ORIGINAL ARTICLE

Soil moisture determines the effectiveness of two urease inhibitors to decrease N_2O emission

Alberto Sanz-Cobena · Diego Abalos · Ana Meijide · Laura Sanchez-Martin · Antonio Vallejo

Abstract Among the mitigation strategies to prevent nitrogen (N) losses from ureic fertilizers, urease inhibitors (UIs) have been demonstrated to promote high N use efficiency by reducing ammonia (NH_3) volatilization. In the last few years, some field experiments have also shown its effectiveness in reducing nitrous oxide (N_2O) losses from fertilized soils under conditions of low soil moisture. An incubation experiment was carried out with the aim of assessing the main biotic mechanisms behind N_2O emissions once that the UIs N-(n-butyl) thiophosphoric triamid (NBPT) and phenil phosphorodiamidate (PPDA) were applied with Urea (U) under different soil moisture conditions (40, 60 and 80 % water-filled pore space, WFPS). In the same study we tried to analyze to what extent soil WFPS regulates the effect of these inhibitors on N_2O emissions. The use of PPDA in our study allowed us to compare the effect of NBPT with that of another commercially available urease inhibitor, aiming to see if the results were inhibitor-specific or not. Based on the results from this experiment, a WFPS (i.e. 60 %) was chosen for a second study (i.e. mesocosm experiment) aiming to assess the efficiency of the UIs to indirectly affect N_2O emissions through influencing the pool of soil mineral N. The N_2O emissions at 40 % WFPS were almost negligible, being significantly lower from all fertilized treatments than that produced at 60 and 80 % WFPS. When compared to U alone, NBPT+U reduced the N_2O emissions at 60 % WFPS but had no effect at 80 % WFPS. The application of PPDA significantly increased the emissions with respect to U at 80 % WFPS whereas no significant effect was found at 60 %. At 80 % WFPS, denitrification was the main source of N_2O emissions for all treatments. In the mesocosm study, the application of NBPT+U was an effective strategy to reduce N_2O emissions (75 % reduction compared to U alone), due to a lower soil ammonium (NH_4^+) content induced by the inhibitor. These results suggest that adequate management of the UI NBPT could provide, under certain soil conditions, an opportunity for mitigation of N_2O emissions from fertilized soils.

Keywords Urease inhibitors · WFPS · Ammonia · Nitrous oxide · Urea · NBPT

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1 Introduction

Productivity of intensive agricultural ecosystems is highly dependent on nitrogen (N) inputs from fertilizer application. Nevertheless, the low efficiency of N from fertilizers to reach crops represents a threat to environmental quality due to the release of reactive N to the environment in the form of, among others, atmospheric ammonia (NH_3) (Rana and Mastrorilli 1998) and the greenhouse gas (GHG) nitrous oxide (N_2O) (IPCC 2007). Agriculture represents 58 % of anthropogenic N_2O emissions (IPCC 2007) and 20 to 80 % of NH_3 emissions in many countries (Jantalia et al. 2012). Urease inhibitors (UIs) have been proposed by UNECE (2001) as a means to reduce NH_3 volatilization from urea (U) application (Grant and Bailey 1999; Sanz-Cobena et al. 2008), which is the predominant source of synthetic N fertilizer used throughout the world (Harrison and Webb 2001). Among the various types of UIs that have been identified and tested, derivates of the phosphorodiamidates such as N-(n-butyl) thiophosphorictriamide (NBPT) and phenyl phosphorodiamidate (PPDA) have been studied the most, showing to be effective at mitigating NH_3 emission at relatively low concentrations under both laboratory (Pedrazzini et al. 1987; Gill et al. 1999) and field conditions (e.g. Sanz-Cobena et al. 2008). When the two inhibitors have been compared, NBPT performed much better as a urease inhibitor than PPDA (e.g. Byrnes et al. 1989). During the last years, the effect of NBPT on N_2O emission has been a matter of research (Zhengping et al. 2007; Zaman et al. 2008, 2009; Menéndez et al. 2009). The few field studies that have been carried out up to 2008 indicated that UIs mixed with urea did not have a significant effect on N_2O emission (see Akiyama et al. 2010 for a meta-analysis including data from 3 studies).

Some studies showed contrary results with significant reduction of N_2O emission when urea was applied with NBPT in maize (e.g. Ding et al. 2011; Sanz-Cobena et al. 2012) and pastures (Dawar et al. 2012). Ding et al. (2011) and Sanz-Cobena et al. (2012) showed a 38 and 54 % mitigation of N_2O emissions, respectively, by using NBPT in a maize (*Zea*) crop fertilized with urea and with controlled irrigation (WFPS of 48 and 58 % on average, respectively). The authors of the latter study evaluated, in a second cropping period, the effect of the UI on N_2O emissions in a highly irrigated soil (i.e. soil moisture ≥70 % WFPS) and, in such conditions, the inhibitor did not have any significant effect. Taking into account that nitrification and denitrification are the dominant sources of N_2O in soils (Firestone and Davidson 1989), the reported effect of UI over N_2O losses must be associated with changes in these processes either directly (i.e. by affecting the soil microbiota) or indirectly (e.g. by affecting the N substrates for these processes). Since the observed abating effect of NBPT over N_2O losses was found under soil nitrifying conditions (soil moisture≤60 % WFPS), we first hypothesized that UIs do not affect, neither directly nor indirectly, the denitrification process. Accordingly, it would be expected that UIs will affect N_2O losses from urea-fertilized soils by indirectly influencing the nitrification process. This is based on the fact that, in soils with low ammonium (NH_4^+) content, the nitrification rate depends on the concentration of this N substrate (Xu et al. 2002) (i.e. first order relationship). Since the inhibition of urease activity slows down the production of NH_4^+ by retarding urea hydrolysis (Zaman et al. 2008), the soil nitrification rate could be decreased by the use of UIs. The overall effect on N_2O losses might, therefore, be similar to that of using nitrification inhibitors, where decreased emissions of N_2O are associated with reduced nitrification rates (Vallejo et al. 2005). However, in the case of nitrification inhibitors the reduction is a direct effect on nitrifying microorganisms (Akiyama et al. 2010). Our final question regarding UIs is derived from the fact of PPDA having been shown to be less efficient than NBPT inhibiting the urease activity (Byrnes et al. 1989). We hypothesized that the extent of the abatement of N_2O emissions is inhibitor-specific, with NBPT leading to lower emissions (Abalos et al. 2012).

Trying to answer our research questions, two laboratory experiments were carried out. To date, given the inherent high variability of field experiments where soil moisture content changes rapidly and spatial variability is high, it has been impossible to understand the underlying mechanisms responsible for the suppression of N_2O emissions associated with the use of NBPT and its dependency on soil WFPS. Therefore, the objectives of the first study (i.e. incubation experiment) were (i) to determine the main biotic mechanism behind N_2O emission when UIs are applied under different soil moisture conditions (40, 60 and 80 % WFPS), and (ii) to analyze to what extent the soil WFPS regulates the effect of the inhibitor over N_2O emissions. The second study (i.e. mesocosm experiment) was designed, based on the results from the incubation study, aiming (iii) to assess the efficiency of NBPT for abating N_2O emissions through influencing the pool of soil mineral N. Additionally, PPDA was also used in both experiments (iv) in order to compare the effect of NBPT with that of another commercially available urease inhibitor; this may allow us to see if the effect of NBPT was inhibitor-specific or not.

2 Methods

2.1 Soil

The experiments were carried out using the same soil as that of Sanz-Cobena et al. (2012). The soil was sampled from the A_p horizon of a Calcic Haploxerepts according to the USDA soil taxonomy system (Soil Survey Staff 1992) from 'El Encín' field station, 38 km east of Madrid, Central Spain (latitude 40°32′N, longitude 3°17′W). Some of the physico-chemical properties of the top 0–28 cm of the soil layer, measured by standard methods of soil analysis (Burt 2009), were: total organic C, 8.4 ± 0.4 g kg^{-1}; pH_{H2O}, 7.9; bulk density, 1.4 ± 0.1 g cm^{-3} and; $CaCO_3$, 13.2 ± 0.3 g kg^{-1}. The soil has a sandy clay loam texture (clay, 28 %; silt, 17 %; sand, 55 %) in the upper (0–28 cm) horizon.

2.2 Incubation experiment

The incubation study was performed to test the effect of urease inhibitors on N_2O emissions at three moisture contents (i.e. 40, 60 and 80 % WFPS). Experimental units consisted of 20 g dry soil in 250 mL glass jars (soil bulk density 1.20 g cm^{-3}). Prior to the experiment, the soil was air-dried, sieved (< 2 mm) and pre-incubated at 40 % water holding capacity for 7 days, to avoid the pulse of respiration associated with wetting dry soils. The fertilizer treatments were: U, NBPT+U (0.15 % w/w) and PPDA+U (2 % w/w); UIs were applied at rates recommended in previous studies (e.g. Joo and Christians 1986; Sanz-Cobena et al. 2011). A control (C) treatment without N addition was also included. All units, except for C, received equal total amounts of U (120 kg N ha^{-1}). To eliminate the substrate limitation for denitrifying microorganisms, nitrate was applied to fertilized treatments in the form of KNO_3 at a rate of 50 kg N ha^{-1}. Therefore, the total N application rate was 170 Kg N ha^{-1}. The fertilizers were added as a 4 mL solution, and subsequently distilled water was added to all units up to reach the humidity defined for each soil water content. The glass jars were then sealed with a rubber stopper and separated into two groups for further additions of 0 and 0.01 % of acetylene (C_2H_2). For N_2O analysis, following the method proposed by Zhu et al. (2013), headspace gas samples (vol. 10 mL) were taken with syringes after 36 h and stored in evacuated vials. Three replicates per treatment were used. Nitrous oxide (N_2O) was analyzed by gas chromatography as described below.

2.3 Mesocosm experiment

2.3.1 Experimental design

An additional 20 day mesocosm experiment under controlled conditions was carried out to quantify N_2O emissions as a function of different fertilizer treatments with and without UIs. The fertilizer treatments were the same as in the incubation experiment. Urea, at a rate of 17 g N m^{-2} (170 kg N ha^{-1}), and UIs were surface-applied in solution to the soil. Mesocosms were constructed of PVC (18 cm width, 18 cm length), filled with 2 kg of air-dried soil. The experiment was carried out at a constant temperature (20 1°C) and at the moisture content at which NBPT showed to be effective mitigating N_2O emissions in the incubation experiment (60 % WFPS). Soil moisture was kept constant during the experimental period, adding distilled water every other day. Six replicates per treatment were used, three for N_2O measurements and three to follow the evolution of mineral N (i.e. availableNH_4^+ and nitrate (NO_3^-)) and urease activity. Fluxes of carbon dioxide (CO_2) and NH_3 were also measured in order to test the effect of the two inhibitors on soil respiration and NH_3 volatilization.

2.3.2 Soil analysis

Soil cores (7 cm depth, 3.1 cm^2 surface area) were collected 11 times during the mesocosm experiment (0, 1, 2, 3, 4, 6, 8, 10, 13, 15, 20 days after fertilization, DAF). NO_3^- and NH_4^+ were determined by extracting 4 g of fresh soil with 50 mL 1 M KCl solution. After soil extraction, NO_3^- concentration was determined based on its reduction to nitrite (NO_2^-) and subsequent colorimetric determination of this ion with a diazo-coupling reaction (Griess reaction) (AOAC 2000). Nitrite and NH_4^+ were colorimetrically analyzed using a Technicon AAII Auto-analyzer (Technicon Hispania, Spain). Net nitrification rate was calculated from the net increase in NO_3^- occurring during two consecutive soil sampling dates (e.g. Cheng et al. 2004). According to these authors, these changes could be fitted to two types of models. One follows a zero-order reaction model (Eq. 1), where the nitrification rate is not substrate (i.e. NH_4^+) dependent, and the other can be adjusted to a first-order reaction model, where NH_4^+concentration is a determinant factor over the nitrification rate (Eq. y2):

$$N_t = k_z t + N_i \tag{1}$$

$$N_t = N_0\{1 - \exp{-k_f t}\} + N_i \tag{2}$$

where N_t is the amount of NO_3^- present in the soil in day t (mg N kg^{-1} soil); N_i is the amount of NO_3^- present in the soil at the beginning of the experiment (mg N kg^{-1} soil); N_0 is the potential amount of N that could be nitrified (mg N kg^{-1} soil) in a first-order reaction model; and k_z and k_f are nitrification rate constants (d^{-1}) for zero- and first-order reaction models, respectively.

Urease activity was determined following Nannipieri's method (Nannipieri et al. 1980), which involves the incubation of soil with buffered urea solution, the extraction of NH_4^+ with 1 N KCl and the colorimetric NH_4^+ determination as previously described. Although both data on urease activity and nitrification rate were measured at 11 sampling dates within the experimental period (i.e. 20 days), only the results from 1, 2, 4, 6, 8, 10 and 13 DAF were included in Table 1, since the values of nitrification rate and urease activity remained constant after the latter date.

Table 1 Urease activity (μmol NH_4^+-N g^{-1} h^{-1}) and nitrification rate (mg NO_3^--N kg^{-1} h^{-1}) measured in the mesocosm experiment at several sampling events corresponding with different days after fertilizing (DAF)

Treatments/DAF	1	2	4	6	8	10	13
Urease activity (μmol NH_4^+-N g^{-1} h^{-1})							
U	7.45±0.03b	9.72±0.12b	10.92±0.33b	12.62±0.42b	10.45±0.23b	5.92±0.56b	5.06±0.37a
NBPT+U	3.83±0.05a	3.48±0.64a	3.50±0.11a	3.08±0.37a	4.31±0.09a	3.21±0.04a	4.44±0.02a
PPDA+U	4.08±0.03a	4.13±0.07a	8.00±0.81b	7.32±0.18ab	9.11±1.81b	7.04±0.04b	3.28±0.05a
Nitrification rate (mg NO_3^--N kg^{-1} h^{-1})							
U	0.61±0.03b	0.04±0.02b	0.78±0.02b	2.55±0.01c	0.00±0.11a	0.00±0.03a	0.15±0.12a
NBPT+U	0.52±0.09a	0.33±0.02c	0.32±0.01a	0.44±0.02a	0.12±0.17a	0.18±0.01b	0.26±0.11a
PPDA+U	0.59±0.02ab	0.00±0.01a	0.63±0.02b	0.93±0.15b	0.00±0.21a	0.00±0.15a	0.51±0.15a

Different letters within columns indicate significant differences by applying Fischer's Least Significant Difference test at $p<0.05$, $n=9$

2.3.3 N_2O, NH_3 and CO_2 flux measurements

Fluxes of N_2O, NH_3 and CO_2 were measured using 0.45 L manual chambers (soil surface area: 0.038 m^2). All chambers were equipped with inlet and outlet holes, which allowed chambers to be closed during GHG sampling and open for NH_3 measurements. A gas flow-through system was used to measure NH_3 fluxes. Several times per day (i.e. 3–4), the lids were placed on the chamber for an hour and air was passed through using an air pump ($Q=1$ L min^{-1}). The NH_3 was trapped in an oxalic acid solution (3 % w/v) in 25 mL Erlenmeyer bottles. The final solution was then analyzed for NH_4^+-N by spectrophotometry using the nitroprussiate method (Searle 1984). The control for the measurement of NH_3 was obtained by bubbling the air (from the laboratory where measurements were taken) directly from the pump to the acid solution. For the measurement of N_2O and CO_2 fluxes, the lids of the chambers were closed, and gas samples were taken at times 0, 15 and 30 min after closure. Gas samples were stored in 10 mL glass vials and analyzed by gas chromatography (HP6890, Hewlett-Packard, Palo Alto, USA), using a flame ionisation detector (FID) equipped with a methanizer and a ^{63}Ni electron-capture detector for direct CO_2 and N_2O measurements (Abalos et al. 2012).

### 2.4 Calculations and statistical analysis

Statistical analysis was performed using Statgraphics Plus 5.1 (Manugistics 2000). The normal distribution of the data was checked using the Kolmogorov–Smirnov test. Differences between treatments were analyzed using analysis of variance (ANOVA, $p<0.05$). The least significant difference (LSD) test was used for multiple comparisons between means. For non-normally distributed data, the Kruskal–Wallis test was used on non-transformed data to evaluate differences at $p<0.05$. Schaich–Hamerle analysis was then used as a post hoc test.

For the incubation experiment, N_2O produced in + C_2H_2 (0.01 %) was attributed to denitrification, whereas N_2O generated via nitrification was calculated by subtracting the N_2O produced in +C_2H_2 (0.01 %) from that produced in –C_2H_2 (Zhu et al. 2013). For the mesocosm experiment, linear regression analyses ($p<0.05$) were performed to determine relationships between NH_3-N, N_2O-N and CO_2-C fluxes with soil NH_4^+-N, NO_3^--N and urease activity. Total NH_3-N, N_2O-N and CO_2-C fluxes per treatment were estimated by successive linear interpolations of the flux measurements.

3 Results

3.1 N_2O emission from the incubation experiment

Nitrous oxide emissions at 40 % WFPS were almost negligible, being significantly lower ($p<0.005$) from all fertilized treatments than those produced at 60 and 80 % WFPS (Fig. 1). Compared with urea alone, NBPT+U reduced N_2O emissions at 60 % WFPS but had no effect at 80 % WFPS. The application of PPDA significantly increased the emissions with respect to U at 80 % WFPS, whereas no significant effect was found at 60 % WFPS. At 80 % WFPS denitrification was the main source of N_2O emissions for all treatments. The N_2O emissions from nitrification (ammonia oxidation) were significantly lower ($p<0.05$) for all treatments at 40 % WFPS. The same occurred with C (for all moisture contents), U at 80 % and NBPT at 60 % WFPS compared with the other treatments and moisture conditions, which were not different between them. Differences from denitrification (heterotrophic denitrification) were statistically significant only at the 10 % significance level ($p=0.068$).
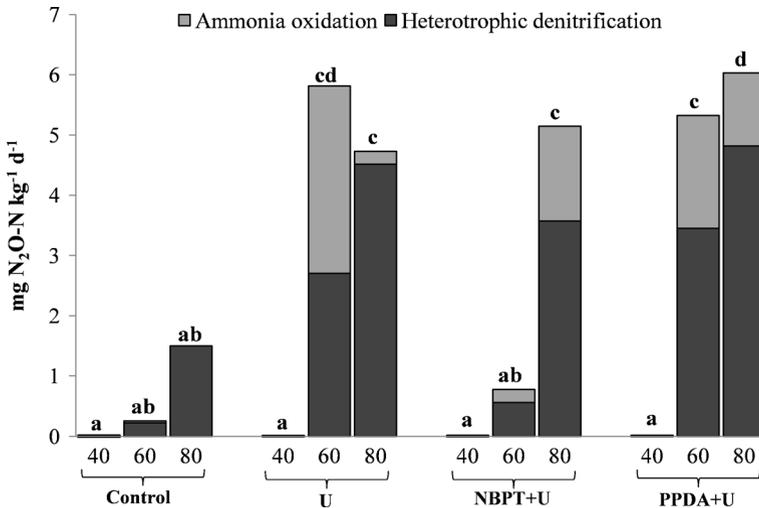
Fig. 1 Effect of urease inhibitors on N_2O emissions (mg N_2O-N kg^{-1} d^{-1}) at three moisture contents (i.e. 40, 60 and 80 % WFPS). Different letters indicate significant differences by applying Fischer's Least Significant Difference test at $p<0.05$

3.2 Mesocosm experiment

3.2.1 Urease activity, mineral N and nitrification rate

Urease activity showed the highest values in U-fertilized soils during the entire sampling period (max. of 12.62 μmol NH_4^+-N g^{-1} h^{-1}, 6 DAF). NBPT+U and PPDA+U significantly decreased urease activity during the 2 days following application (Table 1). From that date, urease activity significantly increased in the PPDA+U fertilized mesocosms up to 9.11 μmol NH_4^+ g^{-1} h^{-1} 8 DAF. These values did not significantly differ from those measured in U (Table 1). NBPT significantly reduced urease activity until day 10 after fertilizer application.

Ammonium in the upper soil (0–7 cm) increased sharply after fertilization in the U treatment, with a maximum value of 144 mg NH_4^+-N kg^{-1} (2nd day). By contrast, the lowest NH_4^+-N concentration was measured in the NBPT+U-treated soil (Fig. 2a). The PPDA+U soil exhibited a similar behavior to NBPT+U during the first 2 days following application. An increase in NH_4^+-N of the PPDA+U soil was observed after that period, reaching values 42 % lower than those of U (87 % lower in the case of NBPT). After 4 days, a continuous decrease in the concentration of soil NH_4^+-N was observed in all treatments, with no significant differences between them at the end of the experiment (i.e. 15–20 days).

Soil NO_3^--N sharply increased for U up to 177.38 mg NO_3^--N kg^{-1} 6 DAF (Fig. 2b). Nitrate in the PPDA+U- and NBPT+U-treated soil showed a similar trend with the lowest NO_3^--N concentration measured at the end of the first week and then a continuous increase to 91 and 70.35 mg NO_3^--N kg^{-1} (i.e. 13 DAF), respectively. Net nitrification rates were affected by inhibitors, with the highest values observed in the U-treated soil (Table 1). The mean nitrification rate during the first 8 days after fertilization was 0.78±0.52; 0.29±0.09 and 0.37±0.21 mg NO_3^--N kg^{-1} h^{-1} for U, NBPT+U and PPDA+U, respectively. Net nitrification rate fitted significantly to a first-order model for this fertilized calcareous soil ($r^2=-1.04$ and $r^2=0.24$ for the zero-order model in U), similarly to Cheng et al. (2004) who found a good fit to a first-order model for calcareous soils and zero-order for acid soils. Nitrification rate was
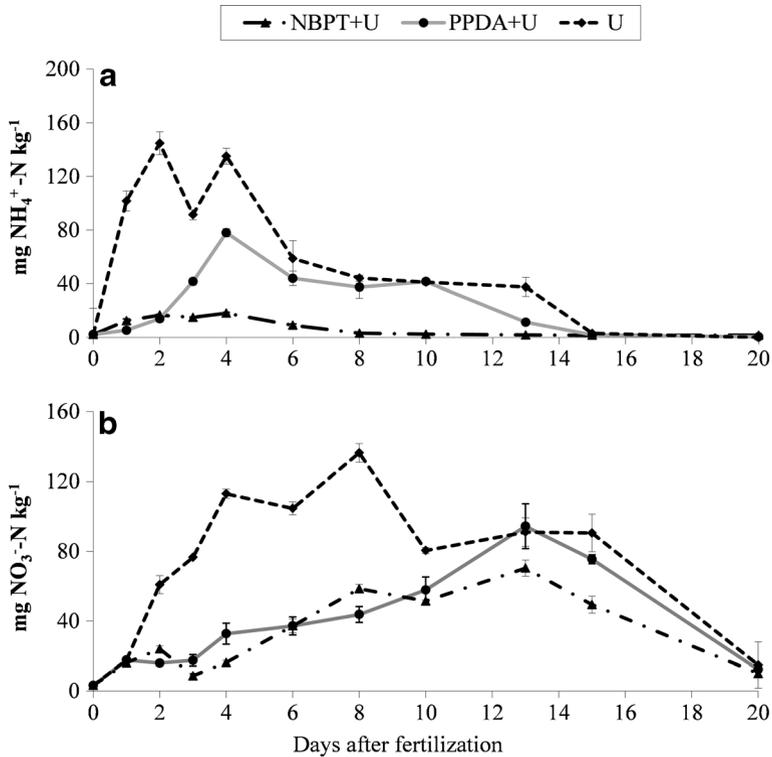
Fig. 2 **a** NH_4^+ (mg NH_4^+-N kg^{-1}) and **b** NO_3^- (mg NO_3^--N kg^{-1}) concentrations in the 0–7 cm soil layer throughout the measurement period. Vertical lines indicate standard errors

also well correlated in the inhibitors treated soil (r^2=−0.96 and 0.23 in NBPT+U and r^2=−1.05 and 0.31 in PPDA+U, for first and zero-order model, respectively). The net nitrification rate constant (k_f) was not significantly (p>0.05) affected by the inhibitors (i.e. 1.3±0.2, 1.1±0.2 and 1.1±0.1 mg kg^{-1} d^{-1} on average for U, NBPT+U and PPDA+U, respectively).

3.2.2 NH_3 volatilization

Ammonia fluxes peaked during the first 48 h following treatment application (Fig. 3a), with the highest fluxes measured in U-fertilized soils (8.8 kg NH_3-N ha^{-1} d^{-1}). By contrast, NBPT+U and PPDA+U produced the lowest NH_3-N fluxes. After peaking, NH_3 emission decreased sharply, to almost zero in all treatments. A positive linear regression was found between NH_4^+-N concentration and measured NH_3-N emission (p<0.01, r^2=0.92, n=90). The highest cumulative NH_3-N emissions were measured in the soil fertilized with U only (20±0.6 kg NH_3-N ha^{-1}) (Table 2). Cumulative NH_3-N emissions were reduced by the application of NBPT+U and PPDA+U by 58 % and 49 %, respectively.

3.2.3 N_2O emission

Daily N_2O-N emissions increased immediately after fertilization for all treatments except for NBPT+U for which low emission rates were maintained during the entire experimental period
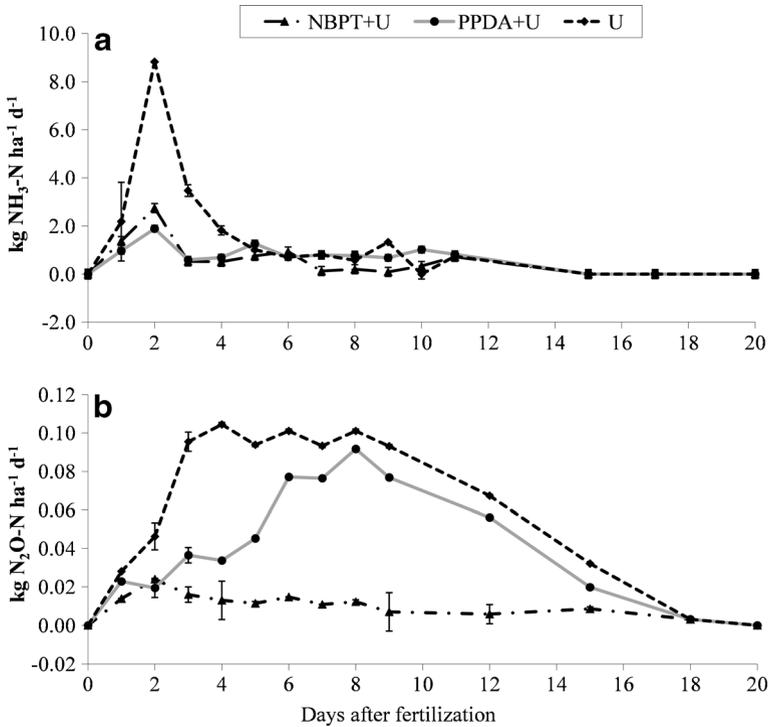
Fig. 3 **a** Ammonia emission rates (kg NH_3-N ha^{-1} d^{-1}) and **b** fluxes of N_2O (kg N_2O-N ha^{-1} d^{-1}) throughout the measurement period. Vertical lines indicate standard errors

(Fig. 3b). Emissions from the PPDA+U-treated soils were similar to those produced by NBPT+U in the first 72 h after fertilizing. After this period, an increase in N_2O-N fluxes was observed in the PPDA+U soil, up to its highest value, 1 week after fertilization (9.1 mg N_2O-N m^{-2} d^{-1}). U produced the highest fluxes of N_2O-N with an initial peak 3 DAF (10.4 mg N_2O-N m^{-2} d^{-1}) (Fig. 3b). After 5 days with stable values, emissions dropped to levels close to zero by the end of the experiment for all treatments. Negative correlations ($p<0.01$) were found between N_2O-N emissions and NO_3^--N concentration in the soil. By contrast, concentration of NH_4^+-N and N_2O-N fluxes were positively correlated ($r^2=0.92$, $n=90$).

Cumulative N_2O-N emissions are shown in Table 2. Application of NBPT+U was the most effective strategy to reduce N_2O-N from U within the measurement period, with a reduction in emissions of 75 %.

Table 2 Cumulative N_2O-N, NH_3-N and CO_2-C fluxes during the measurements periods (20 days)

	N_2O (kg N_2O-N ha^{-1})	NH_3 (kg NH_3-N ha^{-1})	CO_2 (kg CO_2-C ha^{-1})
U	1.01±0.06c	20±0.63c	158.64±7.14c
NBPT+U	0.17±0.01a	8.5±0.31a	98.05±7.25a
PPDA+U	0.67±0.01b	10.3±0.61b	147.07±1.34bc

Data are the averages of means from three replicates ± standard deviation. Different letters within columns indicate significant differences by applying Fischer's Least Significant Difference test at $p<0.05$

4 Discussion

Our results show that the two UIs used could be an effective option to mitigate N_2O emissions (and NH_3 volatilization) under nitrification favoring soil conditions (e.g. soil moisture contents of c. 60 % WFPS). This effect was inhibitor-specific, as NBPT was more effective than PPDA reducing N_2O emissions (83.2 and 33.7 % of reduction for NBPT and PPDA, respectively). However, under denitrifying conditions (80 % WFPS), none of the inhibitors reduced N_2O emissions. Below, we discuss the mechanisms behind the effects observed.

4.1 Effect of urease inhibitors on denitrification

In contrast to our first hypothesis, we found during the incubation experiment that NBPT reduced the emissions from denitrification (average reduction for the 3 moisture contents of 42 %). The differences were statistically significant at the 10 % significance level ($p=0.068$), probably due to the low number of replicates used. Given that NO_3^- was added in order to eliminate the substrate limitation for denitrifying microorganisms, the reduction must be a consequence of a direct effect of the inhibitor on these microbial populations. Although there is no easy explanation for this, similar results have been previously found. For example, Abalos et al. (2012) observed a lower denitrification potential for soils treated with NBPT. This could be supported by the lower soil respiration (i.e. CO_2 fluxes) measured in our study for U+NBPT compared with the other treatments. This potential negative impact on a key soil ecological function such as denitrification should be the focus of further research on the use of this chemical product and its bioavailability. However, the effect of NBPT on denitrification was not large enough to decrease the total N_2O emissions when moisture conditions favored this microbiological process (80 % WFPS). This might partially explain why this inhibitor did not reduce N_2O emissions in several studies (Akiyama et al. 2010). The UI PPDA did not reduce the N_2O emissions from denitrification. We speculate that the differences between the UIs could be a consequence of the sulfur added with NBPT but not with PPDA, as this element is known to affect denitrification (Hasegawa-Kurisu et al. 2010). Further research is also needed to confirm this hypothesis.

4.2 Effect of urease inhibitors on nitrification

At 60 % WFPS, NBPT consistently reduced the N_2O emissions produced from nitrification ($p<0.05$). Whether the N_2O reduction was directly or indirectly induced was tested in depth during the mesocosm experiment, where urease activity was significantly reduced by NBPT during 13 DAF. PPDA also reduced the urease activity but in this case the effect lasted only 3 days. For NBPT, this inhibition period was much longer than observed in a previous experiment carried out in a similar soil, in which NBPT was found to be effective at inhibiting urease activity during the first 9 days following urea application under field conditions (Sanz-Cobena et al. 2008). The longer inhibition of urease activity by NBPT in this study was probably caused by a more homogeneous distribution of the inhibitor within the soil volume due to the application method (i.e. dissolved in water). This would have allowed the inhibitor to reach most of soil microsites and therefore allowed it to act on more urease enzymes. In contrast, when the inhibitor is applied coating the fertilizer granules, as in the Sanz-Cobena et al. (2008) study, the inhibition might have affected a smaller volume of soil.

Inhibition of urease activity delayed urea hydrolysis, as shown by the lower concentration of NH_4^+-N measured in the upper soil layer for the NBPT and PPDA treated soils. Application of NBPT produced the lowest NH_4^+-N concentrations throughout the experiment. Four days

after fertilizing the soil NH_4^+-N content increased for PPDA+U as a consequence of the lower effectiveness of this UI to inhibit the urease activity compared with that of NBPT. The reduction of urea hydrolysis caused by both inhibitors was in accordance with previous research (Zhengping et al. 1991; Zaman et al. 2009). The quick decrease of the PPDA inhibitory effect over urease activity has been observed previously (e.g. Pedrazzini et al. 1987) and is associated with a degradation of the inhibitor as a result of increasing pH. The soil pH in this study was relatively high and the hydrolysis of urea could have increased it even more in the soil micropores without inhibitor.

Nitrification rates, which are potentially high in soils under Mediterranean climates, such as the soil used in our study (Aguilera et al. 2013), would have been enhanced due to the presence of readily available NH_4^+-N in the soil solution after U application. NBPT slowed urea hydrolysis, reduced NH_4^+-N concentration and thereby limited the nitrification rate, NO_3^- supply and ultimately N_2O production. Since the net nitrification rate was lowered by NBPT and PPDA compared with U, and k_f was not significantly affected by the inhibitors, the effect of these compounds over nitrification would have been produced due to their effect on the concentration of NH_4^+-N potentially nitrified (indirect effect) and not by affecting nitrifying bacterial populations (direct effect). Although there is no scientific evidence to link k_f and the community of nitrifiers from the results of this study, the aim of this statement was to clearly differ between N availability and other factors (summarized in k_f) probably affecting the microbial community and/or its nitrifying activity. Further research on the effects of these chemicals over microbial communities is needed. In any case, the effect of this type of inhibitors on N_2O fluxes is different from that observed for nitrification inhibitors (Vallejo et al. 2006), where N_2O emissions were mitigated due to the direct inhibition of the nitrifying activity, maintaining a high concentration of NH_4^+ in the soil.

At 80 % WFPS the inhibitors did not reduce N_2O emissions from nitrification. This is probably because NBPT is not an effective urease inhibitor per se; it needs to be converted to its oxygen-analog form (NBPTO) first (Sanz-Cobena et al. 2011). The high moisture conditions of these treatments could have reduced the NBPT to NBPTO conversion rate thereby reducing the potential of NBPT to affect N_2O emissions. This could be another explanation for why NBPT did not reduce N_2O emissions in some field and lab experiments. The lower effectiveness of NBPT in flooded rice has been attributed in previous studies to that effect (Chien et al. 2009).

4.3 Effect of inhibitors on NH_3 volatilization

Cumulative NH_3 emission from fertilization with U was significantly reduced by NBPT at 60 % WFPS. The urease inhibitor PPDA was also effective mitigating these losses. Urease activity inhibitors retarded urea hydrolysis, which may have affected NH_3 losses through volatilization by two different mechanisms. Firstly, urea molecules could have more time to reach a deeper position in the soil profile due to their infiltration with the water flow, as would be the case during rainfall/irrigation events under field conditions. The subsequent formation and release of NH_4^+-N into the soil solution would therefore have occurred at a depth below the soil surface where diffusion of NH_3 to the atmosphere would have been more difficult (Grant et al. 1996). Secondly, since urea hydrolysis was retarded by UIs, soil pH changes would have been moderated by the inhibitors, favoring the prevalence of NH_4^+ over NH_3 (Zaman et al. 2009). The smaller reductions of NH_3 volatilization (i.e. from day 4 to 11 after fertilizing) as well as N_2O emissions induced by PPDA compared with NBPT are probably the result of the lower urease activity inhibition period of this inhibitor (3 *vs* 20 days). Given that the NH_3 measurement technique used in this study was not micrometeorological (i.e. sensitive

to changes in (e.g.) wind variables), the results shown here should be understood as a qualitative comparison between treatments.

4.4 Implications of our study

Caution must be exercised extrapolating results from controlled mesocosm studies to field-scale processes. For instance, our observed effects may depend on soil type (e.g. structure, nutrient availability) and moisture contents within the ranges evaluated. Mesocosms may also constrain to some extent the N dynamics due to wall effects. However, our study clearly shows that adequate management of the urease inhibitor NBPT may provide an opportunity for climate change mitigation. Under wet conditions (near-saturation) produced by traditional irrigated systems (i.e. surface or sprinkler), denitrification prevails, and the inhibitor should not be recommended. This is because, as shown in this study, N_2O emissions would not be suppressed and high water addition may have the same mitigation effect on NH_3 volatilization as NBPT (Sanz-Cobena et al. 2011). Conversely, its use could be promoted as "best management practice" when irrigation is applied efficiently according to crop requirements. This would have several benefits: lower N_2O emissions and NH_3 volatilization, and a potentially higher N use efficiency and crop yield (Zaman et al. 2009). Nevertheless, the cost efficiency of this technology is still under debate. To our knowledge there are no economic assessments of using UIs as a strategy to mitigate N losses. Apparently when NBPT-treated U is used as a fertilizer under conditions of enhanced NH_3 volatilization (i.e. dry soil with pH$\geq$7) the N application rate may be reduced, but determination of the optimal N rate to achieve a yield increase will require further experimentation (Turner et al. 2010). A lower rate of N application and increased grain yield or protein concentration in the crop may lead to cover the higher cost of NBPT-treated U (priced at 21 % more than granular urea; Turner et al. 2010).

5 Conclusions

Both NBPT and PPDA were effective in the abatement of NH_3 emission from fertilization with U at 60 % WFPS. In the incubation experiment, NBPT+U reduced N_2O emissions at 60 % WFPS but had no effect at 80 % WFPS compared with U alone. The application of PPDA significantly increased the emissions with respect to U at 80 % WFPS, whereas no significant effect was found at 60 % WFPS. In the mesocosm experiment, a significant abatement of N_2O was also observed in the NBPT+U-treated soil (i.e. 75 % reduction when compared with U). This effect was associated with a reduction in the nitrification rate driven by this UI as a consequence of a lowered soil NH_4^+-N concentration. The nitrification rate constant (k_f) was not affected by urease inhibitors, suggesting that this process was not directly affected. Although caution must be exercised when extrapolating results from controlled mesocosm studies to field-scale processes, our study clearly shows that adequate management of the urease inhibitor NBPT taking into account the soil moisture content may provide an opportunity for mitigation of N_2O emissions from urea-fertilized soils.

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