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REGULAR ARTICLE

The nitrification inhibitor 3,4-dimethylpyrazole-phosphat (DMPP) - quantification and effects on soil metabolism

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Abstract
Aims Nitrification inhibitors (NI) formulated on granulated ammonium sulphate nitrate (ASN) are an option to minimize nitrate leaching into ground waters and emissions of the greenhouse gas N_2O. This paper focuses (a) on the development of an analytic enabling to extract and quantify the NI *3,4-dimethylpyrazolephosphate* (DMPP), marketed since 1999. The efficiency of DMPP has been studied in laboratory and field soils. Here the DMPP analytic and the behaviour of a nitrifying bacterial consortium enriched from a field soil and exposed to zero, field applied and a 10 fold higher DMPP concentration than the recommended one for field application are in the focus.

Methods For extracting DMPP quantitatively from soils a method connected to a HPLC analytic has been developed by us and was standardized in laboratory experiment with a silt clay field soil (allochtone Vega). The method is detailed described here. Its reliability has been tested in a 3 years field trial under varying cropping systems and climatic conditions asides the influence of DMPP on CO_2−, CH_4− and N_2O- emissions, measured by the closed chamber method. Parallel a nitrifying bacterial consortium of the silty clay field soil was enriched and subjected to 0, the recommended DMPP concentration for field applications and a 10 times higher one. In incubation experiments the conversion of ammonium to nitrite and nitrate in presence and absence of DMPP was spectrophotometer determined and pH-shifts with a scaled litmus paper. In sacrificed flasks at the end of incubation morphological changes of the bacteria involved were studied by transmission electron microscope (TEM).
Results The ammonium, nitrite and nitrate determinations and the TEM pictures show that in presence of the field applied DMPP concentration the nitrifying activity returned around 30 days later than in the control and the cells were slightly enlarged. In presence of a 10 times higher DMPP concentration a recovery was prevented. DMPP prolongs, compared with dicyandiamide (DCD), the period of nitrifiers' inhibition and reduced N_2O− and CO_2− the emissions (Weiske et al., Biol Fertil Soils 34:109–117, 2001a, Nutr Cycl Agroecosys 60:57–64, b).

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Conclusions With the method developed by us the stability of DMPP in agricultural soils can be satisfyingly and reproducible studied down to a detection limit of 0.01 μg DMPP g^{-1} dry soil. The morphological changes in the nitrifying consortium due to DMPP concentrations are in agreement with the recovery rate found by nitrite and nitrate formation.

Keywords Nitrification inhibitor · 3,4-dimethylpyrazolephosphat (DMPP) · DMPP quantification · Dicyandiamide (DCD) · Transmission electron microscopy · Microbial soil processes

Introduction

Synthetic nitrogen fertilizers improve the crop productivity worldwide (Ladha et al. 2011). Since about 40 years N-fertilizers stabilized by nitrification inhibitors (NI) as dicyandiamide (DCD) 3, 4 dimethylpyrazolephosphate (DMPP) introduced and are marketed as ALZON and ENTEC, latter with increasing market share (Pasda et al. 2001; Wissemeier et al. 2002). Both products inhibit the conversion of ammonium into mobile nitrate ions whereby the ratio between nitrifying bacteria and archaea, the soil recycler community, may change and the soil organic matter (SOM) content in agricultural fields decrease (Martens-Habbena et al. 2009; Kleineidam et al. 2011). In soils, not receiving NI-stabilized fertilizer, ammonium converts into mobile nitrate ions and gaseous intermediate products as NO_2, NO, N_2O. These reactive oxidized N-products may pollute especially during the plant-free period ground, drinking water and the atmosphere whereby N_2O is seen as greatest threat to the ozone layer in the 21st Century (Benckiser 1994; Ravishankara et al. 2009). N-containing compounds may influence asides the CH_4 emissions (Bollag and Czlonskowski 1973, Weiske et al. 2001a; b). For minimizing N losses, NO_3^-- and N_2O-impacts N-fertilizer split application is an option, another is to choose crops with a high biological inhibition to inhibit the ammonium oxidase (AMO) and a third the use of nitrification inhibitor (NI)-stabilised N fertilizers (Fig. 1; Amberger 1986; Zerulla et al. 2001; Hatch et al. 2005; Subbarao et al. 2012).

DMPP stabilized N-fertilizers contain 1.6 % DMPP related to NH_4^+−N correspondingly to 0.8 mg DMPP L^{-1} culture solution, about 10 times less than DCD-stabilized N-fertilisers (about 16 % DCD) and field studies revealed that DMPP superior to DCD in lowering NH_3-volatilisation, NO_3^--leaching, and NO- and N_2O-emissions, inter alia, because its nitrification inhibition efficacy after heavy rainfall simulations is longer lasting and its plant compatibility seems to be better than that of the more mobile DCD, which even increase N losses under hot climates (Pasda et al. 2001; Wissemeier et al. 2002; Chaves et al. 2006; Mahmood et al. 2011).

