Influence of fungal inoculant type and water potential on degradation of mixtures of simazine, dieldrin and trifluralin in soil-based media

S Fragoeiro, A Elyassi, N Magan
Applied Mycology Group, IBST, Cranfield University, Silsoe, Bedford MK45 4DT, UK
Email: n.magan@cranfield.ac.uk

ABSTRACT

A wide range of basidiomycetes has been screened on soil-based media at two water potentials for the ability to degrade mixtures of simazine, dieldrin and trifluralin. The best treatments were found to be *Trametes versicolor*, *Trametes socotrana*, *Polystictus versicolor* and *Pleurotus ostreatus*. These were generally better than *Phanerochaete chrysosporium* in media containing 5-10 ppm of the pesticides. In soil microcosms with two different water potentials mixtures of *P. versicolor* and *T. socotrana* or *P. versicolor* alone were able to degrade >75% of a 5 ppm mixture when inoculated on straw in field capacity soil over a 75 day incubation period. Under water stress conditions the degradation rates were reduced. These studies suggest that it is important to include varying environmental conditions in bioremediation experiments to identify and optimise fungal inoculants for effective breakdown of recalcitrant chemicals in soil.

INTRODUCTION

While a significant amount of research has been carried out on bioremediation of individual pesticides, less research has focussed on mixtures of pesticides. In the present study we have concentrated on a mixture of three pesticides, simazine (a triazine herbicide), dieldrin (organochlorine insecticide) and trifluralin (a dinitroaniline) herbicide, all on the UK "Red List".

Although the soil system is a dynamic one in which fluctuations of environmental factors, especially of temperature and water potential, occurs continuously, few studies have included an examination of these in relation to the degradation of xenobiotics in soil (Kostowska & Rola, 1984; Carter, 1991). Other important factors are soil pH and nutrient status. Indeed, organic matter amendments and aeration have been used previously to enhance the degradation of simazine (Ahonen & Heinonen-Tanki, 1994). Ritter & Scarborough (1995) suggested that optimum conditions for the biological activity at a bioremediation site included pH values of 6.5-8.5, temperature of between 27-35°C, and an organic carbon:nitrogen:phosphorus ratio of 300:15:1. However, the critical factor of water potential was not considered.

The objectives of the present study were: (a) to screen a range of white rot fungi for tolerance and growth on a range of individual and mixtures of simazine, dieldrin and trifluralin under two different water potential regimes and (b) to evaluate fungal species for remediation in soil microcosms held at different steady state water potentials.

MATERIALS AND METHODS

In vitro studies

Fungal species used in this study were *Pleurotus ostreatus*, *Polystictus versicolor*, *Trametes versicolor* and *Phanerochaete crysosporium.*

Initial screening was carried out on soil extract agar (Lang *et al.*, 1997) which was modified to –0.7 and –2.8 MPa water potentials. The soil extract agar was modified with mixtures of pesticides in the range 0 to 10 ppm by addition to the molten agar, thoroughly mixed and poured into 9-cm Petri plates. These were overlayed with sterile cellophane and centrally inoculated with a 4-mm agar plug from the margin of a growing colony of each fungal species. Care was taken to avoid puncturing the cellophane layer to avoid any direct contact between fungal colony and pesticide containing soil extract agar. The temporal growth was used to obtain the growth rates from the linear regression lines of the linear phases. Experiments were carried out with three replicates per fungal treatment and pesticide condition at 15°C. Pesticides obtained from Merck (dieldrin); Dow Agrosciences (trifluralin) and Riedel-de Haen (simazine) were dissolved in methanol.

Soil studies

A soil moisture adsorption curve was made by thermocouple psychrometry. The sandy loam soil (PT008) was supplied by Levington Agriculture. It contained 15% clay, 19% silt, 66% sand, 1% organic matter, with a pH of 6.2. The pesticides (5 and 10 ppm) were added to soil dissolved in acetone (dieldrin and trifluralin) and in tetrahydrofuran (THF, simazine). After thorough mixing, the appropriate amount of water was added to obtain field capacity, -0.065 and –0.28 MPa water potentials and allowed to equilibrate for 24 hrs at 4°C with regular mixing. Moist sterile straw segments (<1 cm lengths) colonised by *P.versicolor* or *T.socotrana* for up to 12-14 days were used as an inoculum. 1 g of straw was added to 10 g soil. For a mixed fungal inoculum 0.5 g of each fungal inoculant was used. Soil microcosms were incubated at 15 and 20°C at the three different water potentials and triplicate samples destructively sampled after 28, 42 and 70 days.

