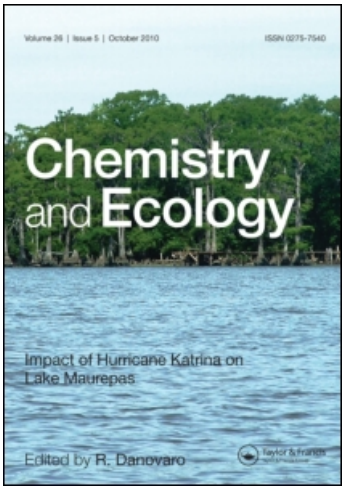

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Mariusz Cycoń[a]; Zofia Piotrowska-Seget[b]; Jacek Kozdrój[c]
[a] Department of Microbiology, Medical University of Silesia, Sosnowiec, Poland [b] Department of Microbiology, University of Silesia, Katowice, Poland [c] Department of Microbiology, University of Agriculture in Krakow, Kraków, Poland

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Dehydrogenase activity as an indicator of different microbial responses to pesticide-treated soils

Mariusz Cycoń[a], Zofia Piotrowska-Seget[b] and Jacek Kozdrój[c]*

[a]*Department of Microbiology, Medical University of Silesia, Sosnowiec, Poland;* [b]*Department of Microbiology, University of Silesia, Katowice, Poland;* [c]*Department of Microbiology, University of Agriculture in Krakow, Kraków, Poland*

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A laboratory study was conducted to assess the impacts of diazinon, linuron or a fungicidal preparation of mancozeb supplemented with dimethomorph on dehydrogenase activity (DHA) in loamy sand (LS) and sandy loam (SL) soils with different features. The pesticides were applied at the maximum predicted environmental concentrations (PEC) under field conditions and at 5 or 100 times the PEC. More distinct effects were observed in LS than SL soils at all sampling times. For PEC, a significant decrease in DHA was found in LS soil treated with diazinon or the fungicidal preparation on day 1 and during the incubation period, respectively. However, DHA did not decrease in SL soil treated with the pesticides at this dosage. For the higher concentrations, decreased DHA was ascertained in LS soil treated with diazinon and the fungicidal preparation at all sampling times, whereas for linuron this effect was evident on days 14 and 28. By contrast, only 100-fold PEC significantly decreased DHA in SL soil amended with diazinon or linuron, compared with the fungicidal preparation that decreased DHA at five-fold PEC. Our results indicate that DHA was particularly sensitive to the fungicidal preparation in both soils, whereas linuron was less harmful than diazinon.

Keywords: dehydrogenase; pesticide treatment; microbial activity; soil contamination

1. Introduction

Millions of tons of different insecticides, herbicides and fungicides are applied each year to control pests, resulting in the contamination of soil and water with these compounds or intermediate metabolites formed during their biodegradation. Diazinon, linuron and a fungicidal mixture of mancozeb and dimethomorph are commonly used in agriculture [1–3].

Diazinon (*O*,*O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidynyl]) is a contact organophosphorous insecticide with a wide range of insecticidal activity. After release into the soil, most diazinon is lost through chemical and microbial degradation [4–6]. In addition, some bacteria belonging to *Pseudomonas*, *Flavobacterium* and *Agrobacterium* can utilise diazinon as a sole source of carbon, phosphorus and energy for growth [7].

*Corresponding author. Email: j.kozdroj@ur.krakow.pl

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Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea), a phenylurea herbicide has been used extensively in the conventional production of corn, cereals, vegetables and fruit to control annual and perennial broadleaf and grassy weeds on both crop and non-crop sites. This herbicide is moderately persistent and mobile in soil, with field half-life values ranging from 22 to 150 days in different soils and under various conditions [8]. It has been found that *Variovorax*, *Hydrogenophaga* and *Achromobacter* establish a microbial consortium of metabolically interacting bacteria, which is involved in degradation of linuron in soil [1].

