Various formulations of a bacterial consortium to optimize diuron and glyphosate mineralization

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Introduction

Phosphonates and phenylurea herbicides are widespread compounds used in France for weed control in crop and non-crop areas. Both herbicides are recovered in soils, sediments and water. Herbicides such as glyphosate (N-(phosphonomethyl)-glycine) and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) were detected in 36 and 34% of the samples respectively from superficial water in the French river basin in 2004 (IFEN, 2006). Microbial degradation i.e. biodegradation, is considered as the primary mechanism responsible of the pesticide mitigation in soil (Araujo et al. 2003). Bioremediation treatments are then suitable because of their low cost along with their low environmental impact compared to chemical treatments.
In this study we aimed at studying a consortium of three bacteria recognized to be able to (i) mineralize glyphosate (*Pseudomonas* 4ASW), (ii) degrade diuron into 3,4-DCA (*Arthrobacter* sp. N4), and (iii) mineralize 3,4-DCA (*Delftia acidovorans*). The experiments were performed in both a synthetic culture medium without phosphorus and a sediment extract medium whose composition was close to the sediment composition used in further bioaugmentation studies. The three strains were co-cultivated as free and/or immobilized cells in Ca-alginate beads to optimize glyphosate and diuron mineralization.

Material and methods

Microbial strains and preculture conditions

A strain of *A*. sp. N4 was previously isolated from a soil contaminated with diuron for many years (Widehem et al. 2002). *D. acidovorans* W34 was previously isolated from a 3,4-DCA-degrading culture originating from a linuron contaminated soil (Dejonghe et al. 2003). *P.* 4ASW was isolated from a soil for its ability to utilize glyphosate as a phosphorus source (Quinn et al. 1989).

Cell immobilization

According to previous experiments (Jezequel et al. 2005), 100 ml of a sterile solution of sodium alginate (30 $g.l^{-1}$) was mixed with the bacterial preculture in order to obtain a final concentration of 1.2×10^7 $cell.ml^{-1}$. Ca-alginate beads of about 3 mm diameter were obtained by dropping the alginate cell mixture into a solution of $CaCl_2$ (30 $g.l^{-1}$).

Mineralization of diuron and glyphosate

Culture medium: experiments were performed in Erlenmeyer flasks filled with 75 ml of (i) a complete mineral salts medium (MM) or (ii) a sediment extract (SE) medium (sediment mixed with the same weight of water (tap water) and autoclaved at 130° C for 1 h. The filtrate was autoclaved at 115° C during 0.5 h). All the flasks were incubated at 28° C and 200 rpm.
Culture conditions: The concentration of diuron and glyphosate in the culture medium was 20 $mg.l^{-1}$ and 80 $mg.l^{-1}$ respectively. The culture media (MM or SE) were inoculated with free (FC) and/or immobilized cells (IC) from the preculture. All combinations of the three microorganisms were tested. For IC, 5 g of alginate beads ml^{-1} were added to each flask. The final concentration of FC and IC was 10^6 cells. ml^{-1} of culture medium.

Analysis

For FC, growth was determined by measuring optical density OD_{600}. For IC, growth was determined by measuring OD_{600} of 10 beads from each flask after dissolving in 3 ml of sodium tricitrate (50 mM). Concentrations of glyphosate, diuron and 3,4-DCA were measured with HPLC. All the experiments were performed in triplicate. Results were shown as means ± confidence intervals.

Results and Discussion

By comparison with the synthetic medium, maximum biomass was reduced when using the sediment extract medium, for all microbial formulations. Regarding *P.* 4ASW cultivated as a pure culture, its biomass was significantly reduced with sediment extract medium compared to synthetic medium (177 vs. 353×10^7 cell.ml^{-1} respectively). It might have been the result of the phosphate presence in the sediment-extract medium compared to the phosphate-free synthetic medium. It was indeed previously reported that the growth of *P.* 4ASW was higher with glyphosate as the sole source of phosphorus (Dick et al. 1995) while the sediment extract medium contained glyphosate and phosphate. In addition, the sediment extract medium was probably poor in some nutrients compared to the synthetic medium. Hence the co-culture of *A.* sp. N4 and *D. acidovorans* W34 exhibited a lower growth in the sediment extract medium. The presence of both glyphosate and phosphate in addition to diuron was not reported to display any negative effect on growth compared to our previous experiments with the same strains, cultivated with a glyphosate-free sediment extract medium only supplied with diuron (Bazot et al. 2007).

