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# Side Effects of Nitrification Inhibitors on Non Target Microbial Processes in Soils

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## ABSTRACT

Agricultural chemicals have been used extensively in modern agriculture and toxicological studies suggest a great potential for inducing undesirable effects on non target organisms. A model experiment was conducted in order to determine side effects of three nitrification inhibitors (NIs, 3,4-dimethylpyrazolephosphate = DMPP, 4-Chlor-methylpyrazole phosphate = CIMPP and dicyandiamide = DCD) on non target microbial processes in soils. Side effects and dose response curve of three NIs were quantified under laboratory conditions using silty clay, loam and a sandy soils. Dehydrogenase, dimethylsulfoxide reductase as well as nitrogenase activity (NA) and potential denitrification capacity were measured as common and specific non target microbial processes. The influence of 5-1000 times the base concentration, dose response curves were examined, and no observable effect level = NOEL, as well as effective dose ED<sub>10</sub> and ED<sub>50</sub> (10% and 50% inhibition) were calculated. The NOEL for microbial non target processes were about 30–70 times higher than base concentration in all investigated soils. The potential denitrification capacity revealed to be the most sensitive parameter. CIMPP exhibited the strongest influence on the non target microbial processes in the three soils. The NOEL, ED<sub>10</sub> and ED<sub>50</sub> values were higher in clay than in loamy or sandy soil. The NIs was the most effective in sandy soils.

**Keywords:** microbial non target processes, nitrification inhibitors, soil enzymes

## INTRODUCTION

Arable land is often amended with agrochemicals like fertilizers, pesticides and other substances to increase plant production, and such practices are an integral part of modern agriculture. 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. A series of studies reported by Di and Cameron over the past few years show a reduction of about 60% in NO<sub>3</sub>- leaching, 70% in N<sub>2</sub>O emissions and an increase of more than 20% in pasture yield can be achieved (Di and Cameron 2005, 2006; Di *et al.* 2007; Moir *et al.* 2007; Sahrawat 2004; Singh and Verma 2007). The use of nitrifications inhibitors expected will be able to control the microbial ammonium oxidation which convert ammonium to nitrate, decrease N leaching, improve efficiency of N use by crops and decrease

the nitrous oxides emission. Thereby N use ecologically will be more efficient. Further, nitrification inhibitors use in agriculture should be recommended in low concentration and capable to control nitrate supply to crop so that avoid the excess of nitrate supply in soils. The nitrification inhibitors have the specific influence that is only inhibit the nitrification (oxidize the ammonia become the nitrite) and not for nitrification (oxidize the nitrite become the nitrate) so that accumulation can be avoid. The Inhibitor should be bacteriostatic and not a bacteriocide which killing certain microorganism in soils like *Nitrosobacter* spp, *Nitrosococcus* sp. Finally, that NI have no negative influence on common microbial activity which is non target in soils (Trenkel 1997; Pasda *et al.* 2001).

The assessment of microbial activity and the role of microorganisms in ecological systems, especially in response to environmental pollution, agrochemicals and cultivation, climatic factors, 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

sulfidoxide reductase (Alef 1995; Debus and Hund 1997; Engelen *et al.* 1998; Caldwell 2005, Ginafreda *et al.* 2005). 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 (Quilchano and Maranon 2002). 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. 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 (Debus and Hund 1997; Engelen *et al.* 1998, Rasool and Reshi 2010). 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 and dimethylsulforeductase activities, denitrification and nitrogenase activity in soils (Stepniewska and Wolinska 2005; Stepniewska *et al.* 2007)

These 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. The NIs, in addition to other agrochemicals, is extensively used in agriculture practice for control of nitrogen supply, but it has the potential to affect the quality of soil, water and air, with attendant risk to humans, flora and fauna in soil. Because of their relationship to soil biology and rapid response to changes in soil management, soil enzymes are recognized as sensitive indicators of soil health and quality (Caldwell 2005). In fact, they have been related to soil physico-chemical characters (Amador *et al.* 1997), microbial community structure (Kourtev *et al.* 2002), and disturbance (Boerner *et al.* 2000). With respect to nitrification inhibitors, however, so little has been done in so few locations that broad generalizations can not be drawn. Thus, the present investigation was aimed to specifically focus on the effects of nitrification inhibitors at different application rates on some key enzyme activities involved in common and specific non target microbial processes in different type of soils such as a clayey, loamy and sandy soils which are commonly found in tropical agricultural soils. With this information, patterns and recommendations for the use of

fertilizers containing nitrification inhibitors can be identified. In addition the NOEL, ED<sub>10</sub>, ED<sub>50</sub> values were determined to evaluate the ecotoxicity of the three NIs.