Ecophysiological tests with DMPP fed to rats showed acute toxic effects only when 5.5 mg L^{-1} 4 h^{-1} have been inhaled (LC_{50}) and fishes (*Brachydanio rerio*), water fleas (*Daphnia magna*), algae (*Scenedesmus subspicatus*) (LC/EC_{50}), and typical heterotrophic soil and water bacteria (*Pseudomonas putida*) (EC_{50}) died first at DMPP concentrations of >100 mg L^{-1} 96 h^{-1}, >100 mg L^{-1} 48 h^{-1}, 67.7 mg L^{-1} 72 h^{-1} and 231 mg L^{-1} 170 h^{-1}, respectively (Andreae 1999). Thus the concentration of DMPP and DCD in NI-stabilized N-fertilisers must be significantly enhanced before under laboratory conditions DMPP side effects are observed (Tindaon et al. 2012). Furthermore, nitrifying archaea seem to be less

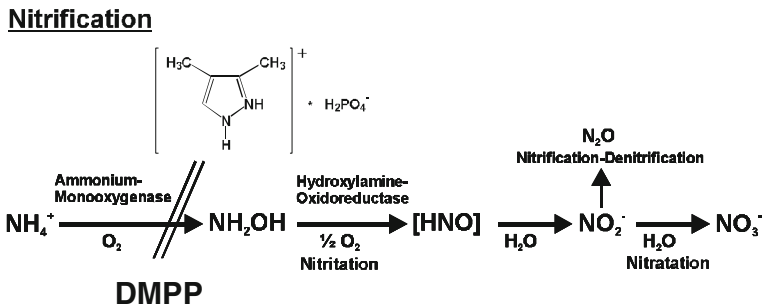

Fig. 1 Chemical structure and specific blocking of NH_3 oxidation by the nitrification inhibitor 3,4-Dimethyl-1H-pyrazoliumhydrogenphospate (DMPP) developed by BASF AG, Germany, and commercialized formulated on granulated, ammonium-based fertilizer under the trade name ENTEC

or not affected by recommended DMPP field application rates and may then numerically outcompete ammonia-oxidizing bacteria (Kleineidam et al. 2011).

NI-effects on soil metabolism can only be studied when a method exist allowing to extract such inhibitors quantitatively from soils after field application. For DCD a method exists (Rajbanshi et al. 1992) and DMPP can be quantified by HPLC in the solution used for stabilizing N-fertilizers (DIN-ISO-5725; Association of German Experimental and Research Stations: Verband Deutscher Untersuchungs- und Forschungsanstalten, VDLUFA; European Committee for Standardization, CEN; VDLUFA-Methodenbuch II.I, 4. Erg., 2008). A reliable method for extracting DMPP quantitatively from the soil matrix we had to develop in order to study the fate of DMPP in agricultural soils.

Here the method, developed by us and tested in a 3 years field experiment, is detailed described. Reported is also how increasing DMPP concentrations influence the bacterial morphology and the NH_4^+ conversion into nitrate.

Material and methods

Experimental design and soil sampling

Soil samples were taken with an auger to a depth of 15 cm in 10×8 m randomly distributed field plots (4 plots per treatment), established within a long-term experiment at the Experimental Station of the Agronomy Faculty of Justus Liebig University, Giessen, Germany. The long-term study was initiated in 1982 to evaluate N_2 fixation in different crop rotation systems. The 3 years field experiment was designed as a Latin square and the soil classified as an allochtone brown earth, derived from river sediments, FAO-classification: Fluvisol, German classification: Vega with a clayey loam topsoil, 0–35 cm: clay 31 %, silt 60 %, sand 5 %, pH_{CaCl2} 6.0–6.4, C_t 1.35–1.48 %, N_t 0.15–0.16 %, P 160–450, K 120–330 mgkg^{-1} dry soil, respectively. On March 24, 1997, the experimental site received 90 kg ammonium sulphate nitrate nitrogen (ASN–N) ha^{-1} in one dose. On the granulated ASN-fertilizer either DMPP or DCD was formulated (ASN, 26 %N, 18.5 % NH_4^+–N and 7.5 % NO_3^-–N; DMPP: 1.6 % to NH_4^+–N and DCD 16 % to NH_4^+–N). The control plots received only ASN without the NI DMPP or DCD. The field plots were cropped with spring barley (*Hordeum vulgare* L., fertilized on 12 March 1997 with 90 kg ASN-Nha^{-1}), with maize (*Zea mays* L., fertilized on May 5, 1998, with 160 kg ASN–Nha^{-1}), and with winter wheat (*Triticum aestivum* L., fertilized on March 18 with 180 kg ASN–Nha^{-1}; for more details see Weiske et al. 2001a; b). In this 3 years field experiment DMPP was regularly quantified by extraction and high pressure liquid chromatography (HPLC) using the method developed by us and detailed described below. Regularly collected random samples of the DMPP treated experimental site were deep-frozen (−20 °C) until DMPP was extracted and quantified from thawed aliquots. Soil samples in the NI-untreated control plots were collected separately for enriching a nitrifying bacteria consortium. The soil dry weight was determined after drying at 105 °C (Schlichting et al. 1995).

