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Toxicity of Nitrification Inhibitors on Dehydrogenase Activity in Soils

Ferisman Tindaon[1]; Benckiser, G [2] and Johannes.C.G. Ottow [2]

[1]. Agroecotechnology Department, Faculty of Agriculture, Nommensen University
Jl. Sutomo No. 4A Medan 20234 Indonesia
Tel. +62614522922 Fax: +62614571426 E-mail: Ferisman_Tindaon@yahoo.com

[2]. Institute for Applied Microbiology, Justus Leibig University
Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
Tel. +49-641/99-37351,37352. Fax +49-641/99-37359

Abstract--The objective of this research was to determine the effects of nitrification inhibitors (NIs) such as 3,4-dimethylpyrazolephosphate=DMPP, 4-Chlor-methylpyrazole phosphate=ClMPP and dicyandiamide, (DCD) which might be expected to inhibit microbial activity, on dehydrogenase activity (DRA) in three different soils in laboratory conditions. Dehydrogenase activity was assayed via reduction of 2-p-Iodophenyl-3-p-nitrophenyl-5-phenyltetrazoliumchloride (INT). The toxicity and dose response curve of three NIs were quantified under laboratory conditions using a loamy clay, a sandy loam and a sandy soil. The quantitative determination of DHA was carried out spectrophotometrically. In all experiments, the influence of 5-1000 times the base concentration was examined. To evaluate the rate of inhibition with the increasing NI concentrations, dose response curves were presented and no observable effect level =NOEL, as well as effective dose ED_{10} and ED_{50}(10% and 50% inhibition) were calculated. The NOEL for common microbial activity such as DHA was about 30–70 times higher than base concentration in all investigated soils. ClMPP exhibited the strongest influence on the non target microbial processes in the three soils if it compare to DMPP and DCD. The NOEL,ED_{10} and ED_{50} values higher in clay than in loamy or sandy soil. The NIs were generally most effective in sandy soils. The three NIs considered at the present state of knowledge as environmentally safe in use.

Key words: toxicity, nitrification inhibitors ,dehydrogenase

I. INTRODUCTION

The assessment of microbial activity and the role of microorganisms in ecological systems, especially in response to environmental pollution, agrichemicals, demand reliable methods for estimating microbial biomass and activity in soils are known, e.g. measurement of ATP contents, substrate induced respiration, dehydrogenase and dimethyl suflidoxide reductase [1],[6],[7]. Soil quality changes resulting from environmental pedoturbation or management practices have been assessed through the use of soil enzyme. One such enzymes is the dehydrogenase whose activity is considered an indicator of oxidative metabolism in soils and thus of the microbiological. It represents the intercellular flux of electron to oxygen due to the activity of several intercellular enzymes catalyzing the transfer of hydrogen and electron from one compound to another [14]. Dehydrogenases are generally present in every upper layer of soils, and essential components of enzyme systems of microorganisms. Dehydrogenase activity can therefore be used as measure of microbial activity in soils [6], [8]. Side effects of agrochemicals use in environment can be measured by perceiving change of

microbial population or with determining the microbial processes activity such as dehydrogenase activity.

Optimization of agricultural resources for improved and sustainable agriculture involves the use of nitrification inhibitors. Addition of nitrification inhibitors (NIs) to fertilizers have beneficial effect on reducing nitrate leaching and nitrous oxide emission and as a result increase plant growth (increase N use efficiency). The inhibitor should be *bacteriostatic* and not a *bactericide* which killing certain microorganism in soils like Nitrosobacter spp, Nitrosococcus sp. Finally, this NI have no negative influence on common microbial activity which is non target in soils [11], [21].

More than 300 type of nitrification inhibitors recently have been well recognized and used in agriculture. Some of these NIs consisted of N-heterocyclic compounds, acetylene derivates, sulphates and also various pesticides and herbicides [10], [16]. The DMPP and ClMPP were developed and produced by Bayerische Acetylen of Soda Fabrik (BASF) Ltd Company, Limburgerhof Germany. Dicyandiamid (DCD) produced by SKW Trotsberg Ag. Trotsberg Germany. Upon these NIs is an ammonium stabilizator in the field and also decrease the accumulation and nitrate leaching, reduce nitrous oxide emissions [2], [3], [7], [9],[26], improve N supply for crop, raise the yields of various crops [13], [17], [27]. Though some researchers have reported the effectiveness of these substances as inhibitors but ecologically, it requires being determined its influence to microbial activity in soils and their residue effects in the environment.