## MATERIALS AND METHODS

This model experiment was conducted in laboratory of Institute for Applied Microbiology, Faculty of Agricultural Sciences and Ecology Management, Justus Leibig University, Giessen Germany. Two tropical soils samples were taken from Soil Experiment Station Institute for Agronomy and Plant Protection in Giessen and a tropical sandy soil from Agrochemical Experimental Station, Bayerische Acetylen Soda Fabrik (BASF, Limburgerhof, Germany). The respective soils were classified as Typic Udorthent (clayey soil) Typic Kandudult (clay and loamy soil), and Typic Paleudult (sandy soil) according to Soil Taxonomy (Soil Survey Staff 1999). These three experiment soil varied in their chemical and physical properties such as texture, pH, C-content (Table 1).

### Nitrification Inhibitors

Three kind of NIs were applied: 3,4dimethylpyrazolephosphate=DMPP and 4-Chlor-methylpyrazole phosphate = CIMPP (Purity 99.9% and 99.7%) were produced by BASF AG, Ludwigshafen Germany. Dicyandiamid (DCD = Purity 96%) was produced by SKW Trotsberg Ag. Germany. These three NIs were applied at recommendation rates 0.36 µg DMPP, 0.25 µg CIMPP and 10µg DCD g<sup>-1</sup> dry soil. These rates were equal to that incorporated in N-fertilizer for 90 kg N ha<sup>-1</sup>. In addition to this recommendation rate, higher NIs concentrations were also included in the experimental set up so as to predict its likely side

Table 1. Chemical and Physical Properties of Experiment Soils.

| Soil properties       | Type of Soil |      |            |
|-----------------------|--------------|------|------------|
|                       | Silty clay   | Loam | Loamy sand |
| Total-C (%)           | 1.35         | 1.30 | 0.70       |
| C-org (%)             | 0.40         | 0.55 | 0.27       |
| Total N (%)           | 0.15         | 0.15 | 0.08       |
| Ratio C/N             | 10           | 9    | 9          |
| pH (H <sub>2</sub> O) | 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         |

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.

### Soil Microbial Enzyme Assays

Dehydrogenase activity (DHA) as an index of microbial activity was determined according to Alef (1995) using 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) as substrate and by spectrophotometric quantification of produced formazan. 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 by using methanol as blank.

Dimethylsulfoxide reductase activity (DRA) was assayed according to Alef and Kleiner (1989) using Dimethylsulfoxide (DMSO). The determination of the DMSO reduction rate exploits the common microbial activity to reduce DMSO to DMS, proven nearly 150 species of bacteria and fungi. The concentrations of DMS was measured on a gas chromatography (Perkin Elmer 8500 equipped with flame-ionization detectors=FID) and a 2.0 m column Hayesep R 1/8, 80-100 mesh. The column was operated at 160°C, and the detector was operated at 220°C. The flow rate of carrier gas, N<sub>2</sub>, was 35 ml N<sub>2</sub>/min.

Potential denitrification capacity (PDC) was measured in four replications according to method of Pell *et al.* (1998). Thawed soil samples (40 g) were placed in 2 liters flasks and kept at room temperature overnight. On the following day, 20 ml of substrate with 1 mM glucose and 1 mM KNO<sub>3</sub> was added and denitrifying conditions were achieved by evacuating and filling flasks with nitrogen gas five times. It takes time about 2-3 minutes. Acetylene was added to reach 0.1 atm partial pressure. The presence of acetylene that blocked up the N<sub>2</sub>O – reductase specifically can be measured as potential denitrification capacity. The soil was incubated at 25°C in a dark room for 6 h, and gas samples were collected every one hour. Nitrous oxide in the gas samples was analyzed on a gas chromatograph (Perkin Elmer 8500 equipped with a flame-ionization detector and a 2.0 m column Hayesep R 1/8, 80-100 mesh). The column was operated at 160°C, and the detector was operated at 220°C. The flow rate of carrier gas, N<sub>2</sub>, was 35 ml N<sub>2</sub>/min.

