennial broadleaf and grassy weeds, and mosses (Giacomazzi and Cochet, 2004). The used of diuron has been prohibited in France for vegetation control since 2008, but it

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continues to be detected in rivers (SOeS, 2011) probably because it is used as an antifouling agent and as an algicide in construction.

These molecules may reach aquatic systems either by accidental or wind driven drift of the herbicide spray, or through transportation by surface runoff (Edwards et al., 1980). Their effects are due to their relative long half-life: one month to a year for diuron (Giacomazzi and Cochet, 2004), a few days for glyphosate, and up to 100 days for AMPA (aminomethylphosphonic acid), the main metabolite of glyphosate. In France, glyphosate has been detected in 32% of surface waters at concentrations ranging from 2.1 to 17 $\mu g\,L^{-1}$, AMPA in 56.7% (2.1–18.8 $\mu g\,L^{-1}$) and diuron in 35%, at concentrations ranging from 2.1 to 36 $\mu g\,L^{-1}$ (IFEN, 2007). These concentrations are a contamination according to the French standard: more than 2 $\mu g\,L^{-1}$ make water unsuitable for drinking. Studies assessing the effect of pesticides on aquatic microbial community have primarily focused on isolated autotrophic organisms and have shown a wide range of EC (effective concentration) or LC (lethal concentration) values, indicating different sensibilities according to herbicide, species used, and experimental conditions (Berard and Pelte, 1999; Lipok et al., 2010). But these studies are not enough to predict significant effects on individual communities and, eventually, on the community as a whole (Geiszinger et al., 2009). For example, one study on a single species and microcosm showed differing responses to the herbicide isoproturon (Traunspurger et al., 1996). The influence of herbicides on autotrophic communities has been investigated (Berard et al., 2003; Berard and Pelte, 1999; Devilla et al., 2005), but the effects on the bacterial compartment have been studied more rarely (Araya et al., 2003; Brummer et al., 2003; Tadonléké et al., 2009; Vercraene-Eairmal et al., 2010), except in soil (el Fantroussi et al., 1999; Imfeld and Vuilleumier, 2011). Likewise, if many studies have focused on the phytoplanktonic compartment (Pesce et al., 2010b; Pérez et al., 2007; Pérès et al., 1996; Vera et al., 2010), some have looked at the overall microbial composition of biofilms (Dorigo et al., 2009; Pesce et al., 2009; Ricart et al., 2009; Tlili et al., 2011; Villeneuve et al., 2011a).

The present study was carried out with water coming from the Angaud, a branch of the Jauron River in Central France, 25 km east of the city of Clermont-Ferrand. This sector was chosen because it combines land under intensive crop cultivation (especially maize and other cereals) and built-up areas (the town of Billom, population 4000). It receives herbicides mostly between April and October (Phyt'eauvergne, 2012) and is located at the outlet of a wastewater treatment plant, where most of the anthropogenic pollution (urban and agricultural) is collected.

The aim of these experimental microcosm studies was to characterize and assess the impact of diuron and glyphosate herbicides, at realistic concentrations (10 $\mu g\,L^{-1}$), on natural periphytic microbial communities collected during an uncontaminated period. We determined the overall composition of natural aquatic biofilms grown in microcosms by enumeration with a cytometric approach and "fingerprint" analysis of the microbial community (DGGE). The specific impact of the two herbicides, diuron and glyphosate, on biofilm communities was investigated using several biochemical criteria, including determination of the amounts of chlorophyll-*a*, polysaccharidic substances (PS), and total proteins. We followed the evolution of both autotrophic and heterotrophic communities.

2. Materials and methods

2.1. Chemical compounds

Diuron (45463, Pestanal, CAS: 330-54-1), glyphosate (glyphosate acid, 45521, Pestanal, CAS: 1071-83-6), was purchased from Fluka Riedel-de-Haën (Germany). For all experiments, diuron and glyphosate were dissolved in pure water.

