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Assessing the safety of *Pseudomonas putida* introduction in the environment: An overview of ecotoxicological tests

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ABSTRACT

Risk assessment guidelines for the environmental release of microbial agents are performed in a tiered sequence which includes evaluation of exposure effects on non-target organisms. However, it becomes important to verify whether environmental risk assessment from temperate studies is applicable to tropical countries, as Brazil. *Pseudomonas putida* is a bacteria showing potential to be used for environmental applications as bioremediation and plant disease control. This study investigates the effects of this bacteria exposure on rodents and aquatic organisms (*Daphnia similis*) that are recommended to be used as non-target organism in environmental risk assessments. Also, the microbial activity in three different soils under *P. putida* exposure was evaluated. Rats did not show clinical alterations, although the agent was recovered 16 h after the exposure in lung homogenates. The bacteria did not reduce significantly the reproduction and survival of *D. similis*. The soil enzymatic activities presented fluctuating values after inoculation with bacteria. The measurement of perturbations in soil biochemical characteristics is presented as an alternative way of monitoring the overall effects of the microbial agent to be introduced even in first stage (Tier I) of the risk assessment in tropical ecosystems.

1. Introduction

The use of agricultural inputs can lead to an environmental imbalance since the anthropogenic actions frequently transform the agroecosystems. This is extremely important in developing countries like Brazil, in which the economy is supported, to a great extent, by agricultural activities. Thus, as a result of growing interest in the conservation of biological resources and their sustainable use, interest in the use of more economically viable and less aggressive alternatives has been growing considerably. In this context, the use of microbial pest control agents has proven to be an effective and appropriate practice for such purposes.

In the case of inoculative releases of parasitoids and predators, low number of them is usually released in the expectation that the organism released will control the target after multiplication. In contrast, in the control of plant pathogens, larger numbers of microbial antagonists are released in the expectation of immediate effects. An example is the use of living *Bacillus thuringiensis* spores for insect control (Eilenberg et al., 2001).

Formulating biopesticides and the evaluation of ingredients that are cost effective and promote product stability has progressed in the case of several microorganisms over the past decade (Hynes and Boyetchko, 2006; Jayaraj et al., 2005) but many questions remain unanswered. Although the vast majority of microorganisms used as biopesticides are considered to be safe and the natural variability of microbial communities can surpass the effects of a microorganism introduction; the biological microbial agents possess the potential ability to disrupt ecological communities even in a transient form (Bakker et al., 2002).

The possible adverse effects produced by the introduction of a pest control agent such as a microorganism, can be summarized as direct and indirect damage to non-target organisms in the local community, including the flora and fauna representing economical, ecological and/or social importance. The possibilities of survival, multiplication, persistence, dissemination, the establishment of pathogenicity and toxicity are all involved in the risk that a microorganism can present to the environment. In looking ahead to the use of biologically based methods, it is important to adequately address these environmental risks. Their significance will depend on how well the regulatory system prevents the high impacts from occurring. Thus the risk analysis of the use of these agents involves carrying out laboratory determinations before their registration and commercialization.

Toxicological testing as a part of regulatory risk assessment of biopesticides aims at providing sufficiently accurate predictions of adverse effects on human health and the environment to guide risk assessment. These studies are performed in a tiered sequence

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based on regulatory guidelines (USEPA, 1996a,b). In spite of the complex microorganism – host interactions, it is believed that the data from the Tier I battery of tests provide a fairly clear evaluation of the potential risks in most cases.

The first tier consists of maximum dose single species hazard testing on non-target organisms. By definition, a worst-case approach always overestimates the likely situations, because the probability of the simultaneous occurrence of unfavorable circumstances in relation to every risk factor is very low (Hoornstra and Notermans, 2001).

In consequence, the impact or the effect of a risk factor on the environment can be determined using worst-case approaches with a dose 100–1000 times greater than agriculturally dose applied. The tests of non-target aquatic organisms are performed in the green algae *Pseudokirchneriella subcapitata*, the microcrustaceum *Daphnia similis* and *Characidae* fishes, respectively, chosen as phytoplankton, invertebrate and vertebrate representatives of Brazilian ecosystem (IBAMA, 1988). The non-target organisms are examined daily to evaluate the mortality, reproduction or any other adverse observable effect (USEPA, 1996b).

For studies on human health security, rodents such as Wistar rats are used. The basic testing protocols are designed to detect non-target effects as allergenicity, toxicity, infectivity and pathogenicity (Castro et al., 1999; USEPA, 1996a). In Tier I, the animals are observed daily to investigate clinical alterations and during necropsy for any lesions in organs at different intervals after administration. It is recommended to test a dose level of at least 10^8 units of the microbial agent per test animal.

Nevertheless, the prediction of the outcomes and interactions of introducing microorganisms into new environments is difficult (Louda et al., 2005). The expression of a microorganism's presence may result in its insertion into a new habitat, and continued propagation in the new habitat. The determination of the environmental effects of a microbial agent when it is introduced into a new environment includes an evaluation of its growth – the way the microbial agent may alter its growth habits, take advantage of new environmental conditions or take advantage of changes in the existing equilibrium among the microbial species. In 1997, Brazilian Corporation for Agricultural Research (Embrapa) adapted the guidelines for biopesticide risk assessment from the international protocols; that have been adopted in Brazilian legislation.

Prior to registration of microorganisms, Tier I data on non-target organisms should be examined. Environmental expression data (Tier II) may also be required on a case-by-case basis for certain microorganisms which are determined to present unique concerns. The effect assessment of foreign microorganisms introduced into soil environment is not included in Tier I international regulatory guidelines. Tier II ecological tests should be carried out in a greenhouse environment to determine whether the microbial agent is able to survive, persist, and replicate in a terrestrial environment consisting of the soil and vegetation representative of the proposed site. Some parameters as temperature, humidity, precipitation (amount, frequency, pH), sunlight, pH (soil and foliar surfaces) and nutrients (soil, vegetation) should be evaluated to determine their effect on the survival and growth of the microbial agent population. These studies are intended to demonstrate whether the released microbial agent is able to survive or replicate in the environment. If results of this study indicate that the microorganism is able to persist in the terrestrial environment, then the appropriate testing in Tier III is required.

Although resource costs restrictions make extensive testing programs impossible to perform for all biopesticides that are subject to regulation, some additional tests would be needed for some of them to subsidize risk management on the best possible scientific evidence. For that, methods for determining possible non-target effects are required; especially in tropics that show proper environmental conditions and have a rich biodiversity. Thus, testing should also be judged according to how they support risk assessment.

To take a careful look at biopesticide environmental effects, we use some of our studies that examined Tier I effects on non-target organisms by *Pseudomonas putida* to serve as examples. They are also used to discuss the results obtained from Tier I tests guidelines and some new approach tests. These tests, as soil microbial studies, could be introduced in Tier I after the legislation review. They would be required depending on the microbial characteristics as possible persistence in the environment, case-by-case; despite the cost and effort required to conduct the analyses.

Pseudomonas species have a variety of commercial environmental applications. *P. putida* is a ubiquitous soil bacterium that has significant potential for use in biochemical processes, such as the production of natural compounds, the bioremediation of numerous compounds in polluted habitats, and the use of strains to control plant diseases (Schneider and Dorn, 2001). *Pseudomonas* may show potential in the degradation of a wide range of xenobiotics (Walia et al., 2002) and in affecting the rhizosphere during plant growth (Johansen et al., 2005). *Pseudomonas* may also be used as a biopesticide for the management of brown rot disease in apricot (Altindag et al., 2006).

2. Materials and methods

2.1. Tier I biopesticide studies – P. putida isolation and characterization

P. putida AF7 was originally isolated from the rizosphere of rice, grown cultivated on soils historically exposed to the herbicide propanil at Massaranduba, Santa Catarina State, Brazil. The strain was previously characterized by the fatty acid methyl esters procedure. In all experiments AF7 was grown in King' B medium for 24 h at 30 °C.

2.2. Tier I test guidelines for mammals (rats) and aquatic invertebrate

2.2.1. Aquatic organisms

For the purpose of investigating possible adverse effects to aquatic invertebrates resulted from the application of *P. putida*, the microcrustacean *D. similis* was tested as a non-target organism. This organism was cultivated in water prepared according to Hosokawa et al. (1991), Elendt and Bias (1990), at 20 ± 2 °C and luminosity of ~2000 lux. The initial number of neonates (less than 24 h old) in each experimental unit (500 mL final volume) was 12. The treatments evaluated in six replicates were: control (without *P. putida*), *P. putida* inactive (10^6 spores mL^{-1} autoclaved at 121 °C for 20 min) and *P. putida* active (10^6 spores mL^{-1}). Suspensions of the microbial agent and control were renewed at 48, 96 and 168 h each week during the total exposure time of 21 days. Before each renewal of the medium, samples were taken in order to evaluate the number of neonates produced per day (NNPD) by each adult and the rate of survival at the end of the exposure period. The mean NNPD were compared by the *F*-test for contrasts (Montgomery, 1991). The survival rates were compared by the Wald test (Stokes et al., 2000).

2.2.2. Rodents

The effects of *P. putida* in rats were investigated in order to evaluate possible toxicopathological effects promoted by exposure to the bacteria. Male and nulliparous female Wistar rats, aged 90 days and weighting 230 ± 15 g, (mean ± standard error) were housed in polycarbonate cages with hardwood chip bedding and given *ad libitum* access to food (brand Purina Lab Chow) and tap water. National and institutional guidelines for housing and treatment were followed. Environmental variables within the facility were controlled

temperature (22 ± 2 °C) at 70% humidity and a 12-h light/dark cycle. All used material and equipments were decontaminated before the experiments. The P-generation breeders in our colony were obtained from University of Campinas, CEMIB facilities, located in São Paulo state, a Brazilian institution recognized as reference by the International Council for Laboratory Animal Science.

One dose level of 10^8 units of *P. putida* AF7 by gavage per test animal was used, and a careful clinical examination of all animals was made daily at least once. Cageside observations included skin and fur, eyes and mucous membranes, respiratory, circulatory and nervous system and behavior pattern. A gross necropsy of all animals was performed at sacrifice 3, 16 and 24 h from the exposure. For microbial quantification, the homogenates of tissues and blood were diluted (10^{-1}, 10^{-2} and 10^{-3}), plated and incubated adequately. All sets of data were checked with the numbers of colonies counted at successive dilutions series. Confirmation of the bacteria taxonomic data was performed with the fatty acid methyl esters procedure (Castro and Melo, 2007); showing 98% of similarity.

2.2.3. Studies on P. putida for soil microorganisms

2.2.3.1. Soils and treatments. The experimental conditions were designed to create controlled experimental environmental conditions close to a field situation. The soils used were Rhodic Hapludox (RH), Typic Hapludox (TH); and Arenic Hapludult (AH). Ten sub samples of each soil were taken at random and collected at 0–10 cm depth. In laboratory, the sub samples were mixed and homogenized to constitute a composite sample, which were air-dried, sieved and maintained under 4 °C until use.

The soil characteristics were: total C_{org} (%) ranged from 2.87 to 80.4, N_{total} concentrations (mg/kg) from 879 to 1952; clay contents from 21.5% to 44%, and pH values from 4.0 to 4.8; concentrations of organic matter from 2.07% to 4.93% and *P* concentrations (mg/kg) from 7.20 to 13.40. The Cation Exchange Capacity (CEC, mmol/dm^3) ranged between 51.1 and 93.2 and the Water Holding Capacity (WHC,%) from 19.7 to 28.9 (Silva et al., 2009).

Before the addition of the bacterium to the experimental units, the soil remained in incubator Erlenmeyer flasks for 7 days, already with humidity corrected to 70% WHC at 27 °C. The lighting regime was set at a photoperiod of 12 h. Then, two concentrations of AF7 (cfu – colony forming units per milliliter) were applied to the soils: 3.5×10^4 cfu g^{-1} dry weight soil (concentration 1) and 3.5×10^5 cfu g^{-1} dry weight soil (concentration 2). The soils moisture was maintained at a standard level with the application of deionized water. Soil samples without inoculation were used as controls. At 7, 14 and 21 days after the incubation, samples were taken to evaluate the activities of β-glucosidase, acid phosphatase and protease at three replications for each treatment.

2.2.3.2. Enzyme assays. Acid phosphatase (Alef et al., 1995) β-glucosidase (Alef and Nannipieri, 1995a) and protease activities (Alef and Nannipieri, 1995b) were determined by standard analytical methods. ANOVA followed by *F*-test for contrasts were performed for quantifying the bacteria concentration effects on enzyme for different soils and evaluation dates.

3. Results

3.1. Aquatic organisms

A significant increment ($p < 0.05$) in the NNPD was observed for the treatments containing both *P. putida* inactive or active (Table 1). Although a reduction of approximately 12% was observed in exposed organisms to active microorganism compared to the control (Fig. 1 and Table 2), no high evidence of bacteria influence on *D. similis* survival rate was found ($p = 0.179$).

Table 1
Effect of *P. putida* AF7 on the number of neonates of *D. similis* produced per day (NNPD).

Treatment	NNPD[a]	*p*-Values for contrasts[*]	
		P. putida AF7 inactive	*P. putida* AF7 active
Control	2.17	0.0268	0.0289
P. putida AF7 inactive	3.11		0.9673
P. putida AF7 active	3.10		

[a] Mean of five replicates corresponding to all intervals for each treatment.
[*] Nominal significance levels arising from *F*-tests for contrasts between treatment means (Control × *P. putida* AF7 inactive; Control × *P. putida* AF7 active and *P. putida* AF7 inactive × *P. putida* AF7 active).

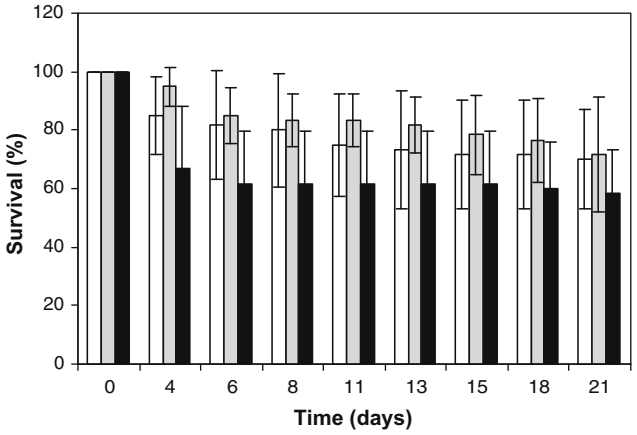

Fig. 1. Survival of *D. similis* after *P. putida* AF7 exposure: (□) control, (▩) *P. putida* inactive and (■) *P. putida* active. Vertical bars represent the standard deviation of five replicates mean.

3.2. Rodents

The animals were observed daily, and showed no clinical alterations at any of the intervals examined after exposure. Although it was not observed *P. putida* colonies after 3 and 24 h of exposure, they were found in lung homogenates 16 h after exposure. The counting of lung sample plates was 3.31×10^4 cfu g^{-1} tissue.

3.3. Effect of P. putida on soil microorganisms

In general, the enzymatic activities presented variation among the soils tested (Table 3). The higher activities of β-glucosidase and acid phosphatase were observed in the RH and AH soils than TH soil. The protease activity was higher in the TH soil. The effect of AF7 concentrations on β-glucosidase activity was observed in RH soil (14 and 21 days) and AH soil (7 and 14 days). In RH soil, a significant reduction ($p < 0.05$) of β-glucosidase activity was observed for

Table 2
D. similis survival after 21 days exposure to *P. putida*. Data are shown in percentage.

Treatment	Survival (%)[a]	*p*-Values for contrasts[*]	
		P. putida AF7 inactive	*P. putida* AF7 active
Control	70.00	0.8408	0.1794
P. putida AF7 inactive	71.76		0.1220
P. putida AF7 active	58.33		

[a] Mean of five replicates.
[*] Nominal significance levels arising from the Wald test for contrasts between treatment (Control × *P. putida* AF7 inactive; Control × *P. putida* AF7 active and *P. putida* AF7 inactive × *P. putida* AF7 active).

Table 3
Enzyme activity in RH, TH and AH soils corresponding to the three *P. putida* AF7 concentration.

Soil[a]	Days	*P. putida* (cfu mL^{-1})		
		0	3.5×10^4	3.5×10^5
Acid phosphatase activity ± STD (μg p-nitrophenol g^{-1} soil h^{-1})				
RH	7	251.7 ± 11.5	268.4 ± 22.6	261.1 ± 19.7
	14	244.4 ± 2.7	238.5 ± 3.4	255.2 ± 8.2
	21	245 ± 2.9	264.2 ± 14	246.9 ± 6.6
TH	7	140.2 ± 27.9	147.4 ± 11	152.4 ± 1.9
	14	108.9 ± 6.8	130 ± 2.5	121.2 ± 14.7
	21	218.6 ± 28.9	224.6 ± 13.8	235.3 ± 53.5
AH	7	324.6 ± 21.3	335 ± 14.6	337.6 ± 21.8
	14	359.2 ± 27.7	326.7 ± 27.8*	353.8 ± 8.7
	21	343.6 ± 20.5	310.9 ± 10*	354.5 ± 12.8
Protease activity ± STD (μg tyrosine g^{-1}. soil h^{-1})				
RH	7	145.5 ± 21.1	196.4 ± 94	196.9 ± 100.1
	14	100.4 ± 8	199.1 ± 9.4	110.5 ± 35.2
	21	68.2 ± 0.7	69.8 ± 13.8	82.9 ± 6.5
TH	7	201.6 ± 6.6	179.6 ± 7.7	267.6 ± 102.5
	14	227.5 ± 27.3	228.1 ± 15.7	230.4 ± 25.1
	21	163.9 ± 21.2	181.5 ± 26.6	177.6 ± 29.5
AH	7	140.4 ± 56.2	135 ± 11.6	122.7 ± 30.8
	14	167.1 ± 45.4	139.1 ± 28.6	169.2 ± 4.1
	21	128.3 ± 79.6	113 ± 14.6	123.1 ± 29.4
β-Glucosidase activity ± STD (μg g^{-1} p-nitrophenol. soil h^{-1})				
RH	7	53.7 ± 16.7	39.5 ± 11.0**	50.2 ± 5.3
	14	63.2 ± 10.9	77.8 ± 8.2	28.5 ± 6.0**
	21	104 ± 17.8	78.3 ± 2.6*	91.2 ± 9.5
TH	7	39.7 ± 3.0	37.8 ± 2.8	39.8 ± 1.8
	14	39 ± 2.1	42.5 ± 3.0	39.3 ± 2.5
	21	50.7 ± 2.7	56.1 ± 9.3	53.7 ± 5.0
AH	7	24 ± 3.8	36.8 ± 4.4	52 ± 0.1**
	14	58.7 ± 12.2	74.5 ± 6.8**	43 ± 0.3
	21	48.5 ± 6.0	43.5 ± 3.8	41.9 ± 3.0

[a] Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH).
* Evidence of concentration effect in relation to control, although no significant.
** significant ($p < 0.05$).

3.5×10^4 cfu g^{-1} dry weight soil (concentration 1 after 7 days) and 3.5×10^5 cfu g^{-1} (concentration 2 after 14 days) when compared to control. In the AH soil, its activity was increased at concentration 2 on day 7 and at concentration 1 on day 14 after the inoculation.

The acid phosphatase activity in the AH soil at concentration 1 was decreased up to 10% on 14 and 21 days after the inoculation. The highest activity was detected in the same soil, varying from 310 to 359 μg *p*-nitrophenol g^{-1} soil h^{-1}. There was no evidence ($p > 0.08$) of acid phosphatase activity temporal variability in the RH and TH soils. For the remaining soil the enzymatic activity varied across time ($p < 0.05$) for at lower concentration of AF7. The available nutrients were similar in all treatments related to concentrations of the bacterium and in the control.

Protease activity show positive correlation with C_{org} ($r = 0.65$; $p < 0.05$) and negative correlation with *N* concentration (−0.64; $p < 0.05$). β-Glucosidase activity show positive correlation with C_{org} ($r = 0.66$; $p < 0.05$) and negative correlation with *P* concentration (−0.74; $p < 0.05$). Acid phosphatase activity show no correlation with these measures ($p > 0.84$) (Silva et al., 2009).

4. Discussion

4.1. Mammals (rats) and aquatic invertebrate

Since bacteria colonies were not found after 24 h of administration, it can be assumed that *P. putida* is rapidly eliminated from the body of mammals and as a consequence apparently does not persist in the organism. Based on these results, there was no evidence of infectivity in the tissues produced by the bacteria. These facts are consistent with the clinical observation of animals, which showed no signs and/or symptoms of damage to their health. The dosed *Pseudomonas* recovered from lungs is possibly due to contamination during the oral exposure, in spite of all disinfection procedures used during the tests. Translocation to other tissues is another hypothesis, but the first step was probably the mesenteric lymph nodes in the spread of microorganisms from the oral exposure. Nevertheless, depending on the dosed *Pseudomonas* strain, translocation to the liver and spleen was observed in mice after 3 till 120 h of dosing (George et al., 1990). Also, even in a much smaller percentage of the initial dose, *P. aeruginosa* strain AC869 was recovered from lungs of mice 2 and 7 days after peroral challenge (George et al., 1996). In any way, it is important to take special care during the handling of samples and asepsis to avoid any contamination. Rats could be overall used to detect adverse health effects when dosed with microbial agents. Then, the primary route of exposure, microbial concentrations and as well overt and indirect health effects should be considered before environmental release of *Pseudomonas*.

Information about environmental risks due the use of microbial agents at long term of exposure is difficult to get. Although *P. putida* seems to not cause deleterious effects in non-target invertebrates like insects (Schneider and Dorn, 2001), the literature related some infections caused by them in fish. In this manner, the histopathological examination of fish tissue infected by *P. putida* revealed macrophagic bacterial peritonitis multifocal in various organs and necrosis in dermis (Smolowitz et al., 1998). According to these authors, *Pseudomonas* sp. are normal inhabitant of fresh and salt water and tend to cause disease in fish under "stress". They have only rarely been identified as a cause of disease in fish in the wild.

Bacterial infections were observed in other species of *Daphnia*, as in the case of the altered levels of the *C*, *N* and *P* in the organisms promoted by *Pasteuria ramosa*. According to USEPA (1998), a moderately toxicity to *Daphnia* sp. was attributed to some strains of *B. thuringiensis*. This biopesticide may affect adversely shrimp and mussels (US National Library of Medicine, 1995).

On view of the results in this work, and based in the fact that the *Cladocerans* are filter feeders of organic detritus including fungi and bacteria, we suggest that *P. putida* functioned as a food supplement which increased the fertilization.

In general, the results of studies on biopesticides have indicated that the protocols currently used have provided useful and necessary information for risk assessment. Together, the elements derived from the protocols can be interpreted to give a measure of the likelihood that an adverse impact on health will result form a given exposure. However, this paradigm fails to cover some key topics because of a lack of clear statutory guidance on how to balance the risks and benefits of biological control. Some new approaches to testing are desirable to better address the risk assessment of target organisms, since ecosystems are complex environments that exhibit intricate interactions between biotic and abiotic factors.

4.2. New approach test – soil microorganisms

The ability of a biopesticide to spread into non-target environments is also of concern. The soil is arguably the most complex habitat within the biosphere, and microorganisms dominate soil-borne communities, accounting for more than 80% of the total biomass (excluding roots) and largely determine the ecosystem functions, such as nutrient cycling and decomposition. Thus the biological process they mediate is fundamental to ecological functioning of the soil ecosystem.

To understand the functioning of a biological system and to test the interaction of a microbial agent with all the organisms in an environment, it is important to identify as many possible non-target ef-

fects associated with the release of any biopesticide. Based on this difficulty some unknown pathogenic relationships with non-target fungi, bacteria, plants or animals could be discovered after their release. Increasing scientific evidence has been published that microorganisms used as biological controls can have significant, measurable effects, both direct and indirect, on non-target organisms (Shaukat and Siddiqui, 2003; Winding et al., 2004; Johansen et al., 2005). The soil environment is the focus of much concern associated with the potential environmental effects of biocontrol agents. Most attempts to monitor the effects of microbial introductions have been centered on the microbial enumeration of specific populations. Transient perturbations have been observed in indigenous bacterial (Natch et al., 1997), fungal (Short et al., 1990; Glandorf et al., 2001), and protozoan (Austin et al., 1990) populations.

Profiles of the relative enzyme activity resulted in insights in the microbial response to the changes in environmental conditions (Corstanje et al., 2007). For example, the fluctuation in values for enzyme activity found after AF7 inoculation may be attributed to a displacement of the communities that produced these enzymes. The first possibility is that the inoculants exclude competitively certain microbial populations. Other possible effect of the inoculants could be in the strain's metabolic activity that might have directly affected the indigenous microbial community (Keel et al., 1992).

The results obtained from enzyme tests suggested that the treatment with AF7 had a significant impact on the carbon cycle on a short-time basis affecting the bioavailability of this element. The strong positive correlation found between β-glucosidase and C_{org} confirmed this observation. Similar results were founded by Landgraf and Klose (2002) and Mawdsley and Burns (1994). The last authors showed that galactosidase and glucosidase levels shifted as a result of the release of *Flavobacterium* sp. into soil. The addition of *P. putida* AF7 caused significant effects upon the β-glucosidase activity. This activity was significant reduced indicating that *P. putida* amendment caused a less demand for C, with a reduction in C_{org} availability.

The higher phosphatase levels in the AH soil when contrasted to other soil types, may have been observed due to the predomination of the soluble form of phosphate.

In the same way, Johansen et al. (2005) studied non-target effects of *Pseudomonas fluorescens* DR54 on the indigenous microbiota in soil and rhizosphere. DR54 soil introduction by seed and soil inoculation displaced indigenous pseudomonads. Since it is difficult to recommend specific techniques that can detect unwanted perturbations of the soil microbiota under all conditions due to the huge genetic and functional diversity of microbial agents; enzyme activity studies can be applied to monitor the microbial non-target effects on the soil microbiota together with other methods.

The use of soil enzymes to determine the extent of perturbations undergone by a soil is a good promise to be a successful approach to study the impact of the different soil management systems, but has received little attention so far with respect to investigating the effect caused by the inoculation of a microorganism (Silva et al., 2009). Based on these issues, appropriate data of the putative impacts on soil microorganisms can represent an important step in improving the scientific basis for risk assessments. It should be included in the Tier I of microbial agent risk analysis in tropical regions since temperature may play an important role in regulating the decay of different soil organic matter fractions due to differences in the relative temperature sensitivities of enzyme activities (Koch et al., 2007).

References

Alef, K., Nannipieri, P., Trazar-Cepeda, C., 1995. Phosphatase activity in methods in applied soil microbiology and biochemistry. In: Alef, K., Nannipieri, P. (Eds.). Academic Press, pp. 335–344.

Alef, K., Nannipieri, P., 1995a. β-Glucosidase activity in methods in applied soil microbiology and biochemistry. In: Alef, K., Nannipieri, P. (Eds.). Academic Press, pp. 350–352.

Alef, K., Nannipieri, P., 1995b. Protease activity in methods in applied soil microbiology and biochemistry. In: Alef, K., Nannipieri, P. (Eds.). Academic Press, pp. 313–315.

Altindag, M., Sahin, M., Esitken, A., Ercisli, S., Guleryuz, M., Donmez, M.F., Sahin, F., 2006. Biological control of brown rot (*Moniliana laxa* Ehr.) on apricot (*Prunus armeniaca* L. cv. Hacıhaliloglu) by *Bacillus*, *Burkholdria*, and *Pseudomonas* application under in vitro and in vivo conditions. Biol. Control 38, 369–372.

Austin, H.K., Hartel, P.G., Coleman, D.C., 1990. Effects of genetically altered *Pseudomonas solanacearum* on predatory protozoa. Soil Biol. Biochem. 22, 115–117.

Bakker, P., Glandorf, D., Viebahn, M., Ouwens, T., Smit, E., Leeflang, P., Wernars, K., Thomashow, L., Thomas-Oates, J., van Loon, L., 2002. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat, Antonie van Leeuwenhoek. Int. J. Gen. Mol. Microbiol. 81 (1–4), 617–624.

Castro, V., Capalbo, D., Moraes, G., Nardo, E., Oliveira, M., 1999. Protocolos de avaliação de agentes microbianos de pragas para registro como biopesticidas – testes toxicopatológicos em mamíferos, vol. 2. Jaguariúna, Embrapa Meio Ambiente Documentos10, pp. 1–40.

Castro, V., Melo, I., 2007. Toxicopathological evaluation of rats exposed to *Pseudomonas putida*. J. Braz. Soc. Ecotoxicol. 2, 1–5.

Corstanje, R., Reddy, K.R., Prenger, J.P., Newman, S., Ogram, A.V., 2007. Soil microbial eco-physiological response to nutrient enrichment in a sub-tropical wetland. Ecol. Indicators 7, 277–289.

Eilenberg, J., Hajek, A., Lomer, C., 2001. Suggestions for unifying the terminology in biological control. BioControl 46, 387–400.

Elendt, B.P., Bias, W.R., 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of *D. magna*. Water Res. 24 (9), 1152–1167.

George, E., Kohan, M., Nelson, G., Schlundt, J., 1996. Determination of potential health effects in the mouse comparing intranasal and peroral exposure to *Pseudomonas aeruginosa* and *Escherichia coli*. Microb. Ecol. Health Dis. 9, 143–156.

George, E., Kohan, M., Whitehouse, D., Creason, J., Claxton, L., 1990. Influence of antibiotics on intestinal tract survival and translocation of environmental *Pseudomonas* species. Appl. Environment. Microbiol. 56 (6), 1559–1564.

Glandorf, D., Verheggen, P., Jansen, T., Jorritsma, J., Smit, E., Leeflang, P., Wernars, K., Thomashow, L., Laureijs, E., Thomas-Oates, J., Bakker, P., Van Loon, L., 2001. Effect of genetically modified *Pseudomonas putida* WC358r on the fungal rhizosphere microflora of field-grown wheat. Appl. Environ. Microbiol. 67, 3371–3378.

Hoornstra, E., Notermans, S., 2001. Quantitative microbiological risk assessment. Int. J. Food Microbiol. 66, 21–29.

Hosokawa, M., Endo, G., Kuroda, K., Horiguchi, S., 1991. Influence of sulfate, Ca and Mg on the acute toxicity of potassium dichromate to *Daphnia similis*. Bull. Environ. Contam. Toxicol. 46, 461–465.

Hynes, R.K., Boyetchko, S.M., 2006. Research initiatives in the art and science of biopesticide formulations. Soil Biol. Biochem. 38, 845–849.

IBAMA, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais, 1988. Manual de testes para a avaliação da ecotoxicidade de agentes químicos. Brasília. Secretaria do Meio Ambiente.

Jayaraj, J., Radhakrishnan, N., Kannan, R., Sakthivel, K., Suganya, D., Venkatesan, S., Velazhahan, R., 2005. Development of new formulations of *Bacillus subtilis* for management of tomato damping-off caused by *Pythium aphanidermatum*. Biocontrol Sci. Technol. 15, 55–65.

Johansen, A., Knudsen, I., Binnerup, S., Winding, A., Johansen, J., Jensen, L., Andersen, K., Svenning, M., Bonde, T., 2005. Non-target effects of the microbial control agents *Pseudomonas fluorescens* DR54 and clonostachys rosea IK726 in soils cropped with barley followed by sugar beet, a greenhouse assessment. Soil Biol. Biochem. 37, 2225–2239.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., Défago, G., 1992. Supression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolic 2,4-diacetylpholoroglucinol. Mol. Plant Microbe Interact. 5, 4–13.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils, Global Biogeochem. Cycles 21, GB4017.

Landgraf, D., Klose, S., 2002. Mobile and readily available C and N fractions and their relationship to microbial biomass and selected enzyme activities in a sandy soil under different management systems. J. Plant Nutr. Soil Sci. 165, 9–16.

Louda, S., Rand, T., Russell, F., Arnett, A., 2005. Assessment of ecological risks in weed biocontrol, input from retrospective ecological analyses. Biol. Control 35, 253–264.

Mawdsley, J.L., Burns, R.G., 1994. Inoculation of plants with *Flavobacterium* P25 results in altered rhizosphere enzyme activities. Soil Biol. Biochem. 26, 871–882.

Montgomery, D.C., 1991. Design and Analysis of Experiments, third ed. John Wiley, New York. pp. 672.

Natch, A., Keel, C., Hebecker, N., Laasik, E., Défago, G., 1997. Influence of biocontrol strain *Pseudomonas fluorescens* CHA0 and its antibiotic overproducing derivative on the diversity of resident root colonizing *Pseudomonads*. FEMS Microbiol. Ecol. 23, 341–352.

Schneider, M., Dorn, A., 2001. Differential infectivity of two *Pseudomonas* species and the immune response in the milkweed bug, *Oncopeltus fasciatus* (Insecta, Hemiptera). J. Invert. Pathol. 78, 135–140.

Shaukat, S., Siddiqui, I., 2003. Impact of biocontrol agents *Pseudomonas fluorescens* CHA0 and its genetically modified derivatives on the diversity of culturable fungi in the rhizosphere of mungbean. J. Appl. Microbiol. 10, 1039–1048.

Short, K., Seidler, R., Olsen, R., 1990. Survival and degradative capacity of *Pseudomonas putida* induced constitutively expressing plasmid mediated degradation of 2,4-dichlorophenoxyacetate (TFD) in soil. Can. J. Microbiol. 36, 821–826.

Silva, C., Castro, V., Oliveira, P., Maia, A., 2009. Influence of *Pseudomonas putida* AF7 inoculation on soil enzymes. Ecotoxicology 18 (5), 1182–1187.

Smolowitz, R., Elizabeth Wadman, E., Chikarmane, H.M., 1998. *Pseudomonas putida* infections of the oyster toadfish (Opsanus tuu). Biol. Bull. 195, 229–231.

Stokes, M.E., Davis, C.S., Koch, G.G., 2000. Categorical Data Analysis Using the SAS System, second ed. SAS Institute Inc., Cary.

USEPA, United States Environmental Protection Agency, 1996a. Microbial Pesticide Test Guidelines – OPPTS 885.3000. Background—Mammalian Toxicity/Pathogenicity/Infectivity.

USEPA, United States Environmental Protection Agency, 1996b. Microbial Pesticide Test Guidelines – OPPTS 885.4000. Background for Nontarget Organism Testing of microbial pest control agents.

USEPA, 1998. *Bacillus thuringiensis* Subspecies Israelensis Strain. EG2215 Fact Sheet. USEPA, Washington, DC.

US National Library of Medicine. Hazardous Substances Databank. Bethesda, MD, 1995.10-19.

Walia, S., Ali-Sadat, S., Brar, R., Chaudhry, G., 2002. Identification and mutagenicity of dinitrotoluene metabolites produced by strain *Pseudomonas putida* OU83. Pest. Biochem. Physiol. 73, 131–139.

Winding, A., Binnerup, S., Pritchard, H., 2004. Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. FEMS Microbiol. Ecol. 47, 129–141.