Nitrogenase activity was determined with the method outlined by Martensson (1993) and modified by Chalam *et al.* (1997). Analysis for acetylene was performed with Perkin Elmer gas

chromatograph (Perkin Elmer 8500 equipped electron capture detectors = ECD) and a 2.0 m column Porapak Q. 1/8 80-100 mesh. The column was operated at 50°C, and the detector was operated at 350°C. The flow rate of carrier gas, N<sub>2</sub>, was 30 ml N<sub>2</sub>/min.

### Statistical Analysis

Non linear regression analysis to drive ecotoxicological value were conducted on untransformed data from assays based on the concentration-response relationship for quantitative endpoint data, using regression model described in Stephenson *et al.* (2000). 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<sub>10</sub> and ED<sub>50</sub> determinations. Differences were considered to be significant at a probability level of P < 0.05. Statistical analyses were performed using Sigma Plot and Sigma Stat Software.

## RESULTS AND DISCUSSION

### 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 ecotoxicological 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 semilogarithmic dose response relationship between three NIs (DMPP, CIMPP, DCD) and dehydrogenase activity in clay soil presented in Figure 1. By using the equation for dose response curve (Richter *et al.* 1996) in Sigma Plot Program where as where as:

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

it is posible to calculate critical value for NOEL, ED<sub>10</sub> and ED<sub>50</sub>. Y= response, a for the maximum response, X<sub>0</sub> and X<sub>t</sub> = log dose of used NIs according to time and b for a constant of the NI-influence.

### Dehydrogenase Activity (DHA)

Figure 1 and 2, shows that the effect of increasing concentrations of the NIs on dehydrogenase activity in clayey soil occur if DMPP and CIMPP used in 50 times of the recommended dose. These were equivalent to 18 µg DMPP, 6.3

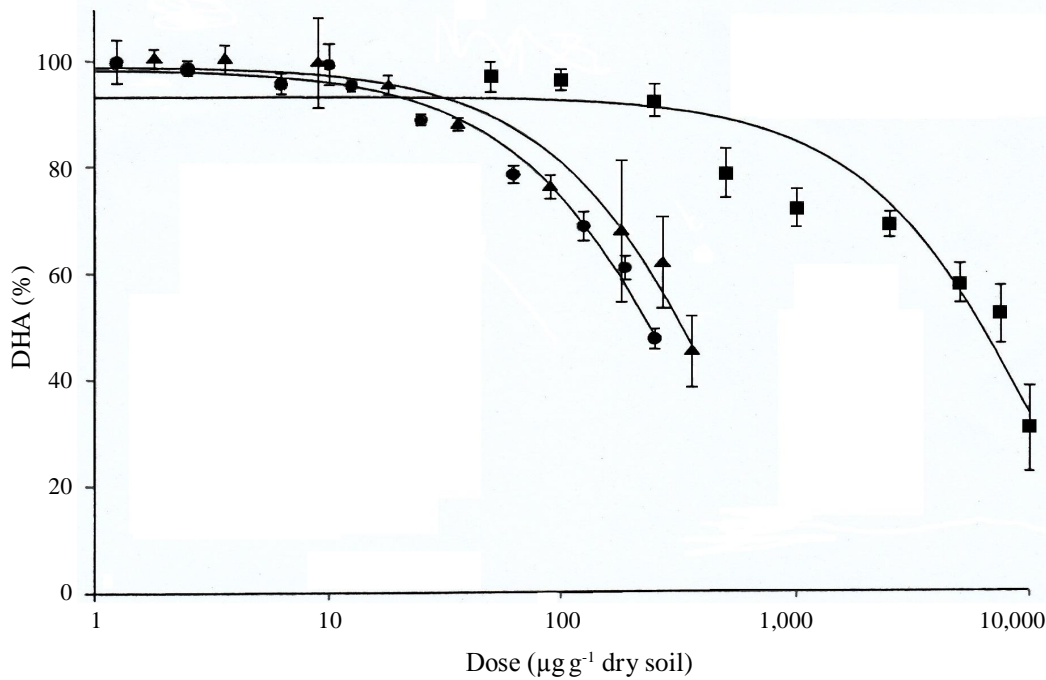


Figure 1. The effect of increasing application rates of NIs DMPP, CIMPP and DCD on dehydrogenase activity (% control) in clayey soil (semilogarithmic, the dose in the log). Dosage recommendations, were 0.36 µg DMPP; 0.25 µg CIMPP and 10 µg DCD per gram dry soil. ▲ = DMPP, ● = CIMPP, ■ = DCD.