Soil samples (10 g) were extracted in 15 ml acetone, sonicated for 10 mins, shaken at 400 rpm for 10 minutes. This was left in the dark at room temperature for 10 mins to allow the soil to settle. The solvent was filtered through a 0.2µm syringe filter prior to analysis by HPLC. A gradient HPLC methods was used using a Envirosep-PP column with a gradient of 75:25% water:acetonitrile for 15 mins, then changed to 50:50 and back to 75:25 in preparation at 30 mins for the next sample. The flow rate was 1 ml min^{-1}. This enabled simazine to be eluted at 4 mins, dieldrin at 17 mins and trifluralin at 20 mins. The acetonitrile phase contained 2% (V/V) THF to help sharpen the peaks. This method gave recoveries of 86.2, 84.8 and 70% for simazine, dieldrin and trifluralin respectively in the same run.

RESULTS

In vitro studies on soil extract agar

Figure 1 shows that all the species grew best at –0.7 MPa water potential on soil extract agar. Overall, *P.versicolor* and *P.ostreatus* and *T.versicolor* were all able to tolerate and grow in the the presence of a mixture of up to 10 ppm of the three pesticides. However, at –2.8 MPa

water potential the growth rates were significantly reduced. These fungal treatments were generally much better than *P.chrysosporium* which is often used as a bioremedial fungal inoculant.

In situ studies in soil microcosms

The effect of temperature of incubation (20 and 30°C) on rate of breakdown of the pesticides is shown in Table 1. Overall, breakdown was more rapid at 30 than 20°C by both *P.versicolor* and *P.versicolor* +*T.socotrana*. However, generally, co-inoculation of the two fungi resulted in a slower degradation rate than *P.versioclor* alone. In the absence of fungal inoculants, natural degradation was also more rapid at 30 than 20°C.

Table 1. The effect of *P.versicolor* or a mixture of *P.versicolor* + *T.socotrana* on degradation of 5 and 10 ppm of the three pesticides after 70 days at 20 and 30°C in field capacity soil.

	Temp (°C)	Conc	Final concentration in soil (ppm)			
			Control	*P. versicolor* (Pv)	Pv+*T. socotrana*	LSD (P<0.5)
Simazine	20	5	2.8	2.0	1.9	0.160
		10	7.8	5.5	5.3	0.272
	30	5	1.0	1.0	1.1	0.169
		10	3.1	1.9	2.1	0.266
Dieldrin	20	5	4.1	2.8	3.1	0.283
		10	8.8	6.4	7.0	0.250
	30	5	4.0	2.5	3.0	0.261
		10	8.4	6.1	6.2	0.266
Trifluralin	20	5	3.2	0 (28 days)	0	0.142
		10	7.2	0 (28 days)	0	0.227
	30	5	1.4	0 (28 days)	0	0.173
		10	3.6	0 (28 days)	0	0.209

Figure 2 shows the impact of changing water potential on degradation of the mixture of pesticides at 20°C over a 70-day incubation period by *P.versicolor*. The relative efficacy of *P.versicolor* was variable with the most significant degradation occurring for trifluralin in field capacity soil. There were also marked differences between the capacity for degradation at different water potentials. With a mixture of pesticides, the degradation of simazine and dieldrin was less than that for trifluralin and also less than that obtained when pesticides were present individually.