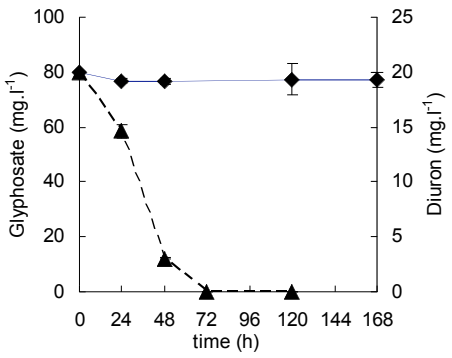

Figure 1: Degradation of diuron (□) and glyphosate (□) by a co-culture of free cells of *D. acidovorans* W34 and immobilized cells of *A.* sp. N4 and *P.* 4ASW cultivated with sediment extract medium supplied with diuron (20mg.l^{-1}) and glyphosate (80mg.l^{-1}). Vertical bars indicated confidence interval (n=3).

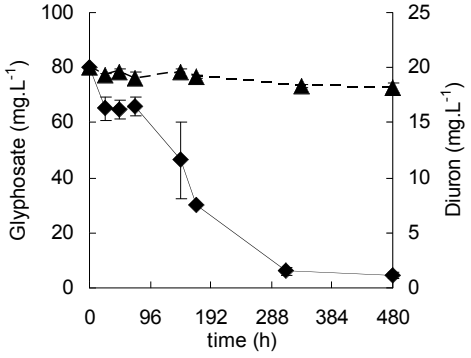

Figure 2: Degradation of diuron (▲) and glyphosate (♦) by a co-culture of free cells of *A.* sp. N4, *D. acidovorans* W34 and *P.* 4ASW cultivated with synthetic medium supplied with diuron (20mg.l^{-1}) and glyphosate (80mg.l^{-1}). Vertical bars indicated confidence interval (n=3).

Glyphosate was mineralized within 72h when *P.* 4ASW was cultivated with the synthetic medium, with a maximum specific degradation and maximum degradation rates of 2.31mg10^6 cells and 3.05 mg. l^{-1}.h^{-1} respectively. Yet it was not degraded in the sediment extract medium even when *P.* 4ASW was immobilized in alginate beads. It was due to phosphate in the medium, as already shown (Hallas et al. 1988). All the diuron, however, was mineralized by co-cultivated *A.* sp. N4 and *D. acidovorans* W34 cells whatever the culture media might have been, as previously shown within sediment extract medium (Bazot et al. 2007). The best performance for the mineralization of diuron, i.e., 72h (Fig. 2) and 0.482 mg. l^{-1}.h^{-1} was observed with co-immobilized sp. N4 and *P.* 4ASW and free *D. acidovorans* W34 cultivated with the sediment extract medium.

The cell growth of the consortium and the herbicide degradation rate of the mixture were closely related to the microbial formulation and the composition of the culture medium; both parameters

acted on the competition among strains. *P.* 4ASW was probably favoured when the three strains were co-cultivated as free cells with the synthetic medium. For only glyphosate was mineralized within 312h at a rate of 0.981 mg. $l^{-1}.h^{-1}$ (Fig. 1), the maximum biomass of the consortium being close to that recorded with *P.* 4ASW cultivated alone (422×10^7 *vs*. 353×10^7 cell.ml^{-1}).

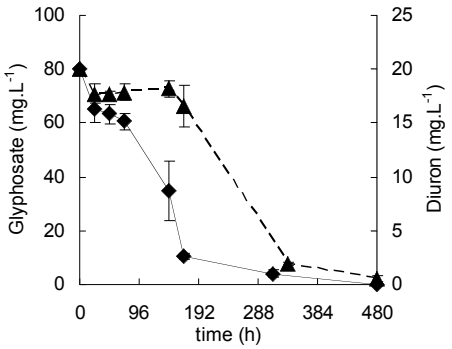

Figure 3: Degradation of diuron (□) and glyphosate (□) by a co-culture of immobilized cells of *A.* sp. N4 and *D. acidovorans* W34 and free cells of *P.* 4ASW cultivated with synthetic medium supplied with diuron (20mg.l^{-1}) and glyphosate (80mg.l^{-1}). Vertical bars indicated confidence interval (n=3).