CO_2−, N_2O−, and CH_4-emissions after application of DMPP- and DCD-stabilised ASN fertilizer, or ASN fertilizer without NIs, were measured with the closed chamber method in the 3 years field experiment (4 replicate plots; Hutchinson and Mosier 1981; Weiske et al. 2001a), DCD according to Rajbanshi et al. (1992) and DMPP as described below.

DMPP extraction from field soils and its quantification

Equipment and chemicals

Centrifuge (4,000 rpm), refrigerator, balance (μg-g-range), overhead shaker, whirl mix, pH-meter, vacuum pump, high-performance liquid chromatography (HPLC) equipped with a Discovery C18 column, 5 μm (15 cm×4,6 mm) + pre-column (Supelco, Cataloguenr. 504955), UV-detector: wave length 220 nm, 50 ml tightly closable, tapered polypropylene centrifuge tubes, 10–15 ml tapered glass centrifuge tubes, HPLC-vials + glass or PVC micro-vials to reduce the volume, drilled plastic stoppers with a hole to accommodate a 1 ml pipette tip, 1 ml pipette tips, 10 ml pipette tips, filter equipment to produce HPLC-suitable water, H_2O (dest), K_3PO_4 (pA), DMPP and DMP (purity 99.9 and 96 %, respectively, obtained from BASF Ag, Ludwigshafen, Germany), DCD (purity 96 %, purchased from SKW Trostberg AG, Trostberg, Germany), $CaCl_2$ (pA), NaOH (pA), butyl-methyl-ether (MTBE, pA), Na_2SO_4 (water free), methanol (HPLC purity), H_3PO_4 (pA), KH_2PO_4 (Merck, Germany).

DMPP extraction procedure

The flow chart (Fig. 2) summarizes the single steps during DMPP extraction from soil samples amended with DMPP stabilized N-fertilizer and how DMPP is quantified by HPLC. For testing the reliability of the developed method deep frozen topsoil field samples (0.5 kg) collected in the plots only having received ASN were thawed, with a spatula thoroughly homogenized and 10 g aliquots were weighted into 50 ml tapered, tightly closable polypropylene centrifuge tubes (Nunc, Denmark; 10 replicates). The soil samples were treated with 0, 0.01, 0.02, 0.04 0.2, 0.4, 0.8 or 1.6 μg DMPP g^{-1} soil (purity 99.9 %), dissolved in 1 ml distilled water, and before it was tried to extract the added DMPP corresponding on dry soil basis to 0, 0.0127, 0.0255, 0.051, 0.2548, 0.5096, 1.0191 or 2.0382 μg DMPP g^{-1} quantitatively from the soil matrix by the following steps DMPP was allowed to interact in a refrigerator for 24 h with the soil matrix.

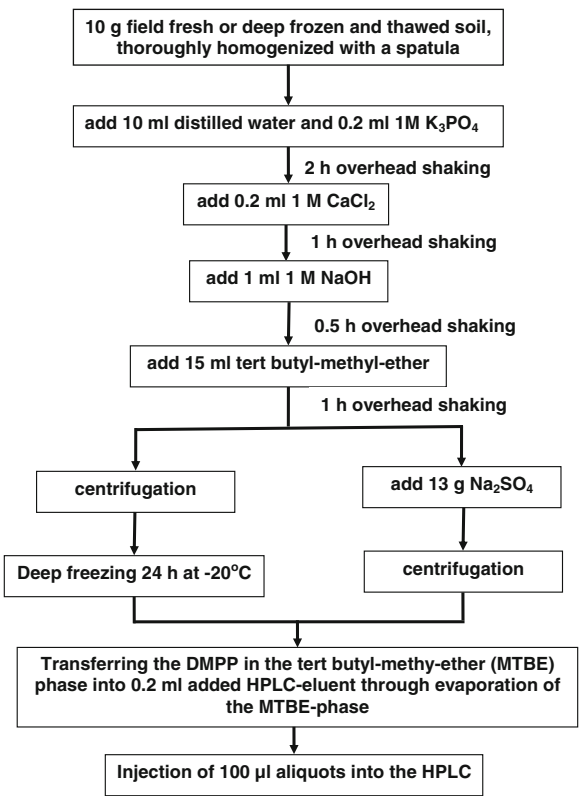

Fig. 2 Flow chart of a newly developed method for quantifying 3,4 dimethyl-pyrazolephosphate (DMPP) in soils