Mancozeb (manganese ethylene[bis]dithiocarbamate polymeric complex with zinc salt) represents dithiocarbamates, the subclass of carbamate fungicides. This fungicide is used to protect many fruits, vegetables, nuts and field crops against a wide spectrum of fungal diseases, including potato blight, leaf spot and scab. In addition, mancozeb is used for seed treatment of cotton, potatoes, corn, safflower, sorghum, peanuts, tomatoes, flax and cereal grains [9,10]. This fungicide has a low half-life: between 1 and 7 days. Dimethomorph ((E,Z)4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)-1-oxo-2-propenyl]morpholine), a systemic morpholine fungicide, protects plants against fungi causing late blight and downy mildew. Furthermore, this fungicide helps to prevent the spread of blight among plants [11,12]. The fate of dimethomorph in soil indicates that it is a moderately mobile fungicide with an aerobic soil metabolism, having a half-life of 66–117 days [10]. To increase the efficiency of plant protection against fungal pathogens and reduce the risk of resistance development, the application of mancozeb supplemented with dimethomorph has been recommended [2].

Different pesticides and their metabolites may affect non-target microorganisms at the population and biochemical activity levels, as indicated by determinations of dehydrogenase activity (DHA) in various soils [3,9,13]. Because intrinsic cellular features are associated with respiratory activity, DHA reflects the catabolic potential of microorganisms involved in carbon turnover, regardless of their susceptibility to cultivation. Hence, any disturbance in microbial mineralisation of different carbon substrates in soil caused by released pesticides may be detected [14].

Soil microorganisms differ in their tolerance to insecticides, herbicides and fungicides; thus DHA is either reduced or stimulated, as indicated by several reports [15–18]. The differences in DHA data depend not only on the type of the chemical compounds, but also on their concentrations. The predicted environmental concentration (PEC) under field conditions and dosages corresponding to 5 or 100 times the PEC have been used to assess the ecological hazards of different pesticides [19–21]. This approach was also applied in this study to compare microbial tolerance to diazinon, linuron and a fungicidal preparation of mancozeb supplemented with dimethomorph in sandy soils by determination of DHA. Because indigenous soil fungi involved in the aerobic mineralisation of organic compounds are potential targets for fungicides, it is assumed that the preparation should be especially inhibitory in relation to DHA.

2. Materials and methods

2.1. *Soil characteristics*

Composite samples of two arable soils, prepared from ten different sub-samples taken from areas of 25 m^2 were collected from the top layer (0–20 cm) of grass-covered fields located at two sites within the area of Pszczyna in Upper Silesia, southern Poland. The sites have not been used for agricultural purposes during the past five years; no plant-protection products or organic and inorganic fertilisers have been used. Detailed physical and chemical characteristics of the loamy sand (LS) and sandy loam (SL) soils are presented in Table 1. Particle size distribution was determined by the areometric method (ISO 11277), and the pH values of the aqueous soil extracts (1:5 w/v) were measured in triplicate with a glass electrode using a Jenway pH-meter at 20 °C (ISO 10390).

Table 1. Comparison of selected properties of different sandy soils

Soil feature	Loamy sand (LS)	Sandy loam (SL)
Sand (%)	83	7
Silt (%)	13	25
Clay (%)	5	8
Density ($g \cdot cm^{-3}$)	1.3	1.6
pH (H_2O)	6.4	6.7
CEC ($cmol + kg^{-1}$) total	11.5	22
Ca^{2+}	7	18.14
Mg^{2+}	0.97	0.99
K^{+}	0.79	0.11
Na^{+}	0.08	0.03
Water holding capacity (%)	33.6	45.8
C_{org} (%)	1.1	2.4
N_{tot} (%)	0.09	0.18
Microbial biomass ($mg \cdot kg^{-1}$)	648	1075

Note: CEC, cation-exchange capacity.

To determine the soil cation-exchange capacity, the barium chloride method (ISO 11260) was used and concentrations of analysed ions were measured by atomic absorption spectrometry (AAS). The water-holding capacity (WHC), organic carbon content (C_{org}) and total nitrogen content (N_{tot}) were determined by a gravimetric method (ISO 14239), dichromate oxidation in the presence of concentrated sulphuric acid (ISO 14235) and the Kjeldahl method (ISO 11261), respectively. The glucose-induced respiration method (ISO 14240-1) was used to assess soil microbial biomass. In the laboratory, the soil was air-dried at 18 °C to ∼10% moisture content and sieved (2 mm) prior to study.