µg CIMPP 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 18 µg DMP, 6.3 µg and 250 µg CIMPP DCD per gram of soil. Therefore the application of these inhibitors in incorporated in N- fertilizer on recommended dose which equivalent to 90 kg N per hectare did not affect dehydrogenase activity in clayey soils.

A comparison with clayey soil (Figure 3) shows that DMPP, CIMPP and DCD inhibited the DHA at 25 times of base concentrations. The NOEL values in clay are 6 µg CIMPP, 9 µg DMPP and 250 µg DCD g<sup>-1</sup> dry soil. It appears that the inhibitory effects of individual NI on general non target microbial activities are significantly larger in clayey soil (Figures 2 and 3). Even in sandy soil (Figure 5) the DHA was less affected. NOEL values for DHA in clay soils is 3.6 µg DMPP; 2.5 and 100 µg DCD CIMPP µg per gram of soil. It turned out that in sandy soil inhibitory effect occurred faster and at lower concentrations. Ecotoxicity of CIMPP was greater than DMPP and DCD, and more effective in inhibiting DHA in sandy soil than clay soil and clay.

#### Dimethylsulfoxide Reductase Activity (DRA)

The effect of increasing the three NI-concentrations on dimethylsulfoxide reductase

activity on clay, loam and sandy soils shown in Figure 3, 4 and 5). Almost similar to the dehydrogenase activity, which the effect of inhibitor on DRA occurred at 25 times the recommended dose. It equals to 9.0 µg DMPP: 6.3 µg CIMPP whereas DCD at 100 µg DCD per gram of dry soil. On loamy and sandy soils, the side effect of the three NIs appear at lower concentrations *i.e.* 3.6 µg DMPP: 2.5 µg CIMPP and 100 µg DCD per gram of dry soil. So it was obvious that DCD provides a smaller effect on DRA activity if compared to DMPP and CIMPP.

#### Potential Denitrification Capacity

Effect of various concentrations of inhibitors nitrifications on denitrification potential capacity has been occurred clearly at 50 times of the recommended dose on clayey soil, and 10 times of recommended dose on loamy and sandy soils. When it compared with the DHA and DRA, the denitrification is more sensitive to all three of these inhibitors. This can be caused by inhibition of nitrate formation by the three inhibitors, because it found that nitrate levels in soils that treated with inhibitors are not much different from the control (separate experiments).

#### Nitrogenase Activity

The use of three nitrification inhibitors DMPP, CIMPP and DCD had no significant effect