DMPP-extraction:

a) 10 ml distilled H_2O (DMPP-extractant) and 0.2 ml of a pA 1 M K_3PO_4 solution (dispersing agent) are added in 3 replications to 10 g clayey loam topsoil in 50 ml tapered tubes. To achieve a good soil aggregate dispersion, prerequisite for a satisfying DMPP-extraction, the stoppered tubes are shaken upside down for 2 h at 30 rpm.
b) The humic acids in the dispersed soil solutions are precipitated with 0.2 ml of a pA 1 M $CaCl_2$− solution, followed by another shaking upside down at 30 rpm for 1 h. The largely dispersed soil samples reach a pudding-like consistence allowing a better DMPP liberation from soil exchangers.
c) Subsequently 1 ml of a pA 1 M NaOH solution is added to the soil suspensions (10 g fresh soil, 10 ml H_2O, 0,2 ml 1 M K_3PO_4 solution, 0,2 ml 1 M $CaCl_2$). The base is homogeneously distributed by an upside down shaking for 30 min at 30 rpm during which the pH in the pudding-like suspension increases to about 8 and DMPP disproportionates into DMP ($[DMP]^+$ and H_2PO_4.
d) For transferring DMP into a t-butyl-methyl-ether phase (MTBE), 15 ml (MTBE) are added to the pudding-like suspensions, the samples for 1 h overhead shaken and afterwards centrifuged for 5 min at 3,000 rpm.
e) The DMP-containing MTBE is separated from the aqueous phase either by freezing or salting out the water. Freezing out the water is achieved by putting the 50 ml centrifuge tubes for 24 h into a deep freezer at −20 °C. Salting out the water is achieved by adding 13 g water-free Na_2SO_4 and shaking the 50 ml centrifuge tubes heavily by hand until the salt is homogenously distributed. Na_2SO_4 clumping must be avoided. A semi-solid salt column forms.
f) The MTBE-water phase is separated by centrifugation (5 min at 3,000 rpm) and the DMP-containing MTBE phase (about 10 ml) transferred into fresh glass test tubes, stoppered, and stored as DMP containing MTBE stock in a refrigerator.
g) From the DMP-containing MTBE stock 2 ml aliquots are pipetted into tapered 5 ml centrifugation tubes containing 0.2 ml HPLC eluent (170 g ≅ 212 ml methanol, 788 ml of a 1 mM H_3PO_4−5 mM KH_2PO_4−buffer L^{-1}, pH 2.8) and the tubes closed with plastic stoppers, in which a

hole is drilled for inserting a 1 ml pipette tip that allows to connect the tube to a vacuum pump.

h) For shifting DMP from the ether into the eluent phase the vacuum pump connected tubes are rotated on a whirl mix under a hood and in presence of a blow-dryer. Is no ether smell detectable anymore the evaporation of the MTBE phase is finished (about 1 min) and a lipophilic component layer becomes visible on the test tube bezel (cell membranes etc. in the ether phase).

i) At expected low DMPP concentrations in the soil samples a second, third or more 2 ml aliquots of the DMP-ether stock can be added to the tubes and evaporated until a HPLC-detectable DMPP concentration is reached.

j) During MTBE-phase evaporation also methanol disappears from the HPLC-eluent. The final volume will be about 160 μl, from which an auto-sampler injects a 100 μl aliquot into the HPLC. For easing the withdrawing of the 100 μl aliquot the addition of a microvial lifting the fluid level may be helpful.

k) The calculation of the DMP concentration on soil dry weight basis affords to determine the weight of the glass test tubes in which proportions of the DMP-MTBE stock are evaporated before and after use. The test tubes can be hanged backwards into a rotating centrifuge at 4,000 rpm for drying them (about 30 s).

DMPP analytic

DMP is quantified by HPLC (Gynkothek, Germany) using a Supelco C 18 Discovery column (5 μm; 15 cm×4.6 mm), a pre-column (Supelco C 18 Discovery; 5 μm; 2 cm×4.0 mm), a pump (Gynkothek P 560 HPG), a detector (Gynkothek UVD 170 S), an auto-sampler (Gynkothek GINA 50), a degassing unit (Gynkothek DG 1310), and a computer software (Chromeleon Version 4.20). The HPLC, DMP, DMPP and the DMPP-stabilised fertilizer were provided by the research partner BASF, which had developed ENTEC and cooperated in finding a way to quantify DMPP in soils. The DMP standards of 0.05, 0.1 and 0.2 $\mu g ml^{-1}$ were made by diluting 0.1001 g DMP (purity 96 %) in 100 ml HPLC-eluent (1,000 μg DMP ml^{-1}). From this stock 25 ml were diluted first in 500 ml eluent (50 μg DMP ml^{-1}) and finally 2 ml in 50 ml eluent, from which 25, 50 and 100 μl proportions were injected into the HPLC. The related DMP-peak areas were integrated by the computer software Chromeleon Version 4.20 (Softron GmbH, Germany) and expressed in μg DMPP g^{-1} dry soil, using the formula

$$\text{DMPP}\left(\mu g\ g^{-1}\text{dry soil}\right) = \frac{(A - c0) \times M_k \times 10 \times 7.5 \times 2.02}{c1 \times S}$$

