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**Science of the Total Environment**

*DOI:*
10.1016/j.scitotenv.2017.12.250

Published: 15/05/2018

Peer reviewed version

# Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil

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## Abstract

Nitrification inhibitors (NIs) such as dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) provide an opportunity to reduce losses of reactive nitrogen (Nr) from agricultural ecosystems. To understand the fate and efficacy of these two inhibitors, laboratory-scale experiments were conducted with $^{14}$C-labelled DCD and DMPP to determine the relative rates of mineralization, recovery in soil extracts and sorption in two agricultural soils with contrasting pH and organic matter content. Concurrently, the net production of soil ammonium and nitrate in soil were determined. Two months after NI addition to soil, significantly greater mineralization of $^{14}$C-DMPP (15.3%) was observed, relative to that of $^{14}$C-DCD (10.7%), and the mineralization of both NIs increased with temperature, regardless of NI and soil type. However, the mineralization of NIs did not appear to have a major influence on their inhibitory effect (as shown by the low mineralization rates and the divergent average half-lives for mineralization and nitrification, which were 454 and 37 days, respectively). The nitrification inhibition efficacy of DMPP was more dependent on soil type than that of

DCD, although the efficacy of both inhibitors was lower in the more alkaline, low-organic matter soil. Although a greater proportion of DMPP becomes unavailable, possibly due to physico-chemical sorption to soil or microbial immobilization, our results demonstrate the potential of DMPP to achieve higher inhibition rates than DCD in grassland soils. Greater consideration of the interactions between NI type, soil and temperature is required to provide robust and cost-effective advice to farmers on NI use.
**Keywords:** Fertilizer use efficiency; Nutrient cycling; Nitrogen losses; $^{14}$C-isotope; NI mineralization; NI sorption.

**Highlights:**

- DCD and DMPP mineralization was slow and did not affect their nitrification inhibition efficacy.
- Relative to DCD, less DMPP was sorbed to the solid phase and more was mineralized.
- Inhibition efficacy of DCD and particularly DMPP decreased in the calcareous soil.
- Both DCD and DMPP mineralization and inhibition efficacy were strongly influenced by temperature.

## 1. Introduction

Nitrification inhibitors (NIs) offer the potential to decrease reactive nitrogen (Nr) losses, which occur when large quantities of ammonium [$NH_4^+$] based fertilizers or urea are applied to agricultural soils. The $NH_4^+$ present in the soil can be oxidized to nitrate ($NO_3^-$) through nitrification, a biotic process which occurs under aerobic conditions (Medinets et al., 2015). This process is one of the major contributors to nitrous oxide ($N_2O$) and nitric oxide emissions (NO; Ussiri and Lal, 2013), which have

severe environmental consequences (Pilegaard, 2013; IPCC, 2014), and are released due to the oxidation of intermediates of the nitrification process, i.e. hydroxylamine ($NH_2OH$) and nitrite ($NO_2^-$; Ruser and Schulz, 2015). Therefore, the inhibition of the first step of nitrification (oxidation of $NH_4^+$ to $NH_2OH$) causes a direct reduction of N oxides ($N_2O$ + $NO_x$) emissions (Akiyama et al., 2010). In addition, the limited availability of the final product of nitrification, prevents losses of $NO_3^-$ via leaching to groundwater (Quemada et al., 2013) and N oxides emissions from denitrification (the heterotrophic reduction of $NO_3^-$ to $N_2$), which has been described as the main $N_2O$ loss pathway (Skiba and Smith, 2000) and also as a source of NO (Loick et al., 2016). The use of NIs in intensive agriculture, therefore, represents a potential management option to reduce the environmental and health costs associated with these water and atmospheric pollutants (Qiao et al., 2015), providing opportunity for increased benefits through enhancement of N use efficiency and crop yields (Abalos et al., 2014; Yang et al., 2016).