2.2. *Soil treatment and analysis*

Linuron (45% active ingredient), a phenylurea herbicide in a soluble concentrated (SC) formulation, was applied at three different dosages of 4, 20 and 400 $mg \cdot kg^{-1}$ soil. The insecticide diazinon (25% active ingredient) in an emulsion formulation (EC) was applied at dosages of 7, 35 and 700 $mg \cdot kg^{-1}$ soil. A fungicidal preparation containing mancozeb (MB) and dimethomorph (DT) (60 and 9% active ingredient, respectively) in a wettable powder (WP) formulation was used at dosages of 15, 75 and 1500 $mg \cdot kg^{-1}$ soil.

The lowest dosage of each pesticide corresponded to the maximum PEC under field conditions, while the higher dosages of the preparations were 5 and 100 times PEC. PEC and five-fold PEC dosages are applied in routine ecotoxicological studies concerning the impact of pesticides on soil microbial activity, to obtain permission to sell and use the plant-protection compounds in agriculture. The highest dosage was used to evaluate the potential hazards of the pesticides on soil biota under accidental discharge of uncontrolled amounts of the chemicals to soil.

The soils used were divided into four portions of equal weight (3000 g) and placed into plastic pots (17 cm diameter × 22 cm height). Three portions of each soil were treated with the corresponding concentrations of water suspension of the pesticide (300 mL), and the fourth portion (control) received the same volume of water. Each treatment was replicated three times, giving a total of 27 pots. The water content of the soils was adjusted to 50% of the maximum WHC. To avoid pesticide photodegradation and evaporation of water from soil, pots were covered with perforated polypropylene sheets and incubated in darkness at 20 ± 2 °C; water losses exceeding 10% of the initial values were compensated by the addition of distilled water.

After 1, 7, 14 and 28 days of incubation, DHA was determined using 2,3,5-triphenyltetrazoliumchloride (TTC) as a substrate. Soil samples (5 g) mixed with Tris buffer (pH 7.6) were then incubated at 25 °C for 20 h. The triphenyl formazan (TPF) produced was

extracted from the reaction mixture with acetone and measured at 546 nm by a Jenway 6300 spectrophotometer. The activity of dehydrogenase was expressed as $\mu g\ TPF \cdot g^{-1} \cdot h^{-1}$ [22].

2.3. *Statistics*

Statistical analyses were performed on three replicates from each treatment on each time-point using three-way ANOVA (Statistica 7.0, PL) and the least significant difference (LSD) test. Values were considered significantly different at the 95% confidence level. In this way, effects of the pesticide dosage, the incubation time and soil type on DHA were assessed.

3. Results and discussion

Enzyme activities have been reported to be long-sensitive indicators of soil ecological stress in both natural and agricultural ecosystems [23,24]. Dehydrogenases contribute to the respiratory activity of microorganisms; hence, DHA has been used to assess microbial activity in soil treated with pesticides [3,25]. However, this assay should be used with caution because of confounding alternative electron acceptors (e.g. nitrate, metals) in soil that can lead to overestimation of DHA [26]. In this study, DHA decreased significantly in all LS treatments shortly after soil amendment with diazinon. Decreased DHAs were also found at the next sampling times, but only in soil treated with 5- and 100-fold PEC of the insecticide (Figure 1). By contrast, diazinon