(A = peak area; c0 = intercept with the ordinate; M_k = DMPP concentration $\mu g ml^{-1}$; 10=factor: injected 100 μl to ml; 7.5=dilution factor by an used ether phase of 2 ml; 2.02=mol weight DMPP/DMP; c1= slope; S = soil dry weight in g)

Nitrifying bacteria enrichments from the soil of the field trial

DMPP affects nitrifying bacteria but how? For a better insight into NI-caused changes 20 g soil from the plots of the field trial which never had received DMPP were weighted in 500 ml Pyrex bottles and for soil dispersion 180 ml of a 0.18 % sterile sodium pyrophosphate solution added. In the soil solution the most probable number (MPN) of autotrophic nitrifying bacteria was determined using the following selective medium: 0.5, 0.5, 3.5, 0.7, 0.1, 0.18 0.014 gL^{-1}, $(NH_4)_2SO_4$, $NaHCO_3$, Na_2HPO_4, KH_2PO4, $MgSO_4$ * 7 H_2O, $CaCl_2$ * 2 H_2O, $FeCl_3$ *6 H_2O, respectively (pH7.8; 3 replicates; Lorch et al. 1995). In separate samples the soil dry weight was determined after drying at 105 °C (Schlichting et al. 1995). After 4 weeks of incubation at 25 °C turbid tubes of the MPN count were checked for nitrite and nitrate formation and from positively tested tubes 4 ml aliquots, transferred into sterile 50 ml of the above fresh, sterile medium containing 100 ml flasks, served as inoculum (3 replicates). The flasks were incubated in the dark on a horizontal shaker (125 UpM, 25 °C) and after 4 weeks the above procedure repeated and this another time. From the third enrichment culture it was assumed that it may contain the nitrifying bacterial consortium present in the allochtone brown earth.

DMPP inhibitory effects on the nitrifying enrichment culture

Four ml of the nitrifying, allochtone brown earth consortium (third soil enrichment) were transferred into sterile 100 ml Erlenmeyer flasks (3 replicates), which contained

46 ml of the above nutrient broth and zero, 0.005 (recommended field applied DMPP-concentration) and 0.05 gl^{-1} DMPP. The flasks were incubated for 75 days on a horizontal shaker in the dark (125 UpM, 25 °C). Weekly the pH was checked with a scaled litmus paper (Merck, Germany) and spectrophotometrically NH_4^+-N, NO_2^--N and NO_3^--N (Benckiser 2007). For eventual morphological bacterial changes, caused by DMPP, transmission electron microscopy (TEM) at 80 kV (Philips EM300) was employed. At the end of the experiment 1 ml of the respective culture solutions was 50 times diluted with water and a small drop (about 1 µl) of these dilutions was transferred to a 0.3 % formvar resin-coated copper net. After about 2 min the water was removed with filter paper and the bacteria inside of the copper net negative-stained with a 2 % uranylacetate ($(CH_3COOH)_2UO_2 \times 2H_2O$) solution before the copper nets were launched into the TEM.

Results

A gram dry soil of the allochtone Vega soil that has never seen DMPP maintains about 10^5 nitrifying bacteria (MPN counts). The bacteria in a nitrifying enrichment culture from the allochtone Vega soil subdivide under the TEM into 2 morphologically distinct cell structures, into rod-shaped bacteria without flagella and stalk bacterial species with an extremely long prostheca-like appending (Figs. 3a, 4a and b). In presence of increasing DMPP concentrations morphological changes of the cell structures exhibited at the end 75 days, altering ammonium into nitrite and nitrate conversions and pH-shifts (Figs. 3a, b; 4 a-d). At non DMPP-stressed conditions the incorporation of NH_4^+ into the biomass and the conversion of NH_4^+ ions into nitrate started between incubation day 6 and 13 (Fig. 3b). Thereby about 40 % of the initial ammonium was oxidized to NO_3^- by the rod shaped bacteria and only small amounts of nitrite released. Around day 20 of incubation nitrate formation reached its maximum and the pH dropped from around 7.6 to 7.0 by reaching pH6.8 at day 75 of incubation.

Slightly enlarged the bacterial cell bodies appeared in presence of the field applied DMPP-concentration (Fig. 4a). The formation of nitrite set in delayed at around day 30 of incubation and reached its maximum at day 37 or about 30 days later (Fig. 4c). The nitrite release was 5 times higher than in the control (Figs. 3b, 4c). Such an increased nitrite accumulation,