Many synthetic (Akiyama et al., 2010) and naturally occurring organic compounds (Subbarao et al., 2015) can act as NIs, with dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) representing two of the most commonly researched and used NIs in Europe (Gilsanz et al., 2016). Both NIs deactivate the enzyme responsible for the first step of nitrification, i.e. the oxidation of $NH_4^+$ to $NH_2OH$. The main proposed mechanisms of inhibition are: i) direct binding and interaction with ammonium monooxygenase (i.e. indiscriminate binding in the case of DMPP and blocking the electron transport in the cytochromes in the case of DCD, Benckiser et al., 2013); and ii) the removal of copper (Cu) as the co-factor of ammonium monooxygenase, thus behaving as metal chelators (Ruser and Schulz, 2015). With regards to their chemical behaviour, DCD is less volatile than DMPP

(Giltrap et al., 2010), while the water solubility of DMPP (125 g $l^{-1}$) is higher than that of DCD (73.2 g $l^{-1}$; Marsden et al., 2016). These authors also observed that the distribution of both NIs within soil columns after a rainfall simulation was similar, except for the top 1 cm (where higher retention of DCD was obtained). DCD has been the main inhibitor employed in several countries such as New Zealand, mainly due to its low cost, although this inhibitor has been voluntarily withdrawn from New Zealand due to the traces of DCD that were found in infant milk exported to China (Pal et al., 2016). On the other hand, DMPP can be added at rates about 10 times less than DCD (Zerulla et al., 2001; Benckiser et al., 2013), with similar, or even higher, reported efficacies (Weiske et al., 2001).

With regards to the inhibition efficiency of DCD and DMPP, published meta-analyses have reported statistically similar average performances of both NIs in mitigating $N_2O$ emissions (Gilsanz et al., 2016) and enhancing N use efficiency or crop yields (Abalos et al., 2014). Conversely, several studies have reported that the efficiency of DMPP surpassed that of DCD in decreasing soil $NO_3^-$ concentrations and/or $N_2O$ emissions, or increasing crop productivities (Pereira et al., 2010; Liu et al., 2013; Kou et al., 2015). In addition to inhibition efficacy, the behaviour of these products in soil is linked to other soil processes (e.g. mineralization, microbial uptake, water extractability) and hence, to soil properties (Zhang et al., 2004; Barth et al., 2008; McGeough et al., 2016). To date, few studies have evaluated these processes (Marsden et al., 2016), and especially in calcareous alkaline soils. Therefore, it is important to understand the key variables affecting the efficacy and fate of NIs, to underpin advice about which product is the most effective under contrasting environmental and edaphic conditions.

Several physical and/or biochemical processes may influence the efficacy of NIs. For example, the water solubility and leaching potential has been shown to be higher for DCD than DMPP (Weiske et al., 2001; Kim et al., 2012), thus resulting in spatial dislocation of soil $NH_4^+$ and the inhibitor, possibly affecting the duration of the inhibiting effect. However, a recent study by Marsden et al. (2016) employing $^{14}C$-labelled NIs, showed that the mobility of both inhibitors were similar. Another possible factor affecting efficacy is the microbial mineralization of NIs. Some studies have found that DCD degrades faster than DMPP in soil, using direct NI measurements by chromatography-based methods (Weiske et al., 2001) or $^{14}C$ labelling of NIs and subsequent measurement of rates of $^{14}CO_2$ emission after application to soil (Marsden et al., 2016). The sorption of NIs to the soil matrix (i.e. clays and organic matter) and immobilization by non-target microorganisms have also been linked to a decrease in the efficacy of NIs. Previous studies demonstrated that sorption (which could reduce the concentration of the NI in soil solution, and effectiveness on nitrifying microorganisms in the short term) is higher for DCD than DMPP (Marsden et al., 2016). A higher microbial assimilation of DCD is also expected, due to the lower degradability and bioavailability of the heterocyclic DMPP compound (Chaves et al., 2006).

In addition, soil temperature has been shown to be a key factor affecting the inhibition effect (Mahmood et al., 2011; Menéndez et al., 2012; McGeough et al., 2016), due to the influence on microbial activity, and hence mineralization and nitrification kinetics. The complex interactions between inhibitor type, soil properties and temperature, however, remains poorly understood. Only a few experiments have reported the simultaneous mineralization of NIs and the effects of the NIs on N cycling, with these often being limited to either measuring the effects of NIs on N cycling without considering the disappearance of the NI itself, or where both have been

123 measured, this has often been for only short periods of time, e.g. hours to days (e.g.
124 Marsden et al., 2015; Marsden et al., 2016).