Therefore the toxicity of the nitrification inhibitors to main microbial processes should be assessed. This standard methods have been recognized to know the side effects of chemicals use to environment which can be checked either in laboratory and also in the field [24].

II. MATERIALS AND METHODS

Soil Samples

In this study three different types of soil samples were used which varied in their chemical and physical properties (Table 1.).

TABLE I
GENERAL PROPERTIES OF EXPERIMENT SOILS

Parameters	Type of Soil		
	Loamy clay	Loam	Loamy sand
C_{total} (%)	1,35	1,30	0,70
C_{H2O} (%)	0,40	0,55	0,27
N_{total} (%)	0,15	0,15	0,08
C/N	10	9	9
pH_{H2O}	6,30	7,00	7,00
pH_{KCl}	6,00	5,50	6,40
Fraction (%)			
Clay	51	24	6
Loam	41	46	19
Sand	8	30	75

Nitrification Inhibitors

Three kind of NIs were applied: 3,4dimethylpyrazolephosphate=DMPP and 4-Chlor-methylpyrazole phosphate=ClMPP (Purity 99,9 % and 99,7 %) were produced by BASF AG, Ludwigshafen Germany. Dicyandiamid (DCD= Purity 96%) produced by SKW Trotsberg Ag. Trotsberg Germany. These three NIs were applied at recommendation rates 0,36 µg DMPP, 0,25 µg ClMPP and 10µg DCD g^{-1} dry soil. These rates were equal to that incorporated in N-fertilizer for 90 kg N per Ha. In addition to this recommendation rate, higher NIs concentrations were also included in the experimental set up so as to predict its likely side effects on microbial non target processes in the events its excessive use. The application rates used in the present study included 1, 5, 10, 25, 50, 100, 250, 500 and 1000 times of base concentrations.

Dehydrogenase Activity Assays

Dehydrogenase activity (DHA) as an index of microbial activity was determined using 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoliumchloride (INT) as substrate and by spectrophotomectric quantification of produced formazan, [16],[21]. The method relies on the reduction of INT by microorganisms to Iodonitrotetrazolium-Formazan (INF). The concentration of INF was measured on a spectrophotometer (Fa. Zeiss PM2-DL) at 436 nm using methanol as blank.

Samples Preparations:

2.5 g of air-dried soil were weighed in 50 ml test-tubes (five parallels) and with 2.5 ml of a Iodonitrotetrazoliumchlorid (INT) buffer solution were added. To produce the INT-buffer solution were 12.11 g Tris (hydroxymethyl) amino-methane (Merck) in 600 ml distilled water and filled up to 1 litre. Then, the pH was adjusted with a few drops of hydrochloric acid (1 mol) up to pH 7.6. INT in Tris buffer (Fluka Chemie AG, Buchs, Switzerland) was dissolved with an ultrasonic probe (Sonorex, RK 100 H, Bandelin Electronic, Berlin). Due to the different sorption capacity of the three test soils, the INT concentration according to the guidelines of [19] was set differently. The optimal concentration for the sandy soil was INT 9 mg ml^{-1} mg, for loamy soil INT 15 mg ml^{-1} and for the clay soil INT 18 ml^{-1} buffer. The soils mixed with the corresponding INT-containing buffer solutions (Whirlmix) for 4 h at 25 ° C incubated (incubator). The violet- Iodonitrotetrazolium formazan(INF) as reduction product then extracted after mixed or shake for 1h with 10 ml of tetrahydrofuran. The extracts kept in the semi-dark room for 2 h and homogenized by hand and filtered. Subsequently, the supernatant INF were measured in the photometer (Zeiss PM2 DL) at the absorbance at 436 nm. The DHA was taken from a calibration curve with INF first and expressed in µg g^{-1} h^{-1} dry soil. The results presented as the averages from at least four parallels.

Statistical Analysis

Non linear regression analysis to drive ecotoxocological value were conducted on untransformed data from assays based on the concentration-response relationship for quantitative endpoint data, using regression model described in [20]. Analysis of variance (ANOVA) and Fisher's least significant difference pair wise comparison tests were applied to litter mass loss and enzymatic activity data for NOEL, ED_{10} and ED_{50} determinations. Differences were considered to be significant at a probability level of $P < 0.05$. Statistical analyses were performed using SigmaPlot and SigmaStat Software.

III. RESULTS AND DISCUSSIONS

Dehydrogenase Activity (DHA)

Figure 1 show that the effect of increasing concentrations of the NIs on dehydrogenase activity in clayey soil occurs if DMPP and ClMPP used in 50 times of the recommended dose. These were equivalent to 18 µg DMPP, 6.3 µg ClMPP per gram of dry soil. While the new DCD visible effects occurred if used as many as 250 µg DCD per gram of soil. Based on absolute doses, it can be concluded that the NOEL values of three NIs for dehydrogenase activity is 18µg DMPP, 6.3 µg ClMPP and 250 µg DCD per gram of soil. Therefore the application of these inhibitors in corporate in N- fertilizer on recommended dose which equivalent to 90 kg N per hectare did not affect dehydrogenase activity in clayey soils.

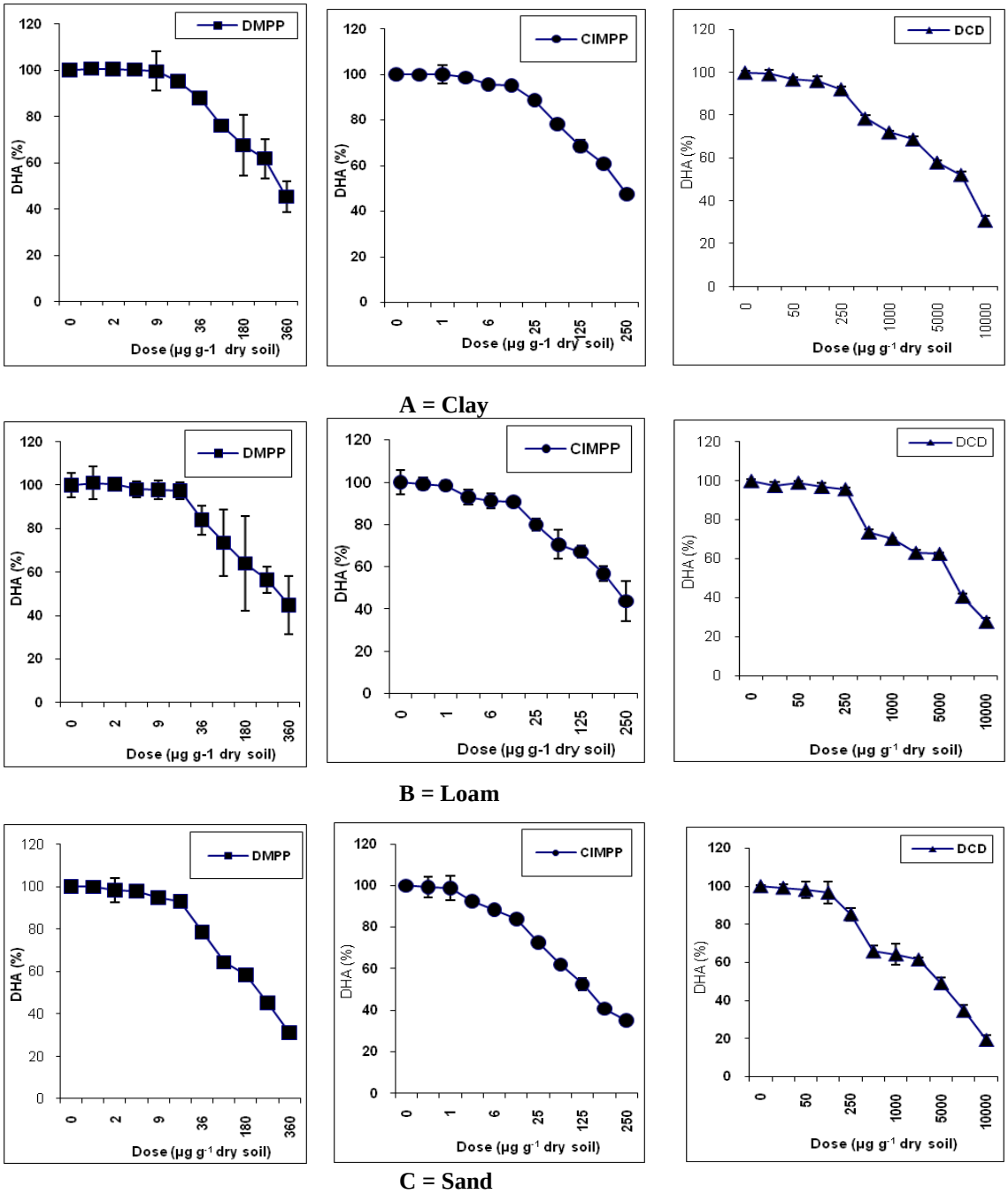

Fig. 1. The effect of increasing the concentration of NI DMPP, ClMPP and DCD on dehydrogenase activity (% control) in clayey soil (A), loamy soil (B) and sandy soil (C). Recommendation dosage were 0.36 µg DMPP; 0.25 µg DCD;ClMPP and 10µg DCD g^{-1} dry soil

A comparison with clayey soil (Fig. 1A) shows that DMPP, ClMPP and DCD inhibited the DHA at 25 times of base concentrations in loamy soil (Fig. 1B). The NOEL values in clay are 6µg ClMPP, 9µg DMPP and 250µg DCD g^{-1} dry soil. It appears that the inhibitory effects of individual NI on general non target microbial activities are significantly larger in clayey soil (Fig.1A. and 1B).

Even in sandy soil (Fig.1C) the DHA was less affected. In contrast to the clay and loamy soil placed in the sandy soil and the DMPP-induced inhibitory effects of DHA ClMPP already at 5 times the concentration of a base (Fig. 1C). NOEL values for DHA in clay soils is 3.6 µg DMPP; 2.5 µg ClMPP and 100µg DCD µg per gram of dry soil. It turned out that in sandy soil inhibitory effect occurred faster and at lower concentrations (Fig.1C). Ecotoxicity of ClMPP was greater than DMPP and DCD, and more effective in inhibiting DHA in sandy soil than loamy and clayey soil. Only at the 750-fold in recommended concentration DMPP or ClMPP should inhibited DHA up to about 50% (ED50). The ED50-values for ClMPP at 160 µg , approximately 250 µg of DMPP or for DCD at 5000 µg g^{-1} dry soil. A comparison with the clay soil (Fig. 1C and Table 2) and the silty clay (Fig. 1B) occurs that the side effects of NI in the sandy soil are not only intense, but also started clearly earlier (Fig. 1C).

The comparison also shows that the DHA of the various soils of DMPP and ClMPP is inhibited significantly more than DCD. The NOEL values for DMPP and ClMPP are significantly smaller than the corresponding values for DCD (ClMPP <DMPP <<DCD). The side effects of the three terms of NI DHA decrease in the order sand> silt> clay (Table 2).

Ecotoxicity of Substances and Dose Response Relationships

Mathematical model for ecotoxicological test on the NOEL, ED10 and ED50 for the three NIs data can be studied constructively by using Sigma plot and Sigma stat. So that approach method showed the threshold of ecotoxocological parameters the substances were determinable (Table. 2). If using ordinary linear equation regression hence determination assess the NOEL from measurement data was not at all enabled. For example, a semilogaritmic dose response relationship between three NIs (DMPP, ClMPP, DCD) and dehydrogenase activity in clay soil presented in Figure 2. By using the equation for dose response curve [16], [20]. in Sigma Plot Program where as:

$$\mathbf{Y = a/(1 + exp (- (X_t - X_0)/b))}$$

it is posible to calculate critical value for NOEL, ED_{10} and ED_{50}. Y= response, a for the maximum response, Xo and Xt = log dose of used NIs according to time and b for a constant of the NI-influence.

There is no clear toxicity difference between each nitrification inhibitors, due to NOEL, ED_{10} and ED_{50}-values. Based on response average values, it can be concluded that ClMPP has more potential side effect on the activities of non target microbes in the soil. This is apparently caused by the effect of halogen element, like chlor, that effectively affects the microbial activity in the soil [7]. Based on the NOEL value, the use of these inhibitors on the dose of 100 times of the recommended dose does not negatively affect the soil environment. All three inhibitors affect non target microbial activities in sandy soils more effectively than in loamy or clayey soils. This is due to the influence of soil clay fraction content that plays a role in adsorption mechanism of inhibitors on the clay surface [3].

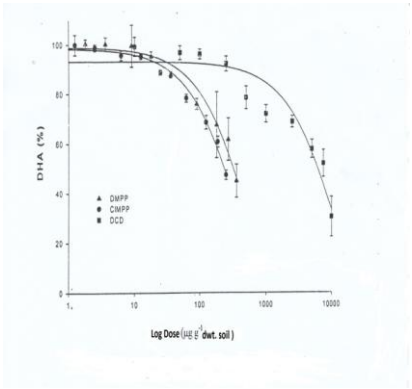

Fig. 2. The effect of increasing the concentration of NI DMPP, ClMPP and DCD on dehyrogenase activity (% control) in clayey soil (semilogaritmic, the dose in the log). Dosage recommendations, were 0.36 µg DMPP; 0.25 µg ClMPP and 10µg DCD per gram dry soil.

Environmental risk threshold value was studied based on [20], that for laboratory trials the average NOEL-value was divided by 10 and for field trials the average NOEL value was divided by 100 (Table 2). The derived from ED_{10} and ED_{50} values Toxicity index (Ti = ratio of ED_{50} and ED_{10} value) describes the intensity decrease with the microbial activities in toxic doses. It turned out that the environmental risk threshold value is still far above the value of 1-50 times N fertilizer recommended dose (inhibitor incorporated with N fertilizer). The current recommended rate for DCD is 10 kg DCD ha^{-1} per application [7] and DMPP was applied as the commercial product Urea with ENTEC™ (1.84 kg DMPP active ingredient/t urea or 0.71 µg DMPP/g soil [4]; [5]. Later, ClMPP was not recommended to be used in agriculture practices.

IV. CONCLUSIONS

Based on the results, conclusion can be summarizes as follows:

- Based on dose response-curves for DHA recorded suggest that DMPP, ClMPP and DCD may affect dehydrogenase activity in soils only at high concentrations.
- The effects of the NIs on DHA were observed if rates about 70-90 times the base concentrations, corresponding to 17 µg ClMPP, 30µg DMPP and 900 µg DCD g^{-1} dry soil were applied (NOEL-value).
- Generally, ClMPP exhibited the strongest influence on non target microbial processes in the three soils compared to DMPP and DCD.
- The NOEL, ED_{10} and ED_{50}-values much higher in clay than in loamy sand or sandy soil. The NIs was generally the most effective in sandy soils.

It should be recommended, that there are no negative effects of ClMPP, DMPP and DCD on dehydrogenase activity in soils at base concentration. DMPP and ClMPP and DCD should be considered as environmentally compatible and safe.

TABLE II
NOEL ASSESSMENT OF ED_{10} and ED_{50} FOR THE THREE NITRIFICATION INHIBITORS IN RELATION WITH DEHYDROGENASE ACTIVITY IN CLAY, LOAM AND SANDY SOIL BASED ON EQUATIONS OF MATHEMATICAL MODELS.

Parameters	Soil Type	Ecotoxicological Value for ($\mu g\ g^{-1}$ dry soil)											
		DMPP				ClMPP				DCD			
		NOEL	ED_{10}	ED50	Ti	NOEL	ED_{10}	ED_{50}	Ti	NOEL	ED_{10}	ED_{50}	Ti
DHA	Clay	91	133	371	3	32	66	255	4	844	1754	6940	4
	Loam	30	72	312	4	28	58	229	4	550	1126	5558	5
	Sand	25	56	230	4	12	33	147	4	167	809	4450	6
	Average	49	87	304	4	24	52	210	4	520	1230	5649	5

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