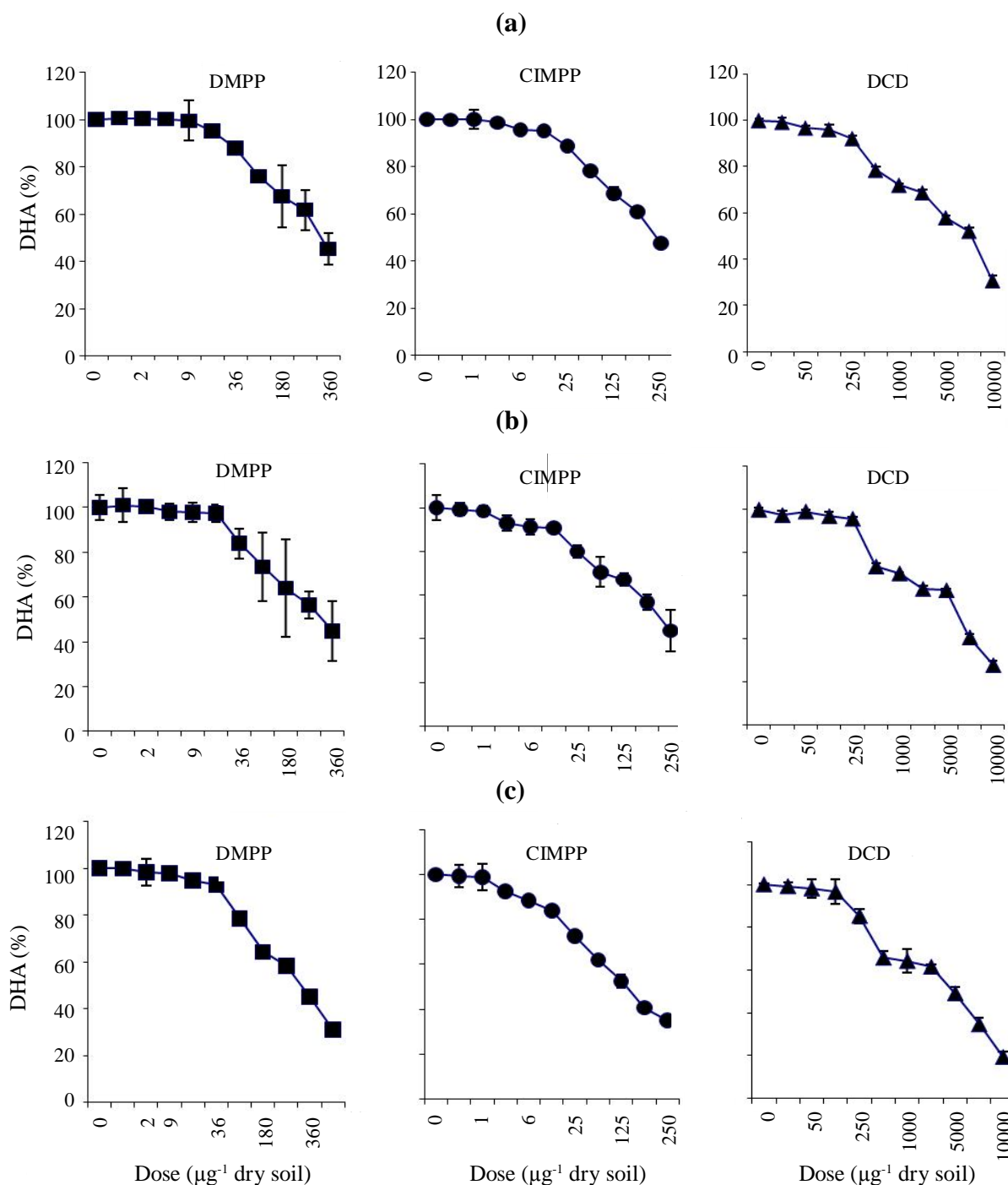


Figure 2. The effect of increasing application rates of NIs DMPP, CIMPP and DCD on the dehydrogenase activity (DHA in % control) in clayey soil (a), loamy soil (b) and sandy soil (c). Recommendation dosages were  $0.36 \mu\text{g DMPP}$ ;  $0.25 \mu\text{g CIMPP}$ ; and  $10 \mu\text{g DCD g}^{-1}$  dry soil.

on nitrogenase activity in the three soil types. So that the NOEL, ED<sub>10</sub> and ED<sub>50</sub> values cannot furtherly be determined. This was considered as positive thing, that the inhibitors do not reduce the activity of nitrogen fixing microorganisms both symbiotic and non symbiotic that was beneficial for plant N availability. Therefore, the study NOEL, ED<sub>10</sub> and ED<sub>50</sub> for the nitrogenase activity (NA) parameter is no longer presented in Table 2.

Generally, there is no clear toxicity difference between each nitrification inhibitors, due to NOEL, ED10 and ED50-values. Based on response average values, it can be concluded that CIMPP 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 (Mc Carty 1999). Based on the NOEL value, the use of these inhibitors on the dose of 100 times of