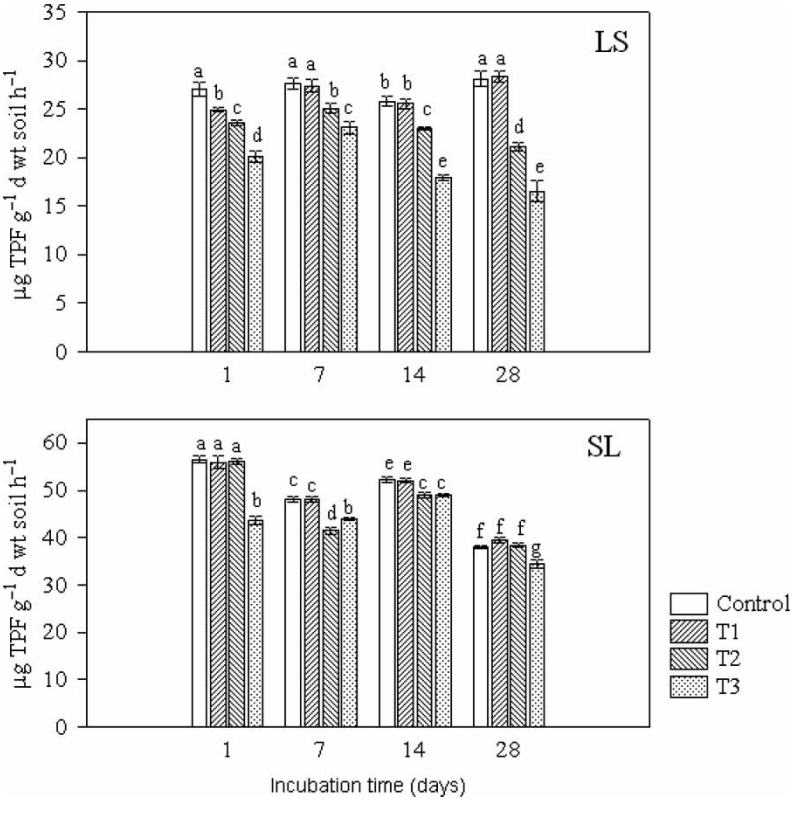

Figure 1. Changes in dehydrogenase activity (DHA) in loamy sand (LS) and sandy loam (SL) soils treated with different dosages of diazinon (T1, $7\ mg \cdot kg^{-1}$ soil; T2, $35\ mg \cdot kg^{-1}$ soil; and T3, $700\ mg \cdot kg^{-1}$ soil). Different letters indicate significant differences ($p < 0.05$, $n = 3$), considering effects of treatments, incubation time and soil type.

decreased DHA at 700 mg · kg^{-1} soil in SL soil during the incubation period, whereas the adverse effect of 35 mg · kg^{-1} was only seen on days 7 and 14. In general, the enzyme activities were significantly higher in SL soil than in LS soil for all dosages and sampling times (Figure 1). The decreased DHA values in soils treated with diazinon might have resulted from the death or metabolic inhibition of a microbial fraction sensitive to the insecticide. In addition, rapid degradation of the enzyme released from microbial cells cannot be excluded, especially in LS soil, where lower clay and organic matter contents could not protect the enzyme to the same level as for SL soil. The increased sensitivity of DHA to other insecticides has also been reported in several studies [18,27]. However, diazinon and other insecticides have also stimulated the enzyme activity in amended soils [3,16]. These discrepancies in the results may be associated with differences in soil characteristics and the composition of microbial communities with regard to the proportions of sensitive to tolerant species.

In this study, the results showed differences in DHA in response to the dosages of linuron added to the soils (Figure 2). In LS soil treated with linuron at 400 mg · kg^{-1} soil, DHA was 5% higher than in controls. However, this effect was transient and was observed only on day 1. At the next sampling times, a significant decrease in DHA was observed. In addition, decreased DHA was also observed for soil treated with linuron at 4 and 20 mg · kg^{-1} soil on days 14 and 28 (Figure 2). By contrast, the inhibitory effect of linuron in SL soil was only observed for the 100-fold PEC on days 14 and 28. Pampulha and Oliveira [28] found a long-lasting negative