125 In this context, a laboratory experiment was conducted to compare the amount of
126 $^{14}C$-labelled DCD and DMPP which is sorbed, mineralized and recovered in the soil
127 extractable pool, in two contrasting soils at three different temperatures. The influence
128 of these three factors on the evolution of soil $NH_4^+$ and $NO_3^-$ contents were also
129 investigated under laboratory conditions, in the absence of plants. We hypothesized
130 that, (1) greater mineralization would occur for DCD (in comparison to DMPP) and at
131 higher temperatures, thus decreasing the inhibition efficacy, and (2) higher sorption and
132 microbial assimilation would be observed for DCD and in the soil with a higher organic
133 matter content and microbial biomass (Marsden et al., 2016; McGeough et al., 2016),
134 affecting the proportion of NIs mineralized and extracted.

135 **2. Materials and Methods**

136 *2.1 Soil properties*

137 Two contrasting soils (from Spain and from UK) were used in this study (Table
138 1). We aimed to compare two soils from different climatic areas and agricultural land
139 use, which mainly differed in pH and organic C content, factors that are known to
140 greatly influence microbial activity. The soil from Spain ('ES soil') was an arable soil
141 collected from the "El Encín" field station (40°32′N, 3°17′W) and was a calcareous
142 sandy clay loam Calcic Haploxerept (Soil Survey Staff, 1992) with vermiculite as a
143 dominant clay mineral. The soil from the UK ('UK soil') was collected from a
144 permanent grassland at the Henfaes Agricultural Research Station, Abergwyngregyn,
145 North Wales (53°14′N, 4°01′W), and was a sandy clay loam textured Typic Eutrudepts
146 (Soil Survey Staff, 1992). At each site, independent replicate soil samples ($n$ = 3; 0–10
147 cm) were collected, sieved to pass 2 mm, and stored at 4 °C in gas-permeable polythene

bags until the start of the experiment. Soil moisture content was determined by oven drying (105 °C, 24 h) and soil organic matter content by loss-on-ignition (450 °C, 16 h; Ball, 1964). Soil pH was measured using standard electrodes in 1:2.5 (w/v) soil-to-distilled water suspensions. Microbial biomass C and N were determined by $CHCl_3$ fumigation-extraction according to Voroney et al. (2008) using $K_{EC}$ and $K_{EN}$ correction factors of 0.35 and 0.50, respectively. Initial $NO_3^-$ and $NH_4^+$ contents in 1:5 (w/v) soil-to-0.5 M $K_2SO_4$ extracts were determined using the colorimetric method of Miranda et al. (2001) and Mulvaney (1996), respectively. Total C and N in soils were determined by elemental analysis with a LECO TruMac CN analyzer®. Total Cu content, which could affect the efficacy of NIs (Ruser and Schulz, 2015; McGeough et al., 2016) was determined by atomic absorption spectrophotometry (AAnalyst 700, PerkinElmer 2000), after treating air-dried soil samples with $HNO_3$ and HF, followed by digestion in Teflon bombs in a microwave oven.

*2.2 DMPP and DCD mineralization within soils*

To determine the mineralization rates of 5-$^{14}$C-DMPP and [U]$^{14}$C-DCD (American Radiolabelled Chemicals, St Louis, MO, USA) in the two soils (ES and UK) at contrasting soil temperatures (10, 20 and 30ºC), a replicated ($n$ = 3) factorial $^{14}$C-labelling experiment was employed. Briefly, 5 g of field-moist soil was weighed into 50 $cm^3$ polypropylene tubes. $NH_4Cl$ was applied to each treatment at a rate of 100 kg N $ha^{-1}$ (i.e. 0.5 ml, 3 g $NH_4Cl$ $l^{-1}$), together with $^{14}$C-DMPP (at a commercial rate of 1 kg $ha^{-1}$, i.e. 0.5 ml 0.03 g $l^{-1}$, *ca.* 2 kBq $ml^{-1}$) or $^{14}$C-DCD (at a commercial rate of 10 kg $ha^{-1}$, i.e. 0.5 ml 0.3 g $l^{-1}$; *ca.* 2 kBq $ml^{-1}$). Subsequently, deionized water was added to each tube to achieve 50% water-filled pore space (WFPS) in each soil, maintaining a suitable soil aeration status for nitrification to proceed. WFPS was calculated by dividing the volumetric water content by total soil porosity. Evolved $^{14}CO_2$ was captured in 1 M

NaOH traps (1 ml; capture efficiency >95 %; Hill et al. 2007), which were changed after 1, 3, 6, 10, 14, 18, 21, 28, 35 and 63 days. The $^{14}C$ activity in the recovered NaOH solution was determined using a Wallac 1404 Liquid Scintillation Counter (Wallac EG&G, Milton Keynes, UK) after mixing with HiSafe 3 scintillant (PerkinElmer, Llantrisant, UK). After the last $^{14}CO_2$ measurement, the remaining activity in the soils was quantified by extracting with ice-cold 0.5 M $K_2SO_4$ (1:5 w/v). Samples were shaken (150 rev $min^{-1}$) for 30 min and subsequently centrifuged (10 000 *g*, 10 min). The activity in the supernatant was measured as described above.