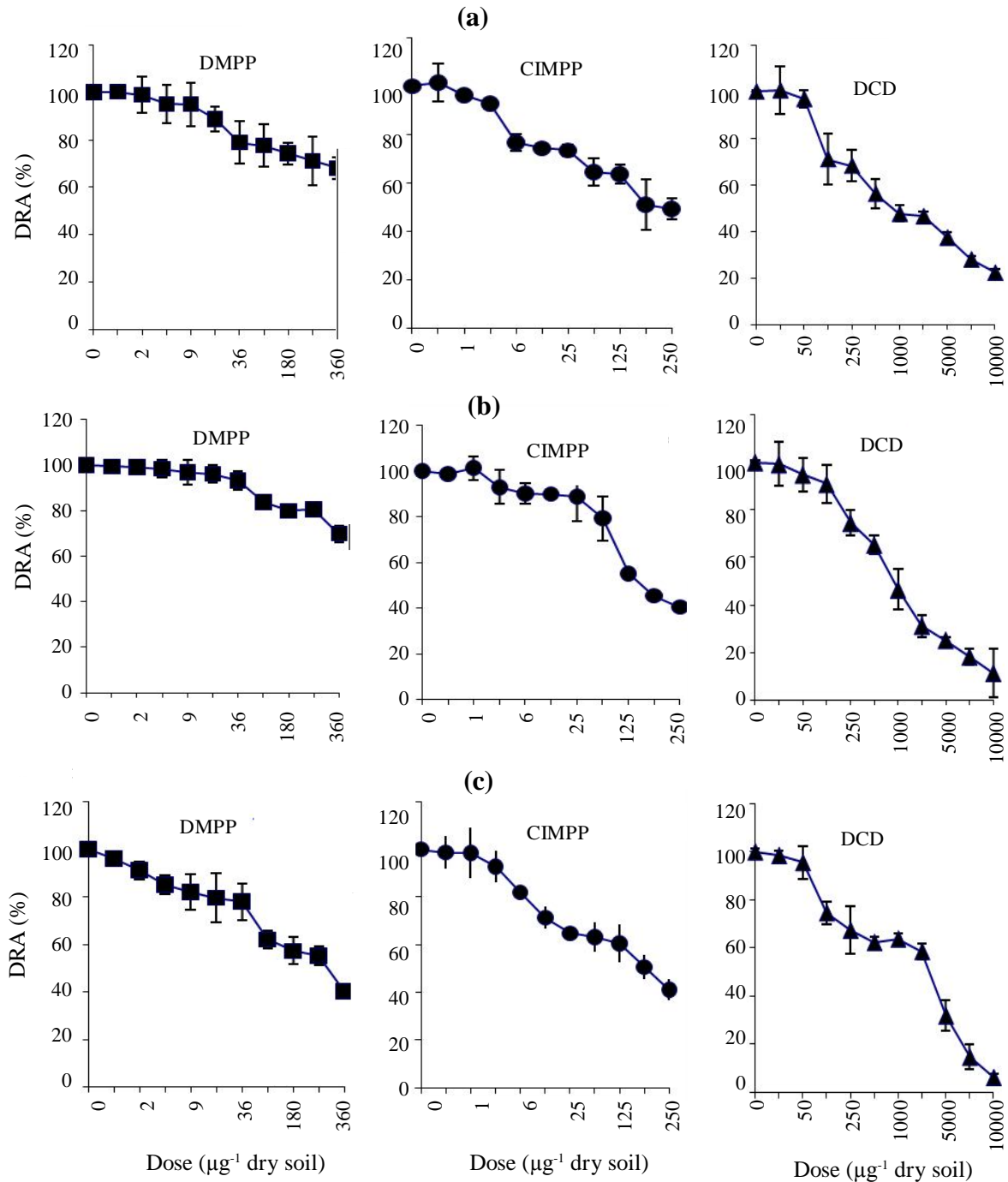


Figure 3. The effect of increasing application rates of NIs DMPP, CIMPP and DCD on dimethylsulfoxide reductase activity (DRA in % control) in clayey soil (a), loamy soil (b) and sandy soil (c). Recommendation dosage were  $0.36 \mu\text{g}$  DMPP;  $0.25 \mu\text{g}$  CIMPP; and  $10 \text{ig DCD g}^{-1}$  dry soil.

the recommended dose did not negatively affect the soil environment. All three inhibitors affected 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 (Azam *et al.* 2001; Barth *et al.* 2001). Once the nitrification inhibitor is in the soil, it is gradually broken down by soil microbes, thus diminishing its effectiveness. The degradation rate of DCD was affected by

temperature (Irigoyen *et al.* 2003; Di and Cameron 2004; Singh and Verma 2007). When applied as solution, increasing DMPP concentrations up to  $7 \text{ mg DMPP kg}^{-1}$  soil had no influence on the inhibition. The effectiveness of DMPP formulated as fertilizer granules was superior to the liquid application of DMPP and  $\text{NH}_4^+$ , particularly in the loamy soil. Without DMPP, a decline in soil matrix potential down to  $-600 \text{ kPa}$  decreased nitrification in both soils, but this effect was more pronounced in the sandy loam than in the loamy soil. DMPP