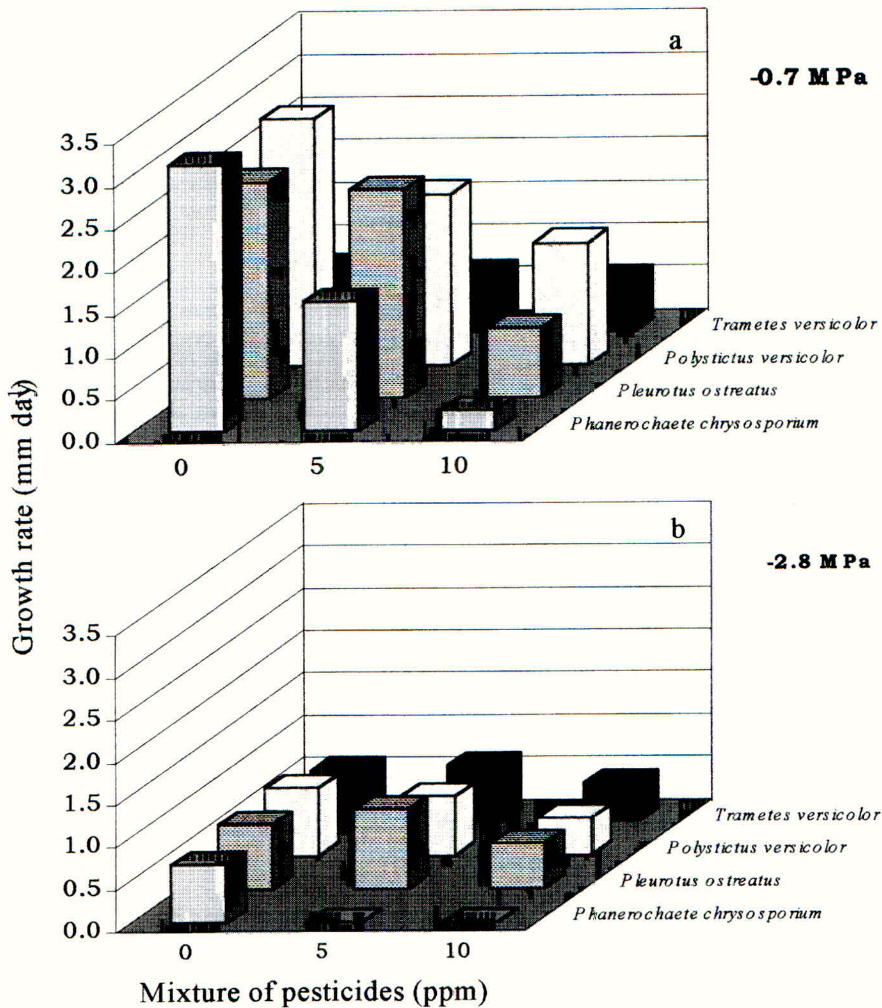

Figure 1. Growth rate (mm day^{-1}) for *Pleurotus ostreatus*, *Polystitcus versicolor*, *Trametes versicolor* and *Phanerochaete crysosporium*, exposed to a mixture of simazine, trifluralin and dieldrin at 0, 5 and 10 ppm at two water potentials (-0.7 and –2.8 MPa).