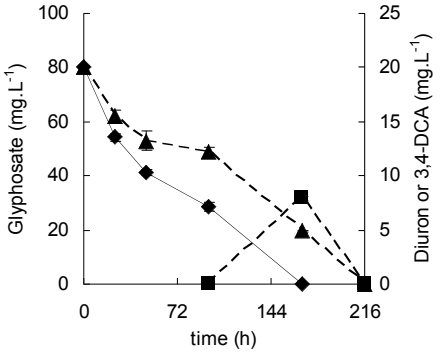

Figure 4: Degradation of diuron (▲) and glyphosate (♦) by a co-culture of free cells of *A.* sp. N4, *D. acidovorans* W34 and immobilized cells of *P.* 4ASW cultivated with synthetic medium supplied with diuron (20mg.l^{-1}) and glyphosate (80mg.l^{-1}). 3,4-DCA (■) was also monitored during incubation. Vertical bars indicated confidence interval (n=3).

All other microbial formulations allowed the mineralization of both glyphosate and diuron. Two different mineralization kinetics were observed depending on whether *P.* 4ASW was cultivated as free or immobilized cells. In the first case, when P. 4ASW was cultivated as free cells, a nearly sequential mineralization was observed, glyphosate being first mineralized (Fig. 3). On the other hand a simultaneous mineralization of glyphosate and diuron was observed in a shorter time compared with free *P.* 4ASW cells and required at least 168h and 216h respectively with immobilized cells of *P.* 4ASW, the two other strains being cultivated in a free mode (Fig. 4). When *P.* 4ASW was cultivated as free cells with at least one of the two other strains being immobilized, the main part of the nutrients were most probably consumed by *P.* 4ASW since the cell concentration was nearly that recorded with *P.* 4ASW alone. Despite this strong competition among strains, populations of *A.* sp. N4 and *D. acidovorans* W34 did not disappear but remained low until glyphosate was mineralized, preventing any diuron mineralization. When glyphosate was exhausted (87% up to 100% within 168h), the population of *P.* 4ASW felt allowing *A.* sp. N4 and *D. acidovorans* W34 to grow and mineralize diuron: 83 up to 91% of diuron mineralized between 168h and 480h. Thus, immobilization of one or both strains, i.e., *A.* sp. N4 and *D. acidovorans* W34 modified the environmental conditions allowing to preserve the cells viability that multiplied when glyphosate was exhausted. Moreover *A.* sp. N4 and *D. acidovorans* W34 most probably benefited from the cells lysis of *P.* 4ASW used as a nutrient supply. Immobilization of *P.* 4ASW strongly modified the microbial equilibrium most probably by lowering the cell growth of this strain to the benefit of *A.* sp. N4 and *D. acidovorans* W34, even when the three strains were co-immobilized. The creation of favorable micro-environments within beads allowed the expression of the three bacteria. This favourable microbial distribution can explain why both diuron and glyphosate were simultaneously mineralized within 216h (Fig. 4).

Thus immobilization is an efficient method to reduce the competition between bacteria growing in the same medium and to optimize pollutant mineralization (Lebeau et al., 1997). More generally bioaugmentation of soil by immobilized inoculants was shown to enhance cell survival (Cassidy et al. 1996). Immobilization in natural polymers such as alginate is currently used for their non

microbial toxicity and the protection of the microorganisms against the toxicity of xenobiotic compounds. Microbial immobilization in alginate beads was also recognized to enhance the degradation rates of pesticides (Cassidy et al. 1997). Immobilized cells exhibited however a lower maximum biomass than free cells most probably as the consequence of the lower diffusivity of nutrients, which modified the nutritional status in beads.
We thus hypothesized that the culture medium acted also on the equilibrium among strains. Diuron mineralization was optimized when at least one of the two strains of *A.* sp. N4 and *D. acidovorans* W34 was immobilized as previously shown (Bazot et al. 2007). Higher diuron degradation rates with the sediment extract medium were most probably due to the lower competitiveness *P.* 4ASW compared with the two other strains.

Conclusions

From these results immobilization can be seen as a relevant method for the implementation of microbial consortia when trying to avoid competition. However, phosphate diffusion into the beads cannot be avoided by alginate beads used in this study, leading to the inhibition of glyphosate degradation. Phosphate was also shown to be responsible of the solubilization of beads (Lebeau et al. 1998). The simultaneous mineralization of glyphosate and diuron thus will require testing appropriate carriers.
To conclude, the co-degradation of glyphosate and diuron, involving many enzymes in organophosphate assimilation and metabolism, appears to be subjected to phosphate regulation. Indeed glyphosate degradation is efficient only in phosphate limited environments (Mc Grath et al. 1997). Nevertheless, although diuron and 3,4-DCA toxicity in environment is higher than glyphosate, their mineralization was easier under natural conditions. Implications for assessing the environmental risk associated with the use of such chemicals should be studied in more details.

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