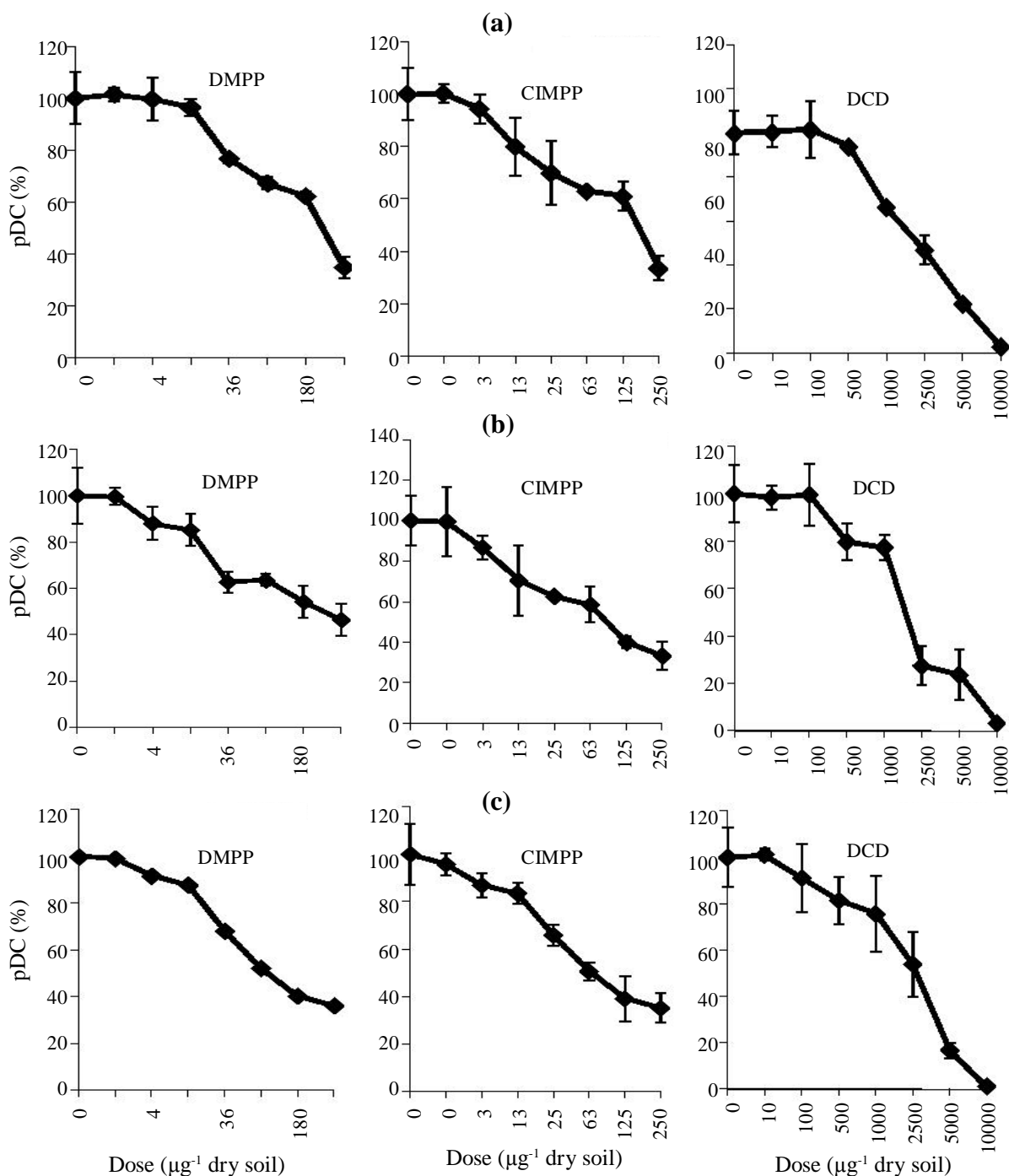


Figure 4. The effect of increasing application rates of NIs DMPP, CIMPP and DCD on potential denitrification capacity (PDC in % control) in clayey soil (a), loamy soil (b) and sandy soil (c). Recommendation dosage were 0.36  $\mu\text{g}$  DMPP; 0.25  $\mu\text{g}$  CIMPP and 10  $\mu\text{g}$  DCD  $\text{g}^{-1}$  dry soil

was most effective in the sandy loam particularly under conditions of higher soil moisture, *i.e.*, under conditions favorable for nitrate leaching (Hua Li *et al.* 2008). However, performance of NIs is highly variable in different agroecosystems as their persistence and activity is strongly influenced by various environmental factors. Adsorption of NIs on soil colloids can particularly reduce their effectiveness in inhibiting nitrification in soil, and this is well documented for the most widely used

inhibitor *viz.*, nitrapyrin. In addition, increasing temperatures significantly reduce the persistence of these inhibitors in soil and this is well established for DCD and DMPP (Irigoyen *et al.* 2003; Di and Cameron 2004). For the vulnerability (sensitive) level of various non-target microbial activity in the soil, it can be sorted that denitrification was more sensitive than dehydrogenase and dimethylsulfoxide reduction (PDC > DRA > DHA, Table 2). Environmental risk threshold value was studied

Table 2. Ecotoxicological criteria of NOEL, ED<sub>10</sub> and ED<sub>50</sub> for evaluating the inhibitor effects of NIs on non-target activity of microorganism (DHA, DRA and PDC) in 3 predominant soils.

| Parametres | Soil types     | Ecotoxicological Value |                  |                  |            |                  |                  |          |                  |                  |
|------------|----------------|------------------------|------------------|------------------|------------|------------------|------------------|----------|------------------|------------------|
|            |                | DMPP NOEL              | ED <sub>10</sub> | ED <sub>50</sub> | CIMPP NOEL | ED <sub>10</sub> | ED <sub>50</sub> | DCD NOEL | ED <sub>10</sub> | ED <sub>50</sub> |
| DHA        | Clay           | 91                     | 133              | 371              | 32         | 66               | 255              | 844      | 1754             | 6940             |
|            | Loam           | 30                     | 72               | 312              | 28         | 58               | 229              | 550      | 1126             | 5558             |
|            | Sand           | 25                     | 56               | 230              | 12         | 33               | 147              | 167      | 809              | 4450             |
|            | Ø              | 49                     | 87               | 304              | 24         | 52               | 210              | 520      | 1230             | 5649             |
| DRA        | Clay           | 47                     | 101              | 365              | 27         | 55               | 215              | 395      | 1083             | 4965             |
|            | Loam           | 11                     | 53               | 287              | 19         | 48               | 197              | 296      | 1102             | 4533             |
|            | Sand           | 5                      | 44               | 266              | 4          | 15               | 133              | 112      | 402              | 2597             |
|            | Ø              | 24                     | 66               | 306              | 17         | 39               | 182              | 268      | 862              | 4032             |
| PDC        | Clay           | 30                     | 62               | 241              | 7          | 29               | 155              | 366      | 761              | 2438             |
|            | Loam           | 17                     | 35               | 193              | 6          | 12               | 105              | 299      | 646              | 2049             |
|            | Sand           | 5                      | 17               | 150              | 2          | 11               | 98               | 159      | 487              | 1724             |
|            | Ø              | 17                     | 38               | 195              | 5          | 17               | 119              | 275      | 631              | 2070             |
| Ø          | Clay           | 59                     | 99               | 326              | 22         | 50               | 208              | 535      | 1199             | 4781             |
|            | Loam           | 19                     | 53               | 264              | 18         | 39               | 177              | 382      | 598              | 4047             |
|            | Sand           | 12                     | 39               | 215              | 6          | 20               | 126              | 146      | 566              | 2924             |
|            | Ø <sup>1</sup> | 30                     | 64               | 268              | 15         | 36               | 170              | 354      | 908              | 3917             |

Note: Ø = Average of every non-target DHA, DRA, and PDK on each soil typw; Ø<sup>1</sup> = Average of non-target DHA, DRA, and PDK on three soil types.

based on USEPA equation (1984), 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). 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<sup>-1</sup> per application (Di and Cameron 2005), and DMPP was applied as the commercial product Urea with ENTEC™ (1.84 kg DMPP active ingredient Mg<sup>-1</sup> urea or 0.71 µg DMPP g<sup>-1</sup> soil (Barth 2006; 2008). Later, CIMPP was not recommended to be used in agriculture practices. This means that the use of these three inhibitors is environmentally compatible and safe.

## CONCLUSIONS

The dose response-curves for DHA, DRA and PDC were generally of sigmoid nature in all investigated soils. The dose response curves recorded suggest that DMPP, CIMPP and DCD may affect non target microbial soil processes only at high concentrations. In general, no side effects of the NIs on parameters DHA, DRA, PDC and NA were observed if rates about 70-90 times the base

concentrations, corresponding to 17 µg CIMPP, 30µg DMPP and 900 µg DCD g<sup>-1</sup> dry soil were applied (NOEL-value).The PDC revealed to be the most sensitive parameter. Sensitivity of three NIs in question decreased in the order of PDC > DRA > DHA > NA. Generally, CIMPP exhibited the strongest influence on non target microbial processes in the three soils compared to DMPP and DCD. Dose response relationships between NIs and microbial non target activities depend on soil types. The NOEL, ED<sub>10</sub> and ED<sub>50</sub>-values were much higher in clay than in loamy sand or sandy soil. The NIs was generally the most effective in sandy soils. In conclusion, there are no negative effects of CIMPP, DMPP and DCD on soil micro flora and soil metabolism at recommended base concentration.

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