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Responses of lake bacterioplankton activities and composition to the herbicide diuron

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ABSTRACT

The direct effects of pesticides on aquatic bacteria are poorly known. We experimentally investigated the direct effects of diuron (herbicide) on the composition and activities of lake bacterioplankton, using Denaturing Gradient Gel Electrophoresis (DGGE), cloning/sequencing, and flow cytometry with dyes that allow detection of dead cells, cells with depolarized membranes and cells with esterase activity (for physiological state). Generally, diuron had negative impacts on bacterial viability and abundance. Bacterial production strongly correlated with ammonium in controls, but not in diuron-treated samples. Moreover the increase in nitrate concentration with the proportion of dead bacteria was significantly higher in controls, providing evidence not previously shown for natural communities, that diuron may alter the mineralization of organic matter and nitrification. A picocyanobacteria and members of the family Flavobacteriaceae, known to degrade complex polymeric organic matter in aquatic systems were negatively affected by diuron. Except that, the DGGE banding patterns in controls and in polluted samples were generally similar, suggesting no perceptible susceptibility of major bacterial groups, and contrasting with previous reports that diuron has a strong impact on bacterial community composition. Our data suggest that diuron may affect functioning of aquatic systems through negative impacts on some bacterial phylotypes and bacterial cycling of nitrogen.

1. Introduction

Phenylurea herbicides are among the most widely used herbicides in non-crop areas, as well as in tree crops (Tomlin, 1994). These herbicides generally degrade slowly in soil and are susceptible to leaching. As a consequence, they have contaminated numerous soil ecosystems and water resources, including surface and ground-waters (Spliid and Koppen, 1998; Strangroom et al., 1998; and references below). Among these phenylurea herbicides, diuron has been one of the most used to kill weeds and mosses. Although authorisations for plant protection products containing diuron have been withdrawn in the European community in 2007, the use of this herbicide in this region was still authorized until 13 December 2008 (articles 1 and 3 of the commission of the European community decision of 13 June 2007). Phenylurea herbicides, including diuron may persist in the environment. Diuron has been detected in many aquatic ecosystems worldwide. For instance, in France, diuron has been detected in 38% of surface waters, with concentrations ranging from 0.05 $\mu g\,L^{-1}$ to 20.3 $\mu g\,L^{-1}$ (IFEN, 2002). Concentrations up to 0.7 $\mu g\,L^{-1}$ have been reported in Mediterranean estuaries (Readman et al., 1993). In coastal waters with ports and marinas in the United Kingdom, concentrations of diuron reaching 6.7 $\mu g\,L^{-1}$ have been found (Thomas et al., 2001). In Japanese aquatic environments, including lakes, 86% of tested samples showed concentration of diuron around 3 $\mu g\,L^{-1}$ (Okamura et al., 2003). There is now evidence from a few studies conducted on soil ecosystems that diuron and other phenylurea herbicides also affect negatively non-targeted communities such as heterotrophic microbes, including bacteria (El Frantoussi et al., 1999; Prado and Airoldi, 2001). Moreover, several studies have been conducted to understand the mechanisms and/or identify the genes involved in the degradation of these herbicides by soil bacteria (Esposito et al., 1998; Turnbull et al., 2001). Comparatively few studies have dealt with the effects of phenylurea herbicides in general, and diuron in particular, on pelagic bacteria in aquatic systems. Furthermore, the studies that have dealt with the effects of herbicides (including diuron) on lake or river bacteria, have seldom examined these effects on the composition of these communities (e.g. Cragg and Fry, 1984; Waiser and Robarts, 1997) or have analyzed the whole microbial communities or the whole plankton without testing the direct effects of these pollutants on bacterial communities (Pesce et al., 2006; Sumpono et al., 2003). Tests of the direct effects of diuron on bacteria are usually performed on bioluminescent-based bacterial biosensors (Strachan et al., 2001). The above remarks are

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also true for most studies testing the effects of other pesticides on aquatic bacteria (e.g. Friberg-Jensen et al., 2003; Knapp et al., 2005). Hence, the direct effects of diuron and many other pesticides on natural aquatic bacteria are poorly known, and it is unclear whether the changes in the dynamics of natural bacteria, reported in the presence of diuron or other pesticides by previous experimental works (Friberg-Jensen et al., 2003; Knapp et al., 2005; Pesce et al., 2006; Sumpono et al., 2003; Waiser and Robarts, 1997), are caused by these pollutants directly or indirectly by virtue of their impacts on the targeted photosynthetic microorganisms, known to fuel aquatic bacteria with dissolved organic matter. Understanding the direct effects of diuron and other pesticides on bacteria is of interest, given that alteration of bacterial communities might affect ecosystem function.

In this study we have experimentally examined the direct effects of the herbicide diuron on lake bacterioplankton. We have analyzed changes in bacterial community composition, and, in contrast to previous studies on this topic, we have examined the physiological state of bacteria at single-cell level for a better understanding of their responses to the herbicide. This was addressed by analyzing bacterial communities using flow cytometry and dyes that allow detection of dead cells, cells with depolarized membranes and cells with esterase activity. We expected that if diuron has negative impacts on bacterial abundance and activity, as found for microbes in soils, then our polluted samples will have more dead bacteria, and this will significantly modify the coupling of bacteria with nutrients, in comparison with the non-polluted samples.