### *2.3 Recovery of $^{14}C$ in soil extract*

The amount of NI which remained extractable in soil was analysed alongside the mineralization assay described above. In this case, 2 g of field-moist soil ($n$ = 3) was added to 20 ml polypropylene vials. Ammonium chloride and NIs were added at the same rates as in the mineralization experiment, and deionized water was added to reach 50% WFPS. Labelled $^{14}C$-NIs were added at 5 kBq $ml^{-1}$ (0.2 ml). The amount of substrate ($^{14}C$-DMPP or $^{14}C$-DCD) remaining in the soil (combination of the soil solution pool and the exchangeable pool) was measured after 1, 3, 6, 10, 14, 18, 21 and 28 days, by extracting the soil with ice-cold 0.5 M $K_2SO_4$, and analysing the activity in the resulting extracts by liquid scintillation counting, as described in section 2.2. The mineralization and recovery in soil extract sub experiments involved the same treatments, i.e. two inhibitors (DCD and DMPP), two soils (ES and UK) and three temperatures (10, 20 and 30 °C), and both were used to calculate the $^{14}C$ mass balance (detailed in section 2.6).

### *2.4 NI sorption*

196 The amount of DCD and DMPP sorbed to either soil was determined as
197 described by Marsden et al. (2015). Briefly, $^{14}$C-DCD or $^{14}$C-DMPP was applied (50 µl;
198 *ca*. 1 kBq) to 1 g ($n$ = 3) of air-dried soil, where a total of 8 concentrations of $^{14}$C-DCD
199 and $^{14}$C-DMPP were used, ranging from 0.08-10 mg NI $l^{-1}$. Subsequently, 5 ml of 0.01
200 M $CaCl_2$ was added to the soils and the soil suspensions were shaken (0.5 h; 150 rev
201 $min^{-1}$) on a rotary shaker. An aliquot (1.5 ml) was then centrifuged (10 000 *g*; 5 min)
202 and the $^{14}$C activity in the supernatant determined by liquid scintillation counting as
203 described above. Sorption isotherms were determined for $^{14}$C-DCD and $^{14}$C-DMPP in
204 the two contrasting soils and the partition coefficient ($K_d$) for the NIs determined via
205 Equation 1, where $C_{ads}$ (µg $g^{-1}$) is the concentration adsorbed to the soil solid phase at
206 equilibrium and $C_{sol}$ (µg $l^{-1}$) is the adsorbate concentration remaining in solution at
207 equilibrium.

208 $$K_d = C_{ads} / C_{sol} \qquad (1)$$

209 This sorption experiment was measured as a one-off complementary
210 measurement to previous assays (i.e. mineralization and recovery by $K_2SO_4$ extract) for
211 each inhibitor and soil.

212 *2.5 Soil mineral N content*

213 Alongside the $^{14}$C experiments described above, a further set of samples were
214 established to monitor the effects of the NIs on the dynamics of soil $NH_4^+$ and $NO_3^-$
215 content over time. 5 g fresh weight of each soil was weighed into 50 $cm^3$ polypropylene
216 tubes, as for the mineralization experiment. The $NH_4Cl$ and the NIs (non $^{14}$C-labelled
217 DCD and DMPP) were applied at the same rates as described in section 2.2 and 2.3.
218 Two additional treatments were included: $NH_4Cl$ at 100 kg N $ha^{-1}$ without NIs
219 (no NI) and a control without $NH_4Cl$ or NIs addition (C). Deionized water was added to
220 bring the soil in all treatments up to 50% WFPS, and then the tubes were incubated at

10, 20 and 30 ºC. All the fertilizer-soil-temperature combinations were replicated three times. After 0, 1, 3, 6, 10, 14, 18, 21 and 28 days replicate samples from each treatment ($n$ = 4) were destructively harvested and their mineral N content determined. A 28 day period was chosen based on known period of active inhibition for DMPP and DCD (Benckiser et al., 2013; Chaves et al., 2006). At each sampling date, $NH_4^+$ and $NO_3^-$ were extracted with 25 ml of 0.5 M $K_2SO_4$, and measured using the same procedure described in section 2.1.