2.2. Biofilm formation within microcosms

To form the biofilms, glass supports (spatulas of 19 cm^2) were immersed for 2 weeks in a homemade aquarium filled with 60 L of water from the Angaud River (Billom, France; X: 726385 -Y: 6515501), renewed twice a week in March and April 2009 for assays involving diuron and glyphosate, respectively. Using a climatic chamber, the experimental environment was maintained at 12 °C with a photoperiod of 12/12 h day/night provided by fluorescent tubes (Cool Daylight 32W/865 TL-D ECO, Phillips) which is an average for the months of March and April. The irradiance level was 80 $\mu E\,m^{-2}\,s^{-1}$. A pump placed at the bottom of the aquarium provided a river-like flow (circa 30 $cm\,s^{-1}$). After the two weeks, each spatula covered by biofilm was individually placed in a microscale bioreactor (micro-fermentor Pasteur) continuously fed with modified BG11 medium mBG11 (BG11 (Fluka, St. Gallen, Switzerland) supplemented with $Na_2SiO_3 \cdot 9H_2O$ 16.33 $mg\,L^{-1}$). mBG11 medium with or without the herbicides was kept in jerrycans in the chamber and prepared every week. Biofilms were then exposed to 0 $\mu g\,L^{-1}$ (control) or 10 $\mu g\,L^{-1}$ of herbicide (diuron or glyphosate) for up to 21 days. At day-0, three spatulas were scrapped and after 2, 7, 14 and 21 days of exposure, the biofilms of 6 spatulas (3 controls and 3 contaminated) were scrapped off, using a natural-rubber scraper, and collected in 20 mL of mBG11 medium each. Homogenization of the samples was realized by repeated passage through a Pasteur pipette and mild sonication (15 min in a Elma S15 sonifier). The resulting suspensions were used to perform the different analyses. The biofilms used in this study were constituted during 2 different periods (March–April 2009) as the climatic chamber was too small to contain both experiments (in triplicate) at the same time. (For details, see Supplementary S1).

2.3. Quantification of total polysaccharides (PS) and proteins

Polysaccharide fractions of the extracellular polymeric substances (PS) were quantified according to a previously described method (Dubois et al., 1956). Briefly, 1 mL of biofilm suspension was added to 1 mL of 5% phenol, and 5 mL of concentrated H_2SO_4 were then added to the mixture. Sugar concentrations were obtained by measuring absorbance at 490 nm using D-glucose as a standard. The quantification of proteins in the biofilm suspension was performed with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories) based on the method of Bradford (1976).

2.4. Measure of total metabolic activity

Nonspecific esterase activities were assessed according to the method described by Bonnet et al. (2003) using a spectrofluorimeter (SFM25, Kontron Instruments) (excitation/emission 485/510 nm) to measure the hydrolysis rate of fluorescein diacetate (FDA). To do so, 200 μL of biofilm suspension were mixed with the FDA solution (5 $mg\,mL^{-1}$) (FDA, Sigma) and fluorescence emitted was measured every minute for 15 min. The slope of each reaction was calculated ($ng\,mL^{-1}\,min^{-1}$) and used to compare and follow each sample. The FDA hydrolysis rate was determined using a standard curve of fluorescein dilution (Fluorescein, Fluka).

2.5. Determination of chlorophyll-a and carotenoid concentrations

Three mL of biofilm suspension were filtered onto a glass microfiber filter GF/F (Whatman), immediately folded in aluminum foil and kept at −80 °C. The day before analysis, the filters were incubated in 3 mL of acetone (90%) and kept overnight at −20 °C. After centrifugation, chlorophyll and pigment levels were determined by spectrophotometry, measuring absorbance at 480, 630, 645, 663, 665 and 750 nm. The amounts of chlorophyll-*a* and carotenoids were determined according to the SCOR-UNESCO (1966) equations.

2.6. Characterization of microbial communities

2.6.1. Flow cytometry

Cell counting was done by flow cytometry. To disperse cells before fixation and staining, the biofilm suspensions were sonicated in presence of pyrophosphate according to Velji et al. (1986). Samples were sonicated 1 min at 0 °C (10 s sonication/20 s cooling x6) with 10 mM pyrophosphate on a Sonopuls (HD2070, Bandelin). Cells were fixed with 2% formaldehyde and stained with fluorochrome SYBER® Green II added to 10^{-2} dilution of commercial stock (Molecular Probes, Eugene, OR). The samples were incubated for 30 min at room temperature before analyzing them with the flow cytometer apparatus (FACSCalibur®), Becton Dickinson), with excitation by the argon laser blue line at 488 nm. Green fluorescent bacterial cells stained by SYBER® Green were analyzed in FL1 channel (530 ± 15 nm). Red fluorescent cells, corresponding to naturally fluorescent chlorophylls, were analyzed in FL3 channel (> 630 nm). Analyses were performed at a high rate setting, with an acquisition period of 1 min. To avoid coincidence, samples were run such that the event rate was below 1000 s^{-1}. The forward

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