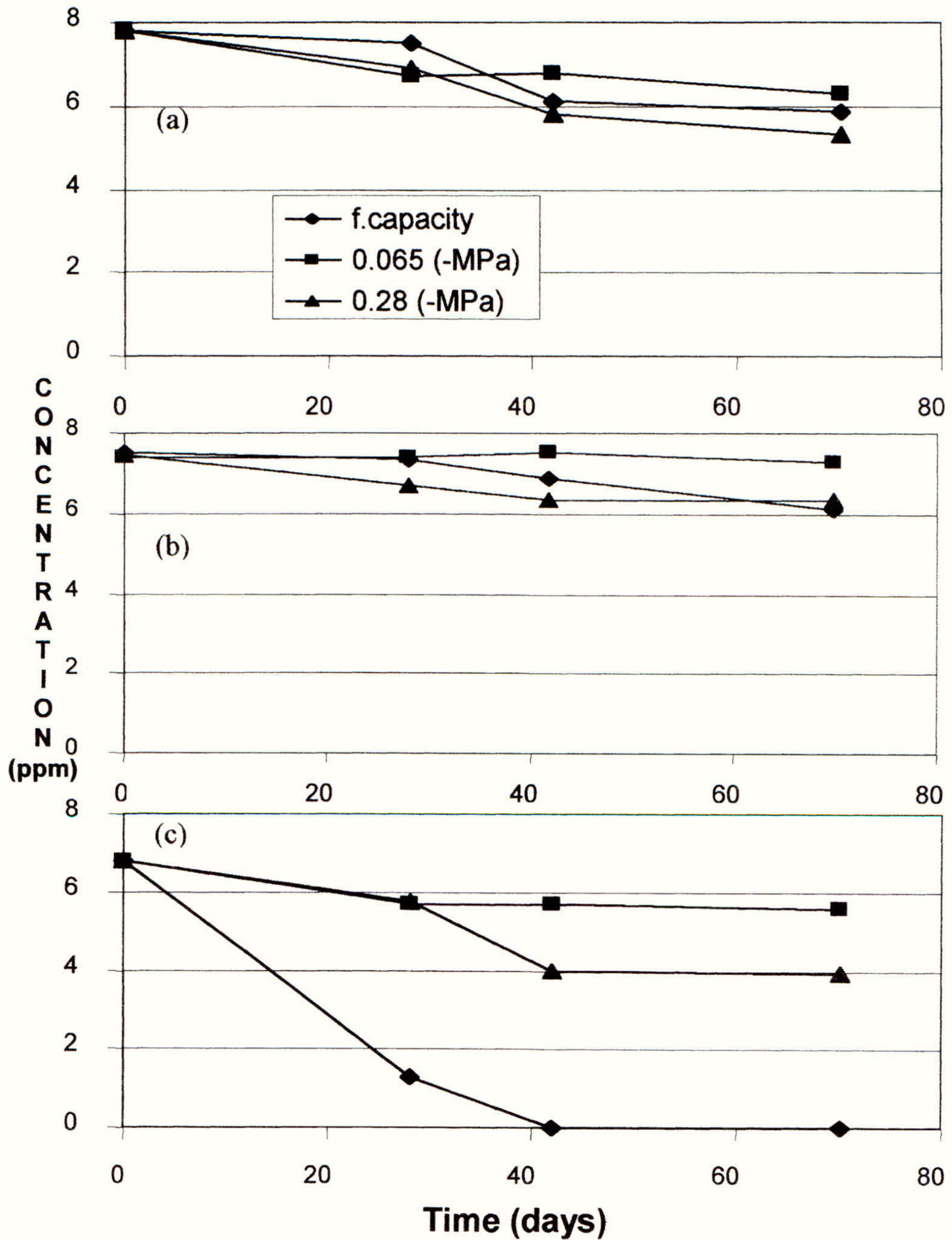

Figure 2. Effect of water potential on the ability of *Polystictus versicolor* to simultaneously breakdown (a) simazine, (b) dieldrin, and (c) trifluralin added at an initial concentration of 10 ppm. The LSDs ($P<0.05$) were 0.19, 0.18 and 0.12 respectively.

DISCUSSION

This study has shown that some basidiomycete fungi are able to produce the necessary extracellular enzymes which enable growth on up to 10 ppm of a mixture of the three pesticides under study under a wide range of water potential regimes. Fungal species examined were better that *P.chrysopsorium*, strains of which are most often used for aerobic bioremediation of pesticides in soil (Cameron *et al*., 2000). The impact of water potential and temperature are significant and it is important that these factors are taken into account when examining potential microbial inoculants.

Studies in soil microcosms suggest that the use of mixed fungal inoculants may not be as effective as individual species because of possible antagonism between species. Where mixtures of microorganisms are not antagonistic, then perhaps additive or synergistic effects may be potentially possible. For example, while bacteria are very sensitive to slight changes in soil water potential, fungal inoculants are much more tolerant. Thus, it may be possible to combine such inoculants for more effective bioremediation of pesticides in soil.

The use of carriers for inoculants is important as cheap and available substrates are needed for effective distribution in soil. We have found a 1:10 ratio effective. However, it may be possible to use carriers such as alginate pellets or other immobilisation systems for incorporation into soil. It is important that environmentally realistic screens are used to identify candidate microbial species for degradation of individual and mixtures of pesticides. This is important if inoculants are to be effectively used practically to enhance degradation of the wide range of xenobiotics in contaminated soils. Studies are now in progress in our laboratories to optimise such systems for optimising the bioremedial effectiveness of such inoculants.

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The microbial degradation of nM-/μM concentrations of linuron estimated by a *Lemna minor* bioassay

K Hulsen, M Höfte
Department of Crop Protection, Laboratory of Phytopathology, Ghent University, Belgium
Email: khulsen@hotmail.com

ABSTRACT

In this work we studied the degradation of linuron by a microbial consortium (isolated from a linuron-treated orchard) and the strain *Variovorax paradoxus*, which is probably the main actor in this consortium. We used a plant-microbial bioassay, in which the aquatic macrophyte *Lemna minor* L. (duckweed) was used to monitor the biodegradation of linuron when this herbicide was applied to liquid cultures in nano- and micromolar concentrations. The toxicity of linuron to *L. minor* was assessed by several growth-related parameters such as total frond number, total frond area and doubling time. Log-logistic based dose-response analysis revealed significant growth inhibition of *L. minor* after 7 days of exposure to linuron concentrations ≥ 80 nM. The relationship between herbicide dose and plant response was described most accurately by using an equation that is modified for growth stimulation at lower doses. The microbial consortium and the *V. paradoxus* strain significantly protected *Lemna* plants from the toxic effects of concentrations up to 1.28 μM linuron. These results suggest that both inocula were capable of degrading nanomolar concentrations of linuron but there was indication that the consortium performed better than the pure isolate. In addition to the growth-related parameters, chlorophyll *a* fluorescence imaging was used to estimate the outcome of the plant-microbe-toxicant interaction by measuring the specific plant metabolic processes affected by linuron.

INTRODUCTION

Lemna species (duckweed) are often used as a bioindicator to assess phytotoxicity of environmental contaminants (Marwood et al., 2001) or herbicides, including phenylurea herbicides such as diuron (Teisseire et al., 1999). Similarly, Siciliano *et al.,* (1997) reported on the use of prairie grass species as bioindicators to evaluate the outcome of the biodegradation of 2-chlorobenzoic acid by a bacterial inoculum.

In this work we show that duckweed can also be used to study biodegradation of herbicides. This investigation forms part of a larger study about the biodegradation of phenylurea herbicides by a bacterial consortium, which was isolated from a pear orchard with a long history of linuron treatment. When investigating the effect of a biological treatment (bacterial inoculation) in a bioassay, it is important to evaluate and choose between different methods and parameters to estimate the bioindicator response. The following questions were addressed: what is the relationship between the herbicide dose and the response of this aquatic macrophyte? Which parameters are the most effective at expressing this relationship? Is it possible to prevent linuron damage to this sensitive plant by inoculating the plant nutrient solution with a linuron-degrading bacterial consortium or a linuron-degrading bacterium?

To our knowledge, this is the first study to use a *Lemna minor* bioassay to estimate biodegradation efficiency of nM-/μM concentrations of linuron.

MATERIALS AND METHODS

Plant material: *Lemna minor* (duckweed) was collected from an artificial pond of the botanical garden of the Faculty of Sciences, Ghent University. A stock culture was maintained on mineral medium in a controlled environment as described by Teisseire & Vernet (2000). The pH of the medium was adjusted to 6.5 and the plants were grown under static conditions in a plant growth chamber at 25 ± 2°C. Light was provided with an intensity of approx. 40 μmol PAR m^{-2} s^{-1}. For the growth experiments, 30 fronds free from any visible chlorosis were taken from the stock cultures and exposed to the according treatment. For each treatment we used 6 repetitions with 5 fronds/ repetition as initial frond number. The experiments were repeated four times independently from each other.

Enrichment culture. A mixed culture of linuron-degrading bacteria was obtained by the enrichment culture technique from a pear orchard at the Royal Research station of Gorsem (Sint-Truiden, Belgium) as described by El Fantroussi *et al.,* (1999). The bacterial consortium and *Variovorax paradoxus,* which is probably the main actor in the consortium responsible for the degradation of linuron (W Dejonghe, personal communication), were maintained on a minimal medium supplemented with 25 mg/L linuron as sole carbon and nitrogen source. The inoculum was prepared after subculturing 10% (v/v) of the initial culture to new flasks, which were incubated for four days at 140 rpm and 28° C. After four days 40 ml of this culture was centrifuged at 5000 rpm for 20 minutes. The supernatant was discarded and the cell pellet was resuspended again with 54 ml of sterile water. From this suspension 1 ml was added to 9 ml of the Lemna nutrient medium (10 ml /repetition).

Chemicals: analytical grade linuron (3-[3,4-dichlorophenyl]-1-methoxy-1-methylurea) with a purity of 99% was purchased from ChemService (West Chester, U K). Linuron was added to the medium as a solution in methanol (25 mg/L) to give a final concentration range between 0.0025 and 0.32 mg/L (10 –1280 nM).

Phytotoxicity of linuron to *L. minor*: the dose-response of the common duckweed was evaluated by various parameters. The corresponding dataset for each parameter was submitted to a log-logistic analysis in order to generate a specific dose-response-curve for each parameter. The log-logistic model as described by Streibig & Kudsk (1993) and Seefeldt *et al.*,(1995) was used to analyze our data. The commonly used sigmoidal model was modified according to Brain & Cousens (1989) to fit our data at the lowest concentrations.

Growth rate was assessed by total number of fronds, doubling time and total frond area. The data represent the mean and standard deviation of measurements performed on six repetitions per treatment. Total frond area was measured by a Windows based MICRO IMAGE (Olympus) software package and the acquired data were submitted to statistical analysis.

Chlorophyll a fluorescence took place in a Chl *a* fluorescence imaging system that was described by Lootens & Vandecasteele (2000). Only far-red fluorescence (730-740 nm) was measured and provided us with images of ground-fluorescence (F_0) upon radiation with a weak irradiance source (1.6 μmol m^{-2} s^{-1}).

Statistical analysis Each concentration was tested in sixfold and four independent experiments were carried out. All data represent mean values for that particular parameter and were calculated/ compared by one way ANOV A or a non-parametric test. Statistical analysis was performed by means of the SPSS 9.0 statistical software package.

RESULTS

Dose-response of *L. minor* to linuron

To asses the toxicity of linuron to *Lemna minor* we used the log-logistic model (Streibig & Kudsk, 1993) which was modified as proposed by Brain and Cousens (1989) to describe the dose-response relationship since our data plot suggested stimulation of growth for the lowest doses (0.0025 and 0.005 mg/L, 10 and 20 nM respectively).

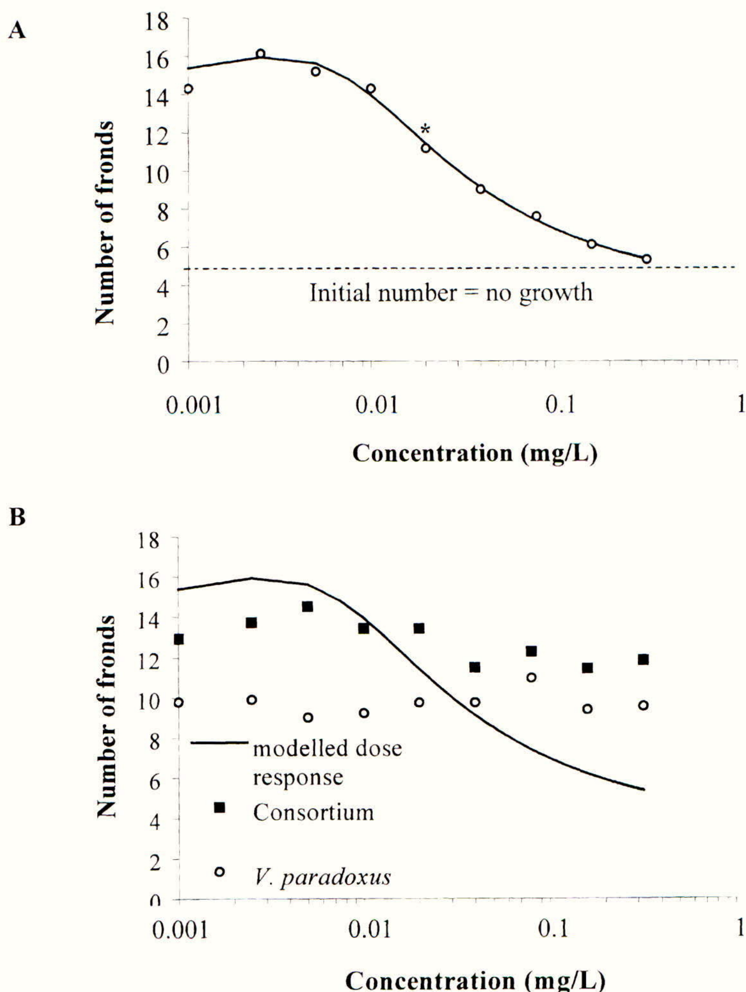

Figure 1. A) Dose-response of *L. minor* (duckweed) to different concentrations of linuron. Asterisk represents the lowest observable effect level, which was found to be 0.02 mg/L. B) Effect of the bacterial consortium and *V paradoxus* on *L. minor* growth.

In total, *L. minor* was exposed to 9 different linuron doses, which were carefully selected to cover the whole range of responses from no visible effect to complete death/ growth inhibition. When various growth parameters were compared according to the model of Brain & Cousens (1989), the total number of fronds parameter gave the most satisfactory fit. Using this parameter it was shown that 0.02 mg/L was the lowest concentration of linuron that caused a significant inhibition of growth after 7 days of exposure to the herbicide (Figure 1A). The circles in this figure represent the experimental data while the curve describes the dose-response calculated according to the model of Brain & Cousens (1989).

In the presence of both bacterial inocula we observed an altered reponse of the *Lemna* plants (Figure 1B). The consortium was capable of protecting *Lemna* plants from the phytotoxic effects of linuron for the whole range of concentrations applied. In contrast, we observed a significant negative effect of the *V. paradoxus* strain when applied together with the lower concentrations of the herbicide. Inoculation with *V. paradoxus* was only beneficial at concentrations ≥ 0.04 mg/L (Figure 2).

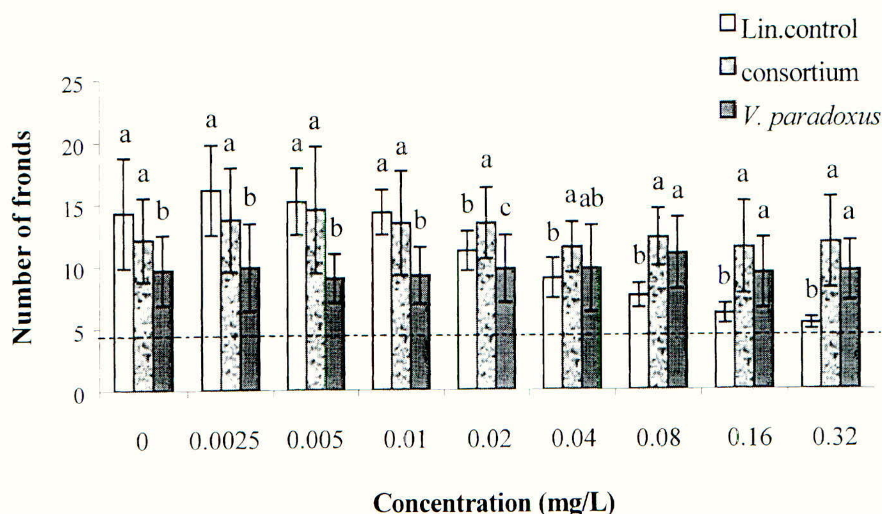

Figure 2. The effect of inoculation with a bacterial consortium or a *Variovorax paradoxus* strain on the number of fronds in a *Lemna* bioassay. Different letters above each column represent significant differences at the 5% level.

Chlorophyll a fluorescence imaging

Chl *a* fluorescence was used to determine if the poor level of protection by *V. paradoxus* could be attributed to a difference in metabolism in comparison to the consortium. For this purpose we followed the initial level of fluorescence F_0 of the *L. minor* plants during the first days of the experiment. Since linuron is known to block electron transport and quantum conversion it can be expected that a loss of photosynthetic function (and an increase in F_0) will occur with increasing exposure time. After 1 h exposure of the plants to 0.32 mg/L (= 1.28 μM) linuron we detected an increased F_0 signal when compared to the untreated control. After 48 h, even the effect of 0.005 mg/L became obvious (Figure 3 picture 1-3).

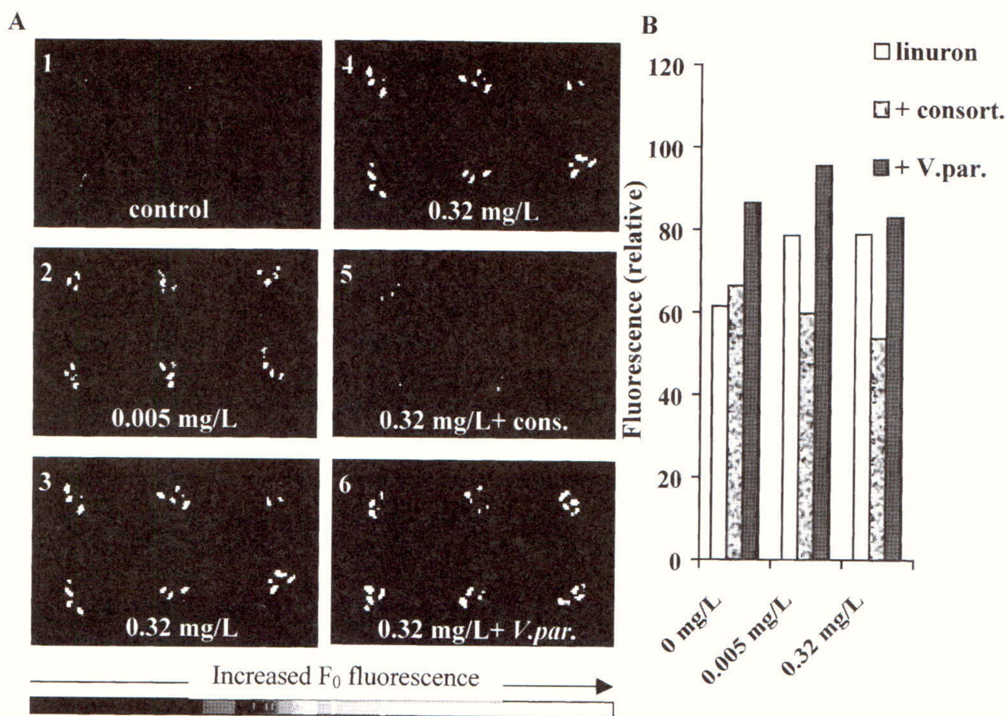

Figure 3. A) Chlorophyll fluorescence images of F_0 taken after 48 h of exposure to the herbicide. B) The graph gives a comparison between the F_0 signal for the different biological treatments measured at 48 h after exposure.

The increasing brightness in the left hand images (1-3) reflects the increased F_0 fluorescence intensity, which is indicative for the herbicide uptake. In an attempt to understand the response of the plants to the different treatments, it became evident that the *V. paradoxus* strain had a negative effect on plant growth. This was in contrast with the observed protective effect when inoculated with the consortium as suggested by a lower F_0 signal (picture 5). Within 48 h exposure of the *Lemna* plants to linuron and/ or the bacteria an increased F_0 was typical for the control plants (without linuron) which were inoculated with *V. paradoxus*. This trend was also observed when the bacterium was inoculated in combinatium with 0.005 and 0.32 mg/L of linuron. In contrast, a significant positive effect on the total number of fronds was observed after 7 days for concentrations > 0.04 mg/L. It appears that the plants were able to recover from the stress caused by residual concentrations of linuron they were exposed to during the first days, which could explain the growth recession when compared to the treatments inoculated with the consortium. A recovery from sublethal doses of a herbicide has previously been shown with *Vicia faba* (Vidal *et al.*, 1995) and in our experiments this suggests a slower or partial degradation of these concentrations in comparison to the bacterial consortium. The origin of the negative effect of *V. paradoxus* on plant growth for concentrations below the L.O.E.L. remains unanswered.

The *Lemna* bioassay inoculated with the bacterial consortium did not show any increase in the ground fluorescence level when the bioassay was spiked with 0.005 mg/L or 0.32 mg/L linuron. This also demonstrates that the degradative capacities of the consortium are more

pronounced in comparison to the *V. paradoxus* strain, even when substrate concentrations are very low.

ACKNOWLEDGEMENTS

We would like to thank P Lootens and P Vandecasteele for providing the chl fluorescence images. The help of L Maeghe with the dose-reponse analysis method is also gratefully acknowledged.

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