2. Materials and methods

2.1. Experimental setup

A 13-day-experiment (16–28 August 2006) was conducted *in situ* in Lake Geneva to test the direct effects of the herbicide diuron on bacterioplankton. After collection, samples were gently (<50 mmHg) filtered on 1 μm pore-size polycarbonate filters to remove grazers and other larger planktonic organisms. The filters were changed regularly to minimize clogging and cell bursting. This filtrate, which contained free-living bacteria and allowed the test of the direct effects of diuron, was sub-sampled for analyses of initial bacterial composition, and thereafter distributed in the experimental flasks (4 L polycarbonate bottles, Nalgene). Removal of larger particles normally eliminates grazer impacts on bacteria and allows observation of the links between these microheterotrophs and nutrients (Pace and Cole, 1994; Tadonléké, 2007). Three treatments with diuron (1 $\mu g\,L^{-1}$, 5 $\mu g\,L^{-1}$ and 50 $\mu g\,L^{-1}$ final nominal concentrations, respectively) and one without diuron (control) were prepared, each in triplicate. The lowest concentration tested here is commonly found and even exceeded in natural aquatic systems (see introduction). The diuron solution was prepared in Dimethyl Sulfoxide (DMSO). DMSO was added to the control at a final concentration nearly identical to that in the diuron-treated samples (<0.001%). The experimental flasks were thereafter tied randomly on a floating incubation system and incubated *in situ*, at the depth 1 m, where initial samples were collected. A probe recording water temperature every 1 h at the depth of incubation was also tied on the incubation system. For analyses, sub-samples were taken a few minutes after the addition of this herbicide (T_0, initial values), and then 48 h (T_{48}), 120 h (T_{120}), 168 h (T_{168}), 192 h (T_{192}) and 264 h (T_{264}) after the beginning of the incubation. Bacterial variables were analyzed at almost all these time points, and included total bacterial abundance, bacterial community composition (not analyzed at T_{168}) and production, the abundance of bacteria with esterase activity, the abundance of dead bacteria, and the abundance of bacteria with depolarized membranes, i.e., with altered membrane potential. Nutrients (nitrates, ammonium, nitrites and dissolved inorganic phosphorus) were analyzed at T_0, T_{120} and T_{264}, while diuron concentrations were measured at T_0, T_{120}, T_{192} and T_{264}.

2.2. Sample analyses

2.2.1. Nutrients

Sub-samples for nutrient analyses were filtered on 0.2 μm pore-size polycarbonate or GTTP membranes. Nutrients were analyzed by standard colorimetric method, using spectrophotometer and auto-analyzers (Metrohm and Alliance Instruments).

2.2.2. Herbicides

Analyses of diuron were performed on a Waters HPLC system (Alliance 2695) coupled to a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) (LC–MS-MS), using a Nucleodur C8 e.c. column (250 mm × 4.6 mm, 5 μm, Machery-Nagel, Hoerdt, France) isocratically with water/acetonitrile 50/50 (v:v) as mobile phase. Samples were kept at 4 °C in the autosampler before injection (5 μL) into the column. The mass spectrometer conditions were as previously described (Perreau et al., 2007). Detection was performed using an electrospray source and the multiple reaction monitoring (MRM) positive ion mode. The limit of quantitation (LOQ) and the limit of detection (LOD) of diuron were 0.17 $\mu g\,L^{-1}$ and 0.05 $\mu g\,L^{-1}$, respectively. These analyses did not reveal the presence of diuron in our control samples. To ensure that diuron was really absent from the control, and to check for the presence of degradation products of this herbicide, further analyses were performed using a homemade coupling of the LC–MS-MS with an on-line solid-phase extraction system. The latter included a preconcentration primary pump P680 (Dionex), a VICI 10-solvent selection valve (Cluzeau-Info-Labo, Sainte-Foy-la-Grande, France) and a Brownlee precolumn holder connected to a Rheodyne 7010 six-port switching valve. HPLC column, LC–MS-MS device and MS conditions were the same as for the previous analyses. Details of the analytical procedure can be found elsewhere (Perreau et al., 2007). Although diuron and one of its metabolites (DCPMU = 3,4-dichlorophenyl-methylurea) were detected during these additional analyses, their concentrations were below the LOQ (LOD and LOQ = 3 $ng\,L^{-1}$ and 10 $ng\,L^{-1}$, respectively, for DCPMU). We thus considered in this study, only the concentrations that were above or equal to the LOQ.

2.2.3. Bacterial production

Bacterial production was estimated from ^{3}H-Leucine incorporation by bacteria (Kirchman, 1993). The saturating concentration used (60 nM) and the incubation time (2 h) were determined prior to the present study (Tadonléké DR, unpubl. data).

2.2.4. Bacterial abundances

Bacterial abundances were determined using a flow cytometer (FacsCalibur, Becton-Dickinson) equipped with a laser emitting at 488 nm. Yellow-green 0.92 μm fluorescent latex beads were used as internal standard. Samples were run at low speed (10–12 $\mu L\,min^{-1}$). Total bacterial abundance was determined using the nucleic acid dye SYBR Green II as described elsewhere (Tadonléké et al., 2005). For the determination of the abundance of bacteria with intracellular esterase activity, the abundance of dead bacteria and the abundance of bacteria with depolarized membranes, the concentration of the dye and the incubation time were optimized prior to the present study (Tadonléké DR, unpubl. data). The abundance of bacteria with esterase activity was determined using the dye CFDA (carboxyfluorecein diacetate, Fluoroprobes) prepared in DMSO (Hoefel et al., 2003; Porter et al., 1995). CFDA is cell permeant; once in the cell, it undergoes