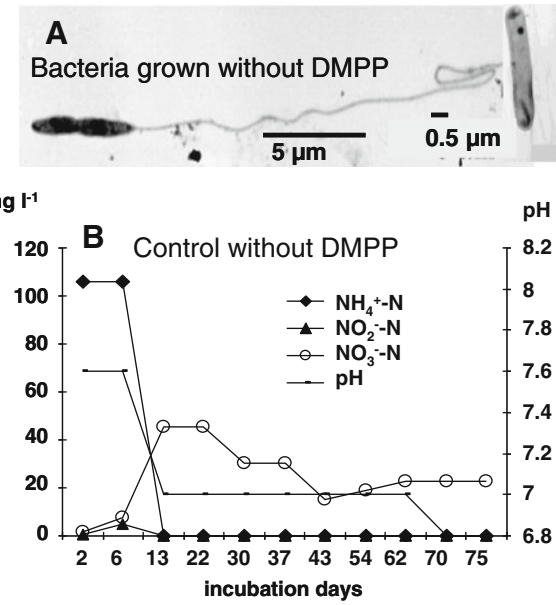

Fig. 3 TEM pictures of bacteria found in a nitrifying enrichment culture from an ammonium-N-fertilizer treated soil of a 3 years field experiment, classified as an allochtone brown earth, derived from river sediments with a clayey loam topsoil (Vega) after 75 days of incubation (Fig. 3a) and conversion of added ammonium to nitrite and nitrate inclusively pH-changes (Fig 3b)

which began to go over into nitrate formation around day 43 of incubation, may affect other metabolic soil processes. The nitrate release peaked significantly delayed compared with the control between day 62 and 70 of incubation and then also the pH began to drop to 6.8 (Fig. 4c). At a 10 times higher DMPP concentration nitrite and nitrate formation was completely blocked, the incurvated bacterial rods considerably enlarged, the prostheca-like attachments widened and broken off and the signalized severe cell damage agrees with the complete inhibition of nitritation and nitratation (Fig. 4b and d).

The schema of the method, developed by us, is shown in Fig. 2 and the data of the Figs 5, 6 and Weiske et al. (2001a; b) unveil that DMPP can be extracted satisfyingly in laboratory and field experiments from the soil matrix. Fig. 5 exhibits that at an expected low DMPP soil concentration the NI can easily be enriched until a HPLC-detectable concentration is achieved and Fig. 6 that the DMPP recovery rate either by freezing or salting out the DMPP extractant, water, is depending on the chosen procedure and on the DMPP-concentration between 50 % and 80 %, somewhat higher by salting out the water indicating possibilities to methodological improvements. During quantifying

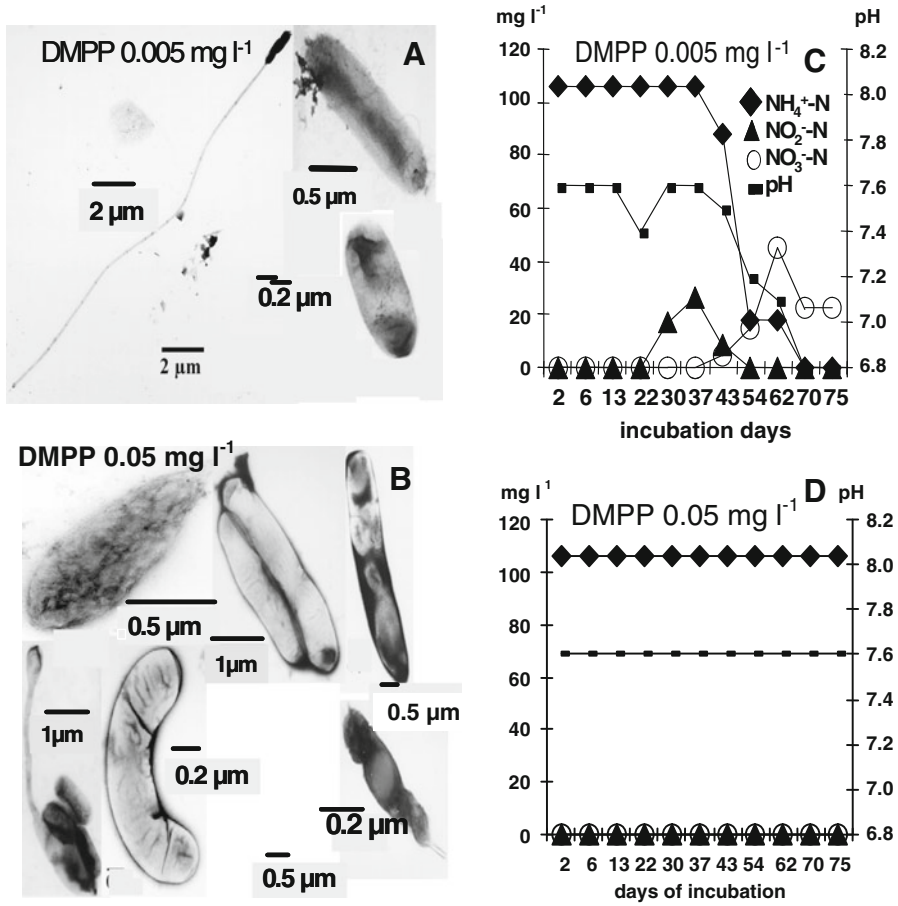

Fig. 4 TEM pictures of bacteria found in a nitrifying enrichment culture from an ammonium-N-fertilizer treated soil of a 3 years field experiment, classified as an allochtone brown earth, derived from river sediments with a clayey loam topsoil (Vega) after 75 days of incubation in presence of 3,4-dimethyl-pyrazolephosphat (DMPP) at field-applied (Fig. 4a) or a 10 times higher DMPP- concentration (Fig. 4b) and conversion of added ammonium to nitrite and nitrate inclusively pH-changes (Fig 4c and d)

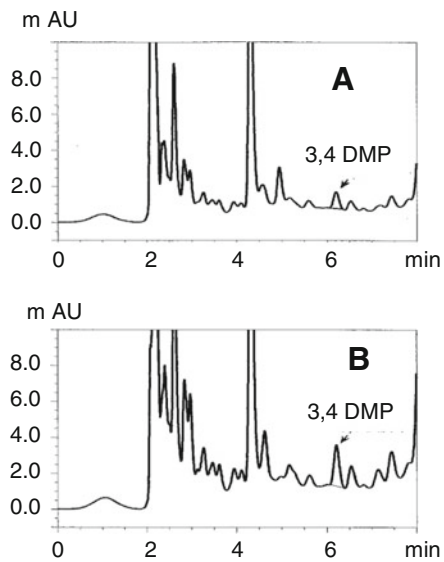

Fig. 5 DMP retention time at a HPLC-eluent pH of 3.4 (**a, b**) (**b**) shows in addition the effect of evaporating another 2 ml DMP containing MTBE from the clayey loam soil extract enriched with 0.01 µg DMPP g^{-1} fresh soil. The peak area almost doubled

DMPP in the field we preferred freezing out the water phase, because deep freezing exhibited no effect on the DMPP recovery rate (data not shown) and freezing out the water is superior to salting out in standardization and sample performance. At Weiske et al. (2001a; b) the disappearing of DMPP and DCD in comparison with

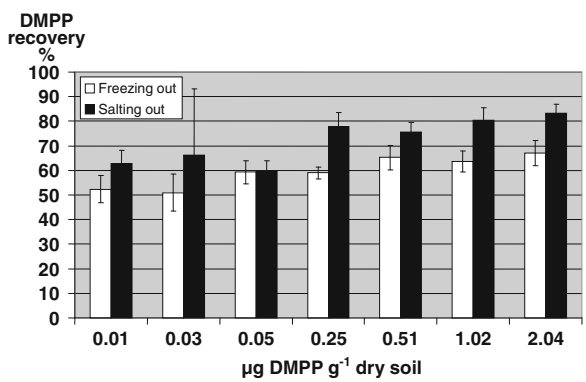

Fig. 6 DMPP (3,4-dimethylpyrazolephosphate) recovery rates after separating the DMP containing MTBE-phase from the DMPP soil extractant water by freezing or salting out the water

CO_2, N_2O and CH_4 emission are presented and reveal that at the end of the vegetation periods in the spring barley field of 1997, the corn field of 1998 and the winter wheat field of 1999 about 16.6 and 15 % of the originally applied DMPP (~ 0.8 mg DMPP kg^{-1} soil), respectively, were still detectable.

Discussion

Most of the nitrogen in the Earth's atmosphere is little reactive N_2 (78 %). It is introduced into soils as organically bound N and converted to more reactive ammonium and oxidized nitrogen intermediates nitrite (NO_2), nitrate (NO_3), nitrogen oxides (NO_x) and nitrous oxide (N_2O; 310 times greater global warming effect as CO_2; IPCC 1995), which may pollute the groundwater or the atmosphere by causing annual costs of around 100 billions US $ (Benckiser 1997; Sutton et al. 2011). DMPP- or DCD-stabilized N-fertilizers are an option to reduce nitrogen impacts (Pasda et al. 2001; Zerulla et al. 2001; Wissemeier et al. 2002; Chaves et al. 2006; Di et al. 2010; Ma et al. 2012). DMPP, binding indiscriminately to the complex of membrane-bound proteins inclusively the ammonium monooxygenase (AMO; Fig. 1), and DCD, blocking the electron transport in the cytochromes of AMO during the conversion of NH_3 to hydroxylamine, could become under hot climate regimes, for example in Pakistani summer crops with soil temperatures of around 35 °C, relatively instable and as found for DCD even increase the N-losses rather than reducing those (Ali et al. 2008; Mahmood et al. 2011). The European Commission, having launched a cost/benefit analysis of farming practices (PICCMAT 2011), has evaluated DCD and DMPP stabilized N-fertilizers and came to the following conclusions: though these relatively expensive fertilizers reduce nitrate formation and losses through greenhouse gas emissions by 26 to 49 %, their N-saving effectiveness is insufficiently tested and their cereal and maize crop yield improvements are not conclusively documented. Thus, the present evaluation is a worthwhile investment (PICCMAT 2011). Also in our summer barley (1997), maize (1998) and winter wheat (1999) field experiments the yields after DMPP-stabilized N-fertilization were more variable within one treatment than among the NI-treatments (Weiske et al. 2001a; b).

DMPP interacts in soils primarily with the nitrifying population on which many other organisms are depending. The nitrifying enrichment from the brown earth soil, we have studied in presence and absence of DMPP (Figs 3 and 4), comprises non-flagellated autotrophic ammonia-oxidizing bacteria (AOB), which may belong to the β- and γ-subgroups of *Proteobacteria*, and bacteria with a narrow, cytoplasm containing extension of the cell wall, a prostheca, which morphologically resemble rather the ubiquitous and frugal α-Proteobacterium *Caulobacter crescentius* family (Stove and Stanier 1962; Staley 1968; Schmidt 1971; Poindexter 1978). A prostheca increases the bacterial surface area, enhances the nutrient uptake, decreases cell sedimentation and is thus helpful for surviving in aquatic and terrestrial environments. Thus, it is not surprising that prostheca carrying species are also found in the archaeal world (Miroshnichenko et al. 1998). AOA seemingly prefer areas low in NH_3 rather than AOB and are in temperate climates evidently less strong inhibited by DMPP than AOB but this needs to be proven (Valentine 2007; Dekas et al. 2009; Prosser and Nicol 2008; Agogué et al. 2008; Di et al. 2010; Fuhrman 2011; Brochier-Armanet et al. 2011; Jarell et al. 2011; Kleineidam et al. 2011; He et al. 2012). Also the accumulation of nitrite in presence of a field applied DMPP-concentration (Fig. 4c) needs to be proven under field conditions, the more because an increased nitrite concentration may interfere with soil respiration (Rinaldo et al. 2008).

On nitrification depends not only the denitrification of nitrifies but also the heterotrophic nitrate respiration, if nitrate is not fertilized. Thus DMPP controls the N_2O release of both bacterial groups by a reduced NO_3^--availability and consequently were in our 3 years field experiment the greenhouse gas N_2O reduced by around 51 % (Fig. 1; Benckiser 1997; Weiske et al. 2001a; b). Reduced N_2O emissions in their dependency of a decreasing NI-concentration were are also observed after field applications of NIs like DCD, nimin, an active agent in the extract of the neem tree *Azadiracta indica*, furanoflavonoid karanjin (3methoxyfurano 2′,3′,7,8 flavone). nitrapyrin or C_2H_2 (Figs 3 and 4; IPCC 1995; Weiske et al. 2001a; b; Majumder et al. 2001 and 2004; Chaves et al. 2006; Benckiser 2007; Di et al. 2010; Abasi et al. 2011; Mahmood et al. 2011; PICCMAT 2011). Oxidized nitrogen intermediates as NO_2^-, NO_3^- or N_2O may also inhibit methane formation while organic bound nitrogen may stimulate this process (Bollag and

Czlonskowski 1973). In our field experiment this process stayed unaffected by DMPP and DCD (Weiske et al. 2001a; b), but all these NI influences on soil metabolic processes and their inhibition efficiency, diffusion behaviour, their fate in soils can only conclusively be understood when a reproducible and routined method exist allowing to extract quantitatively NI-compounds like DMPP from the soil matrix. Our above described method detects DMPP down to a concentration of 0.01 $\mu g g^{-1}$ dry soil and the detection limit is not yet reached as salting out the water implies (Figs 2, 5 and 6). After Azam et al. (2001) had an analytic in hand allowing to study the behaviour of DMPP in soils they found that DMPP in the silty clay soil of the field experiment remains at a water holding capacity of 18 % or 24 % to 80 % within the 0- to 5-mm region around the fertilizer granule and moved only to about 5 to 15 % into the 5− to 20-mm region and to <3 % into the 25− to 40-mm region. In reverse, NH_4^+ diffused about 4 cm in the same 10 days incubation interval and nitrate showed a fairly uniform distribution. Thus, nitrifying/denitrifying soil AOB and AOA can convert ammonium into mobile NO_3^--ions and further into N_2O or N_2 DMPP-uninfluenced from a 4 cm zone on but due to the lower scattering of DMPP compared to DCD the N-fertilizer is prolonged protected and that may explain the extended nitrification inhibiting efficiency of DMPP into the plant growth period as found by Weiske et al. (2001a; b).

Different from temperate climate conditions NIs may behave under soil temperature regimes above 30 °C (Ali et al. 2008; Mahmood et al. 2011). At such conditions the NIs seem to be less stabile and only efficient when their application rates are significantly increased. At a 10 times higher concentration than the recommended field apllied concentrations side effects are observed (Fig. 4), which needs to be studied in more details. Further, there are gaps in our knowledge about (a) the other factors determining the nitrifying potential of soils except NIs, (b) the N_2O emissions rate ratio of AOA and AOB and (c) DMPP soil side effects (PICCMAT 2011).

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