*2.6 Calculations and statistical analysis*

Since it is not possible to determine the $^{14}C$ recovery in the microbial biomass with any reliability (Glanville et al., 2016), we used a mass balance approach to calculate the amount of $^{14}C$ present in the microbial biomass. This microbial pool was calculated as the difference between the starting $^{14}C$ pool (amount of $^{14}C$ added to the soil at $t$ = 0) and the amount recovered as either $^{14}CO_2$ plus that recovered in the $^{14}C$-$K_2SO_4$ extractable pool (Glanville et al., 2016; Marsden et al., 2016). The mineralization rates of the inhibitors and the changes in $NH_4^+$ content with incubation time were modelled with a first- or a zero-order reaction kinetic model, as described in Zhao et al. (2007). Afterwards, the half-life was calculated as $C_0/2k$ or $ln(2)/k$ for zero and first-order reactions, respectively, where $C_0$ was the initial concentration of substrate and $k$ was the kinetic constant. To determine the significance of the effects of NI type, soil type and temperature on mineralization, $^{14}C$ recovery in soil extract, and inhibition efficacy, a three-way ANOVA was conducted. The normality (Shapiro-Wilk test) and homogeneity of variance assumptions (Levene's test) were assessed prior to conducting the ANOVA. Data were arcsin or log-transformed before analysis when ANOVA assumptions were not met with the original data. Means were separated by Tukey's honest significance test at $P < 0.05$. For non-normally distributed data, the

Kruskal–Wallis test was used on non-transformed data to evaluate differences at $P < 0.05$. Linear correlations were carried out to determine relationships between mineralization and recovery in the soil extract with the average $NH_4^+$ and $NO_3^-$ contents, considering also the effectiveness on nitrification inhibition (comparison between $NH_4^+$ and $NO_3^-$ contents in NIs and –NI treatments; $n = 36$). All statistical analyses were carried out with Statgraphics Plus 5.1 (Statpoint Technologies, Inc., The Plains, VA).

## 3. Results

### *3.1 Mineralization and availability of nitrification inhibitors in soil*

Overall, the NI mineralization patterns were linear and stabilized 63 days after NIs and $NH_4Cl$ addition in most treatments (Fig. 1). On average, 6.3 and 11.8% of $^{14}C$-DCD and $^{14}C$–DMPP, respectively, was mineralized 28 days after N addition (Table 2). One month later, the cumulative $^{14}CO_2$ released was 10.7 and 15.3% for DCD and DMPP, respectively. On average for both NIs, the mineralization was highest at 30 ºC, and was reduced by 39% (ranging from 20% for DMPP to 57% for DCD) and 49% (ranging from 26.9% for DMPP to 70.6% for DCD) at 20 °C and 10 ºC, respectively ($P < 0.05$). Conversely, the mineralization differences between temperatures were lower for DMPP and only significant when comparing 30 ºC with 20 ºC and 10 ºC. The mineralization of DMPP was not affected by soil type, while more DCD was mineralized in the calcareous soil than in the non-calcareous soil ($P < 0.05$). The soil×inhibitor interaction effect on NI mineralization for DCD was particularly marked at 30 °C. DMPP mineralization trends were similar in both soils (Fig. 1c, d) at 30 °C. Conversely, a greater mineralization of DCD was observed in the ES than in the UK soil at this temperature (Fig. 1a, b). The mineralization of NIs in all treatments followed a first-order kinetic relationship (Table 3). Results confirmed that the NI half-life decreased with increasing temperature, and was greater for DCD (635 days) than DMPP

(273 days) across both soil types. Average half-lives for UK and ES soils were 570 and 338 days, respectively. The sensitivity of mineralization to temperature was evaluated with the $Q_{10}$ parameter (Table S1; Hill et al., 2015). DMPP mineralization showed a significantly lower sensitivity to temperature than DCD mineralization. Regarding the soil effect, the response of the mineralization to temperature was higher in the ES than in the UK soil for DCD (with similar behaviour in both soils for DMPP).