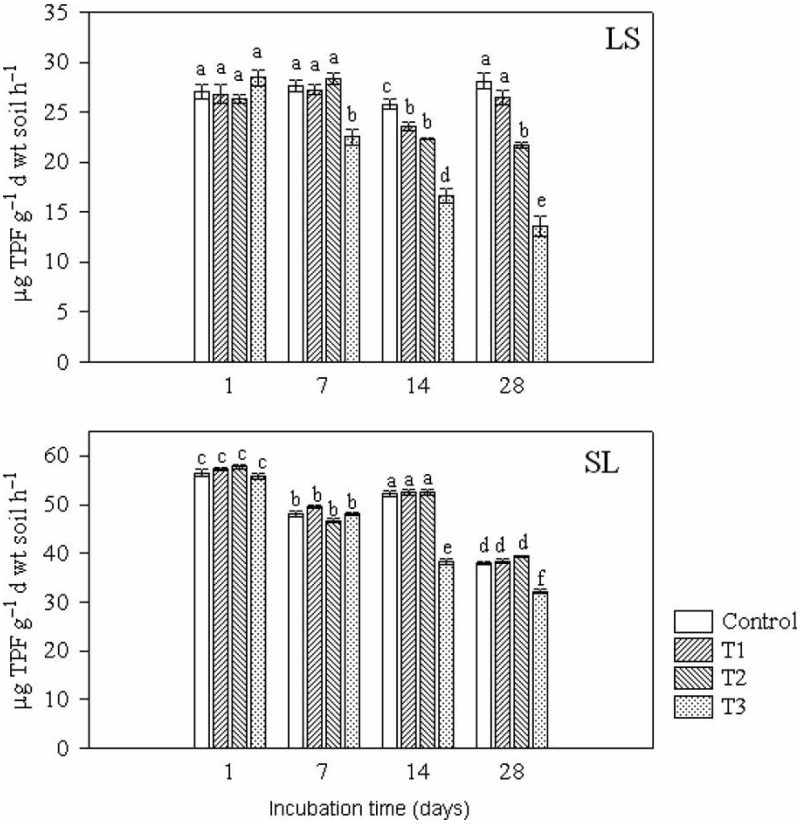

Figure 2. Changes in dehydrogenase activity (DHA) in loamy sand (LS) and sandy loam (SL) soils treated with different dosages of linuron (T1, 4 mg · kg^{-1} soil; T2, 20 mg · kg^{-1} soil; and T3, 400 mg · kg^{-1} soil). Different letters indicate significant differences ($p < 0.05$, $n = 3$), considering effects of treatments, incubation time and soil type.

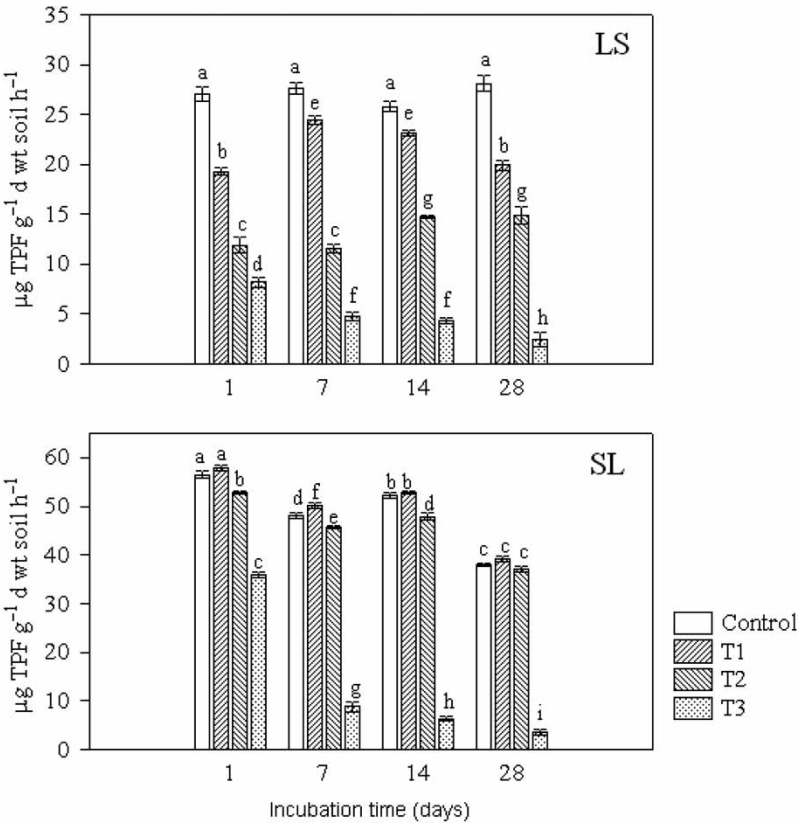

Figure 3. Changes in dehydrogenase activity (DHA) in loamy sand (LS) and sandy loam (SL) soils treated with different dosages of a fungicidal preparation of mancozeb supplemented with dimethomorph (T1, 15 mg · kg^{-1} soil; T2, 75 mg · kg^{-1} soil; and T3, 1500 mg · kg^{-1} soil). Different letters indicate significant differences ($p < 0.05$, $n = 3$), considering effects of treatments, incubation time and soil type.

impact of a herbicide mixture applied at different dosages on DHA in a sandy soil. However, several authors have reported positive effects of glyphosate applied up to 200 mg · kg^{-1} soil on DHA [6,15,29]. Furthermore, Grenni et al. [13] did not find changes in DHA data between non-amended soil and soils treated with linuron at an agricultural rate. It seems reasonably to suppose that the different impact of herbicides on DHA is associated with differences in soil organic matter content, herbicide types, their dosages and farming history [30,31].

The results obtained in our studies indicate that the fungicidal preparation of mancozeb supplemented with dimethomorph had the strongest impact on DHA which declined in both soils (Figure 3). In LS soil, DHA was significantly reduced by all dosages of the preparation during the incubation time. In addition, with higher concentrations of the fungicidal preparation, a greater decrease in DHA was ascertained. By contrast, DHA was not decreased in SL soil treated with the preparation at PEC throughout the incubation time (Figure 3). Furthermore, lower inhibition of DHA was found in SL soil amended with 75 mg · kg^{-1} soil of the fungicidal preparation in comparison with the corresponding treatment in LS soil. For the 100-fold PEC of the preparation, however, DHA decreased to levels similar to those ascertained in LS soil, especially on days 7, 14 and 28 (Figure 3). The large decrease in DHA was also found as a microbial response to soil treatment with mefenoxam and metalaxyl [17], as well as azoxystrobin, tebuconazole and chlorothalonil [32]. In addition, Chen et al. [9] observed harmful effects of benomyl and captan on

DHA in soil treated with these fungicides. Conversely, Megharaj et al. [27] found that fenamiphos did not inhibit or even strongly stimulated DHA in the treated soil.

Given the impact of the pesticides on target organisms that are sensitive to them, DHA data indicate that fungicides may significantly disturb indigenous soil fungi. However, because insecticides and herbicides have no targets among microorganisms, their harmful effect can be noticeable at high concentrations (i.e. pollution events). In addition, soil microbes seem to be less tolerant to insecticides than herbicides, because diazinon reduced metabolic activity shortly after the exposure to the insecticide, whereas linuron affected DHA at all dosages two weeks later. It may be conjectured that some degradation products of the pesticides may be released over time, which may adversely affect microbial activity in the soils. Moreover, because sandy loam soil has higher contents of clay minerals and organic matter than loamy sand, microbial cells as well as dehydrogenases released from dead microbes are better protected against pesticides. Hence, higher values of DHA were ascertained in the sandy loam soil in this study.

4. Conclusions

DHA is a useful marker for the assessment of microbial activity in sandy soils contaminated with diazinon, linuron or a fungicidal preparation of mancozeb supplemented with dimethomorph. Although some nitrate could be released from these pesticides during degradation, the amounts were not large enough (data not shown) to compete with TTC for electrons, thus disturbing the DHA assay [33]. The results clearly show that soil microorganisms are the most sensitive to the fungicides, presumably due to a significant decrease in DHA of fungal origin. In addition, DHA measurements allow comparison of microbial sensitivity to an insecticide and herbicide at the metabolic level. In this way, the potential disturbance of catabolic transformation of soil carbon, which is important for the conservation of soil quality, may be shown. Careful attention should be paid to the application of pesticides, especially when large amounts of these agrochemicals can be released by an undesirable pollution event into soil.

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