Biodegradation (2010) 21:21–29
DOI 10.1007/s10532-009-9278-7

ORIGINAL PAPER

A new concept for reduction of diffuse contamination by simultaneous application of pesticide and pesticide-degrading microorganisms

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Received: 24 March 2009 / Accepted: 11 June 2009 / Published online: 26 June 2009

Abstract Pesticide residues and their transformation products are frequently found in groundwater and surface waters. This study examined whether adding pesticide-degrading microorganisms simultaneously with the pesticide at application could significantly reduce diffuse contamination from pesticide use. Degradation of the phenoxyacetic acid herbicides MCPA (4-chloro-2-methylphenoxyacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) was studied in soil microcosm experiments after simultaneous spraying of herbicide and herbicide-degrading bacteria on an agricultural soil and on a sand with low degradation potential. The latter represented pesticide use on non-agricultural soils poor in microbial activity. Degradation and possible loss of herbicidal effect were also tested in a system with plants and the amounts of bacteria needed to give satisfactory MCPA-degradation rate and the survival of degrading bacteria in formulated MCPA were determined. The results showed >80–99% degradation of 2,4-D and MCPA in soil within 1 day and >99% within 3 days after inoculation with 10^5–10^7 herbicide-degrading bacteria g^{-1} dry weight of soil. Enhanced degradation of MCPA was also obtained in the presence of winter wheat and white mustard without loss of the intended herbicidal effect on white mustard. The survival of an isolated MCPA-degrading *Sphingomonas* sp. in three realistic concentrations of formulated MCPA was very poor, showing that in practical applications direct contact between the microorganisms and the pesticide formulation must be precluded. The applicability and economic feasibility of the method and the information needed to obtain a useable product for field use are discussed.

Keywords Pesticides · Diffuse pollution · *Sphingomonas* · Bioprophylaxis · Bioaugmentation

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Introduction

When pesticides are sprayed onto crops, a large amount of the active ingredient often does not actually reach its intended target (Ravier et al. 2005). These pesticides and their transformation products, plus residues from pesticides that actually reach the target, can subsequently be transported to other environmental compartments, as shown for instance by their frequent detection in groundwater and surface waters (Gilliom 2007; Maloschik et al. 2007; Törnquist et al. 2007; Hildebrandt et al. 2008; Schipper et al. 2008).

Such off-target movement of crop protection compounds is increasingly coming under public and regulatory pressure, necessitating the development of new innovative concepts to obtain sustainable and environmentally friendly pesticide use. To achieve this goal, problems with point sources from handling of pesticides and diffuse sources after spraying in the field must be addressed. One effective and simple biological method to reduce contamination from point sources is the use of biobeds (Castillo et al. 2008). However, contamination from diffuse sources is more complicated to deal with.

Diffuse losses of pesticides after use in the field can occur by several different routes, e.g. as volatilisation, surface transport and leaching through the soil profile to drainage pipes and groundwater (Carter 2000). The magnitude of losses emanating from these processes depends among other factors on the amount of pesticide exposed to the environment and the duration of the exposure. An efficient reduction in diffuse contamination could be obtained if such exposure were to be reduced by introduction of microbial strains that give rapid degradation of the pesticide as soon as it has had its intended effect. The need for such enhanced degradation is particularly pressing for many non-agricultural uses of pesticides, where both pesticide binding and degradation capacity are often low, e.g. on roadsides, railroad embankments, paths, farmyards and in cities (Spliid et al. 2004).

This concept of enhanced contaminant degradation by inoculation with degrading microorganisms is not new and has been extensively studied for bioremediation purposes (Schroll et al. 2004; Singh et al. 2004; Grundmann et al. 2007). However, there are some difficulties with such an approach when dealing with aged contaminants that have been in contact with soil for a long time. Since pesticide availability in soil can be determined by time-dependent processes controlled by non-equilibrium sorption (Steinberg et al. 1987; Johannesen et al. 2003; Jensen et al. 2004), to be efficient such a concept requires desorption and transport of the more or less available compounds towards the applied microorganisms for enhanced degradation to occur. In addition, the microorganisms applied must survive and remain active for extended periods in the natural ecosystem (Schroll et al. 2004). These difficulties are of no concern in our new concept because the bioavailability of non-aged pesticides for degradation is at its maximum immediately after application, concurrent application of pesticide and degrading microorganisms minimises the diffusion paths needed for their contact and the microorganisms only need to be viable and active for the intended short period for complete degradation to occur. However, other concerns could be relevant, for instance the amounts of microorganisms needed to give a satisfactory degradation rate could be so high that the cost becomes prohibitive, the pesticide could be degraded before it has had time to exert its intended effect, and pesticides or their commercial formulations could be harmful to the survival and activity of the microorganisms.

In order to study the potential of our concept and to address the above-mentioned concerns, soil microcosm experiments were performed in which the degradation of the herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid) was studied after simultaneous spraying of herbicide and herbicide-degrading microorganisms. MCPA and 2,4-D are systemic, phenoxy acid herbicides, used for post-emergence control of annual and perennial broadleaved weeds. They are absorbed through the leaves and are commonly used for the control of weeds in cereal crops, grass swards and lawns.

In the first experiment, degradation in an agricultural soil with 2,4-D as test compound and *Cupriavidus necator* JMP134 as test organism was studied. Thereafter, degradation of MCPA was studied in the same soil but now with plants present to study the effect of inoculation with an MCPA-degrading enrichment culture on degradation and herbicidal efficacy. Finally, in order to mimic pesticide use on non-agricultural soils poor in microbial activity, degradation of MCPA in sand was studied. In this case, an MCPA-degrading *Sphingomonas* sp. isolated from the enrichment culture was used as test organism at two different inoculum sizes. In addition, its survival in solutions of formulated MCPA was determined.

Materials and methods

Model herbicides

Technical MCPA (4-chloro-2-methylphenoxy acetic acid, 95% purity) was purchased from Sigma Aldrich Sweden AB and technical 2,4-D (2,4-dichlorophenoxy acetic acid, 98% purity) was obtained from Dr

Ehrenstorfer (Augsburg, Germany). Formulated BASF MCPA 750 (MCPA dimethylamine salt, 750 g a. i. L^{-1}) was obtained from BASF AB, Sweden. All other chemicals and solvents used were of analytical grade.

Microorganisms

The microcosm experiment with 2,4-D was performed using the 2,4-D-degrader *C. necator* JMP134, formerly *Alcaligenes eutrophus* JMP134 (Stenström 1989). The MCPA-degrading consortium used for microcosm experiments with plants was enriched on MCPA (see below for enrichment procedure). The MCPA-degrading strain T51 used for the microcosm experiments in sand was isolated from the MCPA-degrading consortium and was determined by the German Collection of Microorganisms and Cell Cultures (DSMZ) to most probably be a new species within the genus *Sphingomonas*.

Enrichment and isolation of MCPA-degraders

For enrichment and maintenance of the MCPA-degrading consortium, a minimal medium with MCPA as substrate was used (MMM). It contained (per litre of distilled water): 50 mg MCPA, 1.0 g NH_4NO_3, 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.2 g $MgSO_4 \times 7H_2O$, 0.1 g NaCl, 0.026 g $CaCl_2 \times 2H_2O$ and salt and vitamin solutions as previously described (Mandelbaum et al. 1993). The pH was set to 7.3 and the MCPA solution was prepared by dissolving MCPA in 0.1 M NaOH. The stock solution of the magnesium salt was steam-sterilised separately. The salt, vitamin and MCPA solutions were filter-sterilised separately. Two gram of soil from a former herbicide production plant at Teckomatorp, Skåne, Sweden, were added to 20 mL of the MMM medium and the microorganisms were allowed to grow to the stationary phase. One milliliter of this culture was again cultured to the stationary phase in 20 mL of MMM medium. This procedure was repeated three times, after which this enriched MCPA-degrading consortium was diluted in MMM and spread on MMM-agar plates containing 300 μg MCPA mL^{-1} to obtain single colonies. The isolate *Sphingomonas* sp. T51 was selected for further experiments due to its potential to degrade MCPA as sole source of carbon and energy.

Preparation of inocula

Unless otherwise stated, liquid cultures were shaken at 150 rpm in 100 mL Erlenmeyer flasks with a working volume of 20 mL. Determination of colony forming units (CFU) was performed by spreading serial dilutions on a Tryptic Soy Broth (9 g Merck TSB L^{-1}) agar (15 g Oxoid agar L^{-1}) medium and counting the colonies after 3 days of incubation at 25°C.

Inocula of *C. necator* JMP134 for the 2,4-D degradation experiments were grown in a minimal medium with 2,4-D as the sole carbon source. This medium contained (per litre of distilled water): 300 mg 2,4-D, 0.5 g NH_4NO_3, 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.2 g $MgSO_4 \times 7H_2O$ and 0.025 g $FeSO_4$ $(NH_4)_2SO_4 \times 6H_2O$. The 2,4-D solution was prepared by dissolving 2,4-D in 0.1 M NaOH and was filter-sterilised separately. The magnesium and iron salts were autoclaved separately. The pH was set to 7.3 and cells were grown at 25°C to late exponential phase, harvested, centrifuged (10 min, 970*g*), resuspended in phosphate buffer (6 mM, pH 7.3), and adjusted to an optical density corresponding to the desired cell concentration.

Inocula of the enrichment culture and of *Sphingomonas* sp. T51 for the MCPA-degradation experiments were grown at 20°C on MMM overnight, transferred to TSB (9 g L^{-1}) and grown to late exponential phase, harvested, centrifuged (10 min, 970*g*), resuspended in phosphate buffer (6 mM, pH 7.3), and washed twice before being adjusted to an optical density corresponding to the desired cell concentration.

Microcosm setup and sampling

For experiments with soil, 30 g dry weight (dw) of a water-washed sand or a sieved (2 mm sieve size) agricultural loam topsoil (18% clay, 41% silt, 41% sand, 1.9% organic carbon, pH_{water} 6.6) collected in Uppsala, Sweden, were weighed into 9 cm (diameter) glass Petri dishes. Each treatment (control with herbicide only and bacteria added immediately after herbicide addition) contained three replicates for each sampling time. The soil was sprayed with a spray bottle with the herbicide, three replicates were immediately put in the freezer and served to give the initial dose applied, and the inoculated treatments were sprayed

with the bacterial solution. Technical 2,4-D (3 μg g^{-1} dw of soil) and 10^5 cells g^{-1} dw of soil were used in the experiments with the agricultural soil and formulated MCPA (BASF MCPA 750, 3 μg of MCPA g^{-1} dw of soil) and two inoculum sizes (10^5 and 10^7 cells g^{-1} dw of soil) in the experiments with washed sand. The soils were incubated at 20°C in the dark and soil moisture content was kept constant at 60% of water holding capacity (WHC) during the experiment by daily watering of the soil with a spray bottle. Destructive sampling of three replicates for analyses of residual herbicide concentrations was made on days 1 and 3 when the soil was mixed and a sub-sample of 10 g was taken for analysis.

For experiment with plants and soil, 30 g dw of the agricultural soil were weighed into 9 cm (diameter) glass Petri dishes. For each treatment (control without MCPA, control with MCPA only and treatment with MCPA added together with MCPA-degrading bacteria) 5 seeds of MCPA-insensitive winter wheat (*Triticum aestivum*) per dish or 7 seeds of MCPA-sensitive white mustard (*Sinapis alba*) per dish were sown in three replicates for each sampling time. After sowing, the dishes were placed in mini-greenhouses with moist fibre-cloth in the base. The greenhouses were tightly sealed to avoid soil drying and were then placed in cultivation chambers at a constant temperature of 15°C and extra light for 18 h per day. After 5 days, plants were thinned to leave 4 plants per dish. The experiment was initiated 1 week after sowing, when the dishes were sprayed with a spray bottle with technical MCPA to give 4 μg of MCPA g^{-1} dw of soil. Three replicates from each treatment (winter wheat and white mustard) were immediately put in the freezer after removal of plants and roots and served to give the initial dose applied. The soils in the other dishes were sprayed with the MCPA-degrading culture to give 10^5 cells g^{-1} dw of soil. The dishes were reinstalled in the mini-greenhouses and the soil moisture was kept constant at 60% of the WHC during the experiment by daily watering of the soil with a spray bottle. Destructive sampling of three replicates for analyses of remaining herbicide concentrations was made on days 1 and 3. The number of living plants was counted, the plants and the roots were removed, the soil was mixed and a sub-sample of 10 g was taken for analysis. Herbicidal efficacy was determined after 7 days by visual evaluation of the survival of the test plants.

Survival of *Sphingomonas* sp. T51 in formulated MCPA

To determine the ability of *Sphingomonas* sp. T51 to survive in contact with formulated MCPA, three different concentrations of BASF MCPA 750 were used to give 3.75, 7.50 and 30 g L^{-1} of MCPA. Phosphate buffer was used as a control treatment. The experiment was set up in 10 mL of liquid in 20 mL culture tubes inoculated with the strain T51 to a final concentration of 10^7 cells mL^{-1} in two replicates. The CFU count was determined at the start (approximately 5 min after inoculation) and after 1, 4, 8, and 24 h.

Analyses of MCPA and 2,4-D

The soil samples (10 g) were extracted for 24 h with 25 mL of acetone:water:acetic acid (80:19:1), centrifuged at 1,500 rev min^{-1} for 15 min (Sorvall T6000D, Lambda Polynom AB, Sweden) and extracted again for 1 h with 10 mL of the solvent. The extracts were combined and diluted with 500 mL of water and a 70 mL portion was cleaned through 200 mg ENV + columns (Isolute®, International Sorbent Technology, Sorbent AB, Frölunda, Sweden), eluted with 2 × 2 mL of methanol with 5% NH_3 and evaporated under nitrogen gas at 40°C. The samples were derivatised with 8 mL of 0.05 M Na_2HPO_4 (pH 8), 2 mL of 0.1% pentafluorobenzyl bromide in dichloromethane and 150 μL of 0.15 M tetrabutylammonium sulphate, shaken for 30 min and centrifuged at 3,000 rev min^{-1} for 2 min. One milliliter of the dichloromethane phase was transferred into a 2 mL glass vial, evaporated under nitrogen gas and dissolved in 0.5 mL cyclohexane before GC–MS analysis. The analyses were performed using a Hewlett Packard model 6890 gas chromatograph equipped with a HP-5MS capillary column, 30 m × 0.251 mm ID (0.25 μm film thickness) (ChromTech AB, Sundbyberg, Sweden). The oven temperature was programmed to run at 80°C for 2 min and then to increase by 12°C min^{-1} to a final temperature of 280°C. Under these conditions, the retention times for MCPA and 2,4-D were 14.02 min and 14.61 min, respectively. The retention time data were complemented with mass spectral (MS) data obtained from a Hewlett Packard model 5973 Mass Selective Detector. Calibration curves were obtained by plotting peak areas against concentrations for

diluted standards (0.01–1.0 μg mL^{-1}). Recovery rates from the soils spiked with 3–4 μg g^{-1} dw of 2,4-D and MCPA were 90–103%. The limit of quantification was 0.002 μg g^{-1} dw and the limit of detection 0.001 μg g^{-1} dw for both MCPA and 2,4-D.

Results

2,4-D degradation by *Cupriavidus necator* JMP134 in soil

The concept of enhanced degradation was first tested with 3 μg of 2,4-D and 10^5 cells of *Cupriavidus necator* JMP134 g^{-1} dw of soil. This treatment led to very rapid degradation of the 2,4-D, with 99% of the initial amount degraded after 1 day, whereas 90–99% still remained in the uninoculated control (Fig. 1).

MCPA-degradation in the presence of plants

A MCPA-degrading consortium was used as inoculum in a microcosm study in the presence of a MCPA-sensitive plant (white mustard) and a non-sensitive plant (winter wheat). MCPA degradation was markedly improved by the inoculation (Fig. 2). After 3 days, 66–109% of the initial MCPA remained in the control soil, compared with 0.1–2% in the inoculated soil. The herbicidal effect of the MCPA was not diminished or altered, since all MCPA-treated white mustard plants died within 1 week of spraying, while no herbicidal effect was evident in the treatment with the non-sensitive winter wheat (data not shown).

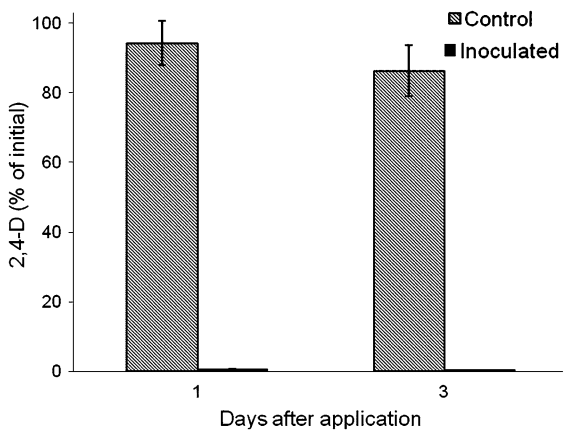

Fig. 1 Degradation of 2,4-D in the arable soil after inoculation with 10^5 cells g^{-1} dw of soil of *Cupriavidus necator* JMP134. Mean values ± SD, $n = 3$. The initial concentration of 2,4-D was 3 μg g^{-1} dw of soil

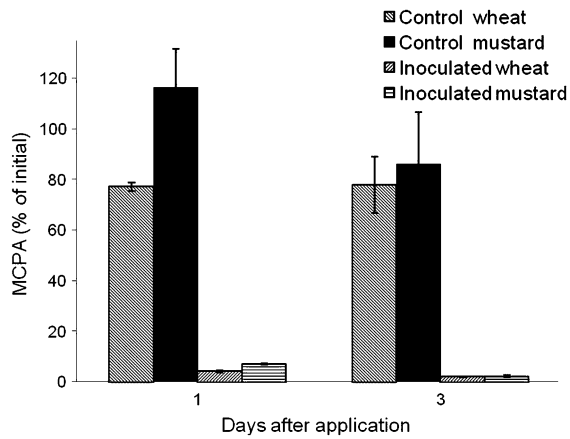

Fig. 2 Degradation of MCPA in an arable soil with plants of white mustard and winter wheat after inoculation with 10^5 cells g^{-1} dw of an MCPA-degrading consortium. Mean values ± SD, $n = 3$. The initial concentration of MCPA was 4 μg g^{-1} dw of soil

Effect of inoculum size on formulated MCPA degradation in sand

Two cell densities (10^5 and 10^7 cells g^{-1} dw of soil) of strain T51 were tested for degradation of MCPA, added as formulated MCPA (BASF MCPA 750) and sprayed on washed sand. The concentration of MCPA was reduced by 79–86% within 1 day and was non-detectable after 3 days in soil inoculated with the lower inoculum cell density (Fig. 3). For the higher cell density, 6–7% of the added MCPA remained after 1 day and the herbicide was non-detectable after 3 days. In comparison, 76–102% of the MCPA remained after 3 days in the treatment without inoculation.

Survival of *Sphingomonas* sp. T51 in formulated MCPA

To determine the ability of *Sphingomonas* sp. T51 to survive in contact with formulated MCPA, 10^7 cells mL^{-1} were mixed with three different concentrations of the formulation BASF MCPA 750 (3.75, 7.50 and 30 g L^{-1} MCPA). The formulated MCPA had a

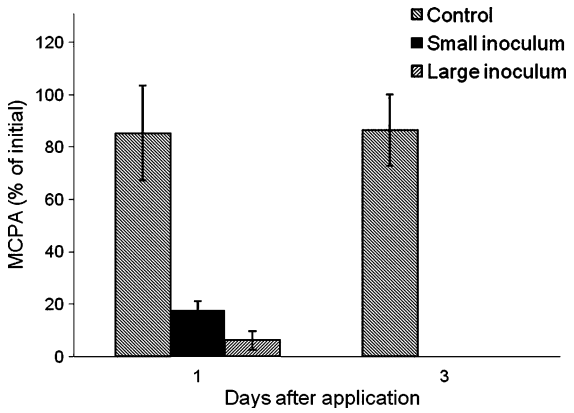

Fig. 3 Degradation of MCPA, added as formulated BASF MCPA 750, in sand after inoculation with a small and a large inoculum size (10^5 and 10^7 cells g^{-1} dw of soil, respectively) of *Sphingomonas* sp. T51. Mean values ± SD, $n = 3$. The initial concentration of MCPA was 3 μg g^{-1} dw of soil

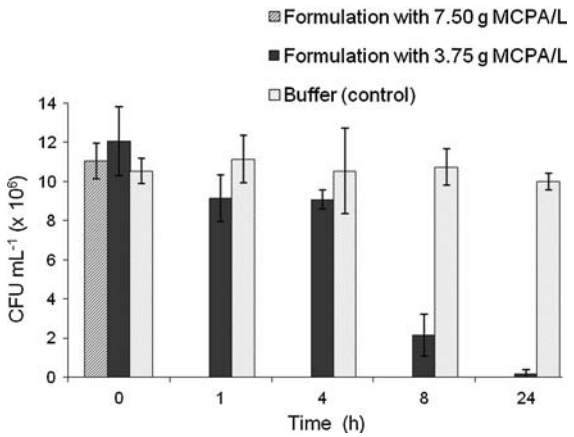

Fig. 4 Survival of *Sphingomonas* sp. T51 in different dilutions of formulated BASF MCPA 750 containing 3.75 and 7.50 g L^{-1} of MCPA. No cells were viable 5 min after mixing into the dilution containing 30 g L^{-1} of MCPA. Mean values ± SD, $n = 2$

concentration-dependent effect on the survival of strain T51 (Fig. 4). At the highest concentration, no cells were viable 5 min after mixing. The number of viable cells in the intermediate concentration was initially unaffected, but decreased rapidly and within an hour no cells were viable. Although the cells did not show a rapid drop in numbers during the initial hours in contact with the lowest concentration of formulated MCPA, the population eventually declined by two log units after 24 h.

Discussion

The use of microorganisms for removal of pesticide contaminants has previously been applied in bioremediation methods, including bioaugmentation, i.e. inoculation with cultures or microbial communities with pesticide-degrading capacity (Singh et al. 2004; Grundmann et al. 2007) or biostimulation, i.e. increasing the pool and activity of indigenous microorganisms in general and thereby also the microbial degradation of contaminants (Moorman et al. 2001). However, these bioremediation methods are intended to facilitate biodegradation of existing contaminants, while our bioprophylactic concept aims to prevent diffuse contamination from pesticide use. The intention is that addition of active microorganisms together with the pesticide at spraying will provide optimum conditions for its degradation, since its bioavailability, the proximity between pesticide and degrading microorganisms and the microbial viability and activity for degradation are greatest immediately after application.

The balance between desirably fast degradation, which reduces persistence and leaching of pesticides, and undesirably fast degradation, which diminishes efficacy, is very delicate. Not all herbicides have as fast a herbicidal action as 2,4-D and MCPA and the same applies for all products with long-time effect based on persistence on target organisms or in soil. Such compounds are thus less suited for our new concept and limit its practical application to products where the active substance is taken up into the target organism. Our main primary focus for the concept is therefore herbicides with a systemic mode of action and comparatively rapid plant uptake.

Enhanced degradation in an agricultural soil was confirmed for 2,4-D by the well-known phenoxyacetic acid degrader *Cupriavidus necator* JMP134 (Fig. 1) (Don and Pemberton 1981; Pieper et al. 1988; Ledger et al. 2006) and for MCPA by an enriched MCPA-degrading consortium (Fig. 2). Enhanced degradation was also confirmed in sand by inoculation with a pure culture of the isolated *Sphingomonas* sp. T51 (Fig. 3).

Enhanced degradation was obtained in the test systems with plants present, with the intended herbicidal effect of MCPA persisting in spite of the MCPA and MCPA-degrading organisms being added almost simultaneously. However, for general practical application with different pesticides and weather conditions, the microorganisms have to be formulated

to ensure that their degrading capacity is not activated before the pesticide has had time to be absorbed into the target organism in sufficient amounts to give its intended effect. In relation to rainfall, this is known as the rainfastness time, i.e. the rain-free period required after application of a pesticide to ensure that its intended effect is not reduced by washing-off. In a compilation of data obtained from pesticide manufacturers, the rainfastness time varies between 10 min and 8 h for 48 foliar herbicide products, with the majority in the time span 1–2 h and with 4 h for MCPA 750 (Swedish Board of Agriculture: http://www.sjv.se/download/18.387a82451207c0cff0880003810/Nr+6.+Regnfasthet+090504.pdf, in Swedish).

To study the relevance of the concept for use in non-agricultural applications, where pesticide degradation rates are potentially low, *Sphingomonas* sp. T51 was tested for its MCPA degradation ability in washed sand (Fig. 3). This material was used to represent the worst case scenario of the coarse and microbiologically poor soils frequently found in railway embankments and other non-agricultural application sites. Herbicides are applied regularly to railway embankments for weed control and environmentally friendly use of herbicides in areas with such soils is a difficult challenge. Studies of the microbiology of railway embankments have shown that MCPA-degraders are often present there, but that both the MCPA-degradation capacity and the general microbial biomass and activity are often very low (Cederlund and Stenström 2004; Cederlund et al. 2007). Fast drainage through coarse soil material and microbial biomass and activity being located mostly in hot-spots, probably due to uneven distribution of organic material (Cederlund et al. 2008), both contribute to increasing the risk of pesticide leaching. Furthermore, the coarse material of e.g. railway embankments favours dry soil conditions and this, together with often low temperatures during large parts of the year in Sweden, limits degradation (Cederlund et al. 2007). In our study, low degradation rates of MCPA were found in the uninoculated sand, with 76–102% of the initial amount still remaining after 3 days. However, inoculation with *Sphingomonas* sp. T51 enhanced the degradation so that MCPA residues were below the limit of detection in 1–3 days.

To reduce the risk of pesticide leaching, the persistence time of a pesticide should ideally not exceed the time for which meteorology can predict with high probability that no rainfall will occur. Nowadays, weather forecasts for the next 24 h in Sweden have an 85% probability of being accurate (the Swedish Meteorological and Hydrological Institute (SMHI), http://www.smhi.se/cmp/jsp/polopoly.jsp?d=9852&a=28411&l=sv, in Swedish). It would thus be desirable for all pesticide residues to be degraded within 24 h of application. Inoculum size is an important factor determining the fate of the target pesticide and can be used as a tool to achieve the required rate of degradation. Furthermore, optimising the amount of inoculum not only ensures rapid and complete degradation, but also sets a limit for the cost of the application. A minimum cell density is often required to achieve enhanced degradation and the degradation rate often increases as the inoculum density increases up to a certain concentration, after which further increases have no or a diminishing effect (Shaw et al. 1997; Labana et al. 2005; Arshad et al. 2008). In the washed sand, both cell densities tested (10^5 and 10^7 cells g^{-1} dw of soil) were sufficiently high to increase the biodegradation rates of MCPA compared with non-inoculated controls and to give persistence times <3 days (Fig. 3). This corresponds to a specific degradation rate of at least 0.5 pg MCPA $cell^{-1}$ h^{-1}, assuming no growth and constant activity of the microorganisms. As a basis for the economic practicability of the concept, the following calculations can then be made: With an initial dose after spraying of 20 μg MCPA g^{-1} in the upper cm of the soil, 10^7 bacteria g^{-1} would degrade this amount in 4 h. With a conservative assumption of 10% active bacteria in the formulation, 40 h would be required. Assuming a soil specific weight of 1 g cm^{-3}, the upper 1 cm layer of soil within 1 m^2 corresponds to a weight of 10 000 g, requiring 10^{11} bacteria, while for one hectare 10^{15} bacteria would be required. With a theoretical fermentation yield of 10^{13} bacteria L^{-1}, 100 L of fermentate would be required for one ha. Thus, with 100% active bacteria, only 10 L of broth would be required per ha for complete degradation in 4 h, or 1.7 L of broth for complete degradation within 24 h at 20°C, or 3.4 L at 10°C, assuming a Q_{10} of 2. This theoretical exercise indicates that given cost-effective fermentation and formulation procedures for pesticide-degrading microorganisms, an economically feasible product can potentially be obtained.

The requirements on the formulation of a microbial product depend on factors such as the intended method for simultaneous spraying of the pesticide and the microbes. One option could be to mix the microorganisms with the diluted pesticide formulation in the spraying tank immediately before spraying, to avoid degradation of the pesticide in the tank. However, in addition to the active ingredient(s), pesticides also contain formulation compounds to optimise the biological activity and to give a product that is safe and convenient to use (Knowles 2008). These components could affect the survival of microorganisms when mixed together, which is why we tested the survival of *Sphingomonas* sp. T51 in formulated MCPA. The three concentrations of MCPA used (3.75, 7.50 and 30 g L^{-1}) are all realistic pesticide concentrations in the spraying tank based on 2 L of MCPA 750 and 50, 200 or 400 L of liquid sprayed ha^{-1}. All three concentrations gave high cell death within 24 h (Fig. 4). Consequently, in practical applications the pesticide and the pesticide-degrading microorganisms should not be mixed within the spraying tank, or if so, the organisms must be formulated to protect them from direct contact with the liquid. Alternatively, the microorganisms could be injected close to or into the spraying nozzles during spraying of the pesticide.

In order to develop the concept of simultaneous spraying of pesticides and their degrading microorganisms, more information and development work are needed. Growth of the degrading microorganisms is not required, due to the large inoculum size used, or expected, due to the high number of organisms in relation to the concentration of the pesticide applied, but the relevant organism must be protected and supported (e.g. by providing water) by the formulation used, and probably also pre-adapted during cultivation in the laboratory to the environmental conditions it can encounter when released in the field (e.g. different temperatures and pH values). The concept must also be tested in the field, e.g. on arable soils and railway embankments. Other important aspects that must be resolved and that are currently being studied at our laboratory are cultivation of microorganisms on an industrial scale to give large, active and efficient biomass; formulation of microorganisms to give a manageable, easily applied and storage-stable product; and methods for safety assessment of the candidate organism to ensure that it is harmless for people and the environment and that it has no properties that would preclude its final use or the registration process for a product containing the organism (Melin et al. 2007).

Acknowledgments This work was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Swedish Foundation for Strategic Environmental Research (MISTRA) by funding the research programme Domestication of Microorganisms (DOM), and the Centre for Chemical Pesticides (CKB) at the Swedish University of Agricultural Sciences.

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