The amount of $K_2SO_4$-extractable DCD and DMPP from soil decreased over time (Fig. 2). The $^{14}C$ recovered in the $K_2SO_4$ extracts after 63 days ranged from 11 to 66% of the initial amount of NI applied (Table 2). Recovery of $^{14}C$ in the extractable pool at both 28 and 63 days was substantially higher for $^{14}C$-DCD than for $^{14}C$-DMPP ($P < 0.001$). This pool was also higher in the ES soil than in the UK soil. In contrast, the $^{14}C$ recovery in the soil extracts was not significantly ($P > 0.05$) affected by temperature at 63 days. A negative correlation between NI mineralization and the amount still present in the soil extractable $K_2SO_4$ pool was found ($P < 0.001$, $n = 36$, $r = -0.63$).

The quantity of $^{14}C$ label which remained unrecoverable (neither mineralized nor extracted by $K_2SO_4$) was significantly higher for DMPP than for DCD (Table 2), and in the UK soil (with lower mineralization and $^{14}C$ recovery in soil extract) than in the ES soil. As for the $K_2SO_4$ extractable pool, temperature had little influence on the size of the non-recoverable $^{14}C$ pool (e.g. the mean values ranged from 47% to 51% for the different temperatures), and it showed less dependence on time than the other pools (data not shown).

### *3.2 Effect of nitrification inhibitors on the net production of mineral N*

Ammonium concentrations for each temperature are shown in Fig. 3a, c, e and 4a, c, e. Both inhibitors resulted in significantly greater $NH_4^+$ concentrations, with respect to the no NI treatment, particularly from days 6 to 21. These increased $NH_4^+$

296 concentrations were particularly clear (and even more long-lasting) at 30 ºC, compared
297 with lower temperatures. In the case of DMPP, $NH_4^+$ concentrations were higher than
298 that in the no NI treatment from the first day after fertilization, while no differences
299 between DCD and no NI were observed during the first 6 days after NIs-$NH_4Cl$
300 addition. After 28 days, all fertilized treatments reached the base $NH_4^+$ levels of the
301 unfertilized control treatment.

302 Nitrate concentrations increased from 3 days after NIs-$NH_4Cl$ addition, reaching
303 maximum values at 17-21 days, remaining nearly constant until the end of the
304 experiment (Fig. 3b, d, f and 4b, d, f). Both inhibitors decreased the measured and
305 average $NO_3^-$ concentrations compared to the no NI treatments, and even the non-
306 fertilized control treatment ($P < 0.05$). A significant correlation between $NO_3^-$
307 concentrations and NI mineralization was also observed ($P < 0.01$, $n = 36$, $r = 0.42$).

308 The effectiveness of nitrification inhibition (Table S2) was calculated through
309 the kinetic constant and the half-life of nitrification, as explained in section 2.6. In all
310 cases, nitrification was best described by a first-order kinetic model ($P < 0.05$), except
311 for the DMPP-UK-10 treatment, which was best described by a zero-order kinetic
312 model. The lower the kinetic constant is (or the longer the half-life is), the more
313 effective the inhibitor (the inhibition of nitrification in this case). The nitrification half-
314 life, which ranged from 8 to 75 days, was lowest at 20 °C, compared to 10 °C and 30
315 °C, for both NIs. As was observed for the recovery of the NIs in the different soil pools
316 ($^{14}C$-$K_2SO_4$, $^{14}CO_2$, $^{14}C$-microbial biomass), significant interactions were observed
317 regarding the half-life of nitrification. DMPP was more effective (longer half-life of
318 $NH_4^+$) than DCD in UK soil, but the opposite (DCD was more effective) was observed
319 in the ES soil (Table S2). Both NIs were more effective in inhibiting $NO_3^-$ formation in
320 the UK soil compared to the ES soil, although differences between soils were higher for

DMPP than for DCD. In addition, Figure 5 shows the increase in the half-life of nitrification due to DCD or DMPP, for each soil and temperature, in comparison to the treatments without NIs added. This figure, which represents the inhibitor×soil×temperature interactions, shows that the lowest efficiency was in the ES soil at 30 ºC, for both inhibitors (even there was no effect of DMPP under these conditions, with respect to the addition of no fertilizer or inhibitor). Both soils showed a different trend with regards to temperature: the lowest efficiency occurred at 20 ºC in the UK soil, and at 30 ºC in the ES soil.

### *3.3 Sorption of nitrification inhibitors to the solid phase*

Sorption isotherms for DCD and DMPP in the two soils are presented in Figure 6. In the DMPP-ES isotherm one outlier (corresponding to 5 mg DMPP $l^{-1}$, 4.2 mg $l^{-1}$ in equilibrium) was removed after applying Dixon's Q test. The partition coefficients ($K_d$, from 1 to 10 mg $l^{-1}$ of initial NIs concentration), calculated as the slope of the sorption isotherms corresponding to both soils and inhibitors, were 4.11, 1.43, 0.49 and 0.90 for ES-DCD, UK-DCD, ES-DMPP and UK-DMPP, respectively. This coefficient was higher for DCD than DMPP, regardless of soil type. Conversely, the soil×inhibitor interaction meant that in the case of DCD, $K_d$ was greater in the ES than in the UK soil, while the opposite was observed for DMPP (higher $K_d$ in UK than in ES soil).

## 4. Discussion

### *4.1 Nitrification inhibitor mineralization in soil*

Studying the mineralization of NIs in different soils and at different temperatures is an issue of major interest. Under optimal conditions, the NIs should be mineralized at a rate that provides a high level of inhibition, whilst also degrading relatively quickly so as not to disturb wider soil functioning (Ruser and Schulz, 2015), minimise loss to

watercourses, or enter the food chain (Marsden et al., 2015; Pal et al., 2016). Controversy surrounds this point, however, since a residual effect could contribute to enhanced efficiency of NIs through subsequent cropping campaigns (i.e. legacy effect; Alonso-Ayuso et al., 2016).

Contrary to our initial hypothesis, more DMPP than DCD was mineralized at 28 and 63 days after N addition (Fig. 1, Table 2). In fact, the average half-life of DCD was 2.3 times higher than that of DMPP (Table 3), and was much higher than that reported by the studies of Kelliher et al. (2008; 64 days at 20 °C) or Barneze et al. (2015; 10 days at 15 °C). Our findings are not consistent with previous studies showing that DCD concentrations decline more rapidly in soil than DMPP e.g. in brown earth Fluvisols (Weiske et al., 2001) and loamy sand soils (Zerulla et al., 2001). Our results may be caused by the different experimental conditions, since in the field study of Weiske et al. (2001), the highly soluble DCD could have been leached within the soil profile (Kim et al., 2012), leading to a loss of DCD. However, similar transport of both inhibitors down the soil profile was observed by Marsden et al. (2016). These authors also measured the mineralization (0-8 h) of both $^{14}$C-labelled NIs, also finding faster mineralization of DCD than that of DMPP. They argued that the characteristics of the molecule (a heterocyclic compound) cause DMPP to be more resistant to microbial attack (Chaves et al., 2006). Although the authors of these previous studies hypothesized that the microbial community degrade DCD faster than DMPP, this was not measured for a period longer than 24 hours.

The effect of temperature on NI mineralization was largely independent of soil type and inhibitor: with increasing temperatures, a higher percentage of NIs was mineralized. The previous studies of Rajbanshi et al. (1992) or Kelliher et al. (2008) found that the mineralization of DCD increased with temperature, supporting our

findings. The $Q_{10}$ values revealed that the mineralization of DMPP was much less influenced by temperature than that of DCD. Similarly, Menéndez et al. (2012) also found that the persistence of DMPP in soil did not greatly depend on temperature. Kelliher et al. (2008) quantified the relationship between temperature and DCD mineralization, observing that at higher temperatures (e.g. 25 °C), a 1 °C increment caused a disproportional decrease in DCD half-life with respect to the same increase at lower temperatures (e.g. 5 °C). Accordingly, in our experiment, the largest differences occurred between 20 ºC and 30 ºC, rather than between 10 ºC and 20 ºC (Table S1).

*4.2 Recovery of $^{14}$C-labelled nitrification inhibitors in soil extracts*

The amount of $^{14}$C-NIs extracted by $K_2SO_4$ was barely influenced by temperature, particularly in the case of DMPP (Table 2), in agreement with Menéndez et al. (2012). The soil extractable pool was significantly larger for DCD than for DMPP. As $K_2SO_4$ removes the compound from the exchange phase, this result suggests that more DCD may have been left in the soil as less remained unrecoverable (Table 2). This result could also indicate a higher potential of DCD to move within the soil solution and therefore, to be translocated or leached down the soil profile (Kim et al., 2012). Conversely, Marsden et al. (2016) did not find significant differences between the mobility of DCD and that of DMPP, also showing that the solubility of DMPP was higher than that of DCD. With regards to the soil type effect, greater amounts of $^{14}$C were recovered in the soil extracts in the calcareous ES soil than in the non-calcareous UK soil, regardless of temperature or type of inhibitor. This suggests there is a greater potential for microbial immobilization in the non-calcareous soil (Marsden et al., 2016), which is consistent with its higher microbial biomass.

*4.3 Sorption of nitrification inhibitors*

In support of our hypothesis, the sorption isotherms and $K_d$ values revealed that more DCD was sorbed to the soil matrix than DMPP, regardless of soil type. The higher sorption of DCD was consistent with the significantly higher recovery of this inhibitor in the soil $K_2SO_4$ extractable pool. There is not a clear explanation for these results, since DMPP is positively charged, so a higher sorption of this compound would have been expected (as opposed to DCD), particularly at high soil pH (which causes amphipathic DCD to be negatively charged). Conversely, these results confirmed those of the previous laboratory experiment carried out by Marsden et al. (2016) in contrasting mineral and organic soils. As found by Zhang et al. (2004), higher sorption of DCD was found in the soil with higher pH. These authors suggested that at a more alkaline soil pH, negatively charged DCD becomes sorbed to metal oxides. However, the sorption of DMPP was higher in the UK soil, possibly indicating the key role of negatively charged domains within organic matter in adsorption processes (Marsden et al., 2016).

### *4.4 Non-recoverable nitrification inhibitor pool in soil*

On average, the amount of NIs in the non-recoverable $^{14}C$ pool was much lower for DCD (average 32%) than for DMPP (average 63%, being the main pool for this inhibitor). This pool could be associated with, i) microbial uptake, as suggested by Marsden et al. (2016); or ii) strong quasi-irreversible binding of NIs into the clays or organic matter matrix, preventing recovery with $K_2SO_4$. The hypothesis of microbial assimilation is consistent with the higher amount of NIs in the non-measured $^{14}C$ pool which was obtained in the UK soil, with higher C and N microbial biomass (Table 1). If the non-measured $^{14}C$ pool is associated with microbial immobilization, our results show that DMPP was more likely to be taken up by microbes than DCD. In contrast, Marsden et al. (2016) observed a similar microbial uptake for both DCD and DMPP. As

opposed to DCD, the greatest proportion of DMPP remained non-measured (Table 2), so further research is needed to determine the fate of DMPP which is not mineralized or extracted by $K_2SO_4$. This is particularly important considering the possible negative or positive effects of NIs on non-target microbiota (Kou et al., 2015; Florio et al., 2016; Wang et al., 2017).

*4.5 Linking NIs fate and efficacy*

Our results showed that the effectiveness of DCD and DMPP in delaying nitrification activity differed between both inhibitors, and was highly influenced by soil type and temperature. Contrary to our initial hypothesis, mineralization did not seem to have a major influence on the inhibitors efficacy, as shown by the average half-life of inhibitors (454 days; Table 3), which was much higher than that of the substrate of nitrification ($NH_4^+$; 36.7 days; Table S2). Moreover, differences in the mineralization rates at 63 days between each NI-soil-temperature combination did not surpass 15% (in absolute values).

The efficiency of DCD and DMPP was mainly driven by the interaction with soil type i.e. DMPP was more effective than DCD in the UK soil (Fig. 5 and Table S2), while DCD was more effective than DMPP in the ES soil. The differences between both soils may be explained by the effect of the contrasting physico-chemical properties in both soils. One of these properties was soil pH, which was acidic in the UK soil and alkaline in the ES. Several studies under acidic soil conditions also found that DMPP efficacy was higher than that of DCD (e.g. Weiske et al., 2001; Chaves et al., 2006; Fangueiro et al., 2009; Di and Cameron, 2011). In addition, the specific composition of organic matter and clays, which affects soil CEC, have been shown to affect DCD and DMPP efficacy (Zhang et al., 2004; Wu et al., 2007; Barth et al., 2008; McGeough et al., 2016). The lower recovery rates associated with DMPP and the UK soil may suggest

444 a greater interaction with the organic fraction (Shi et al., 2016). Otherwise, the
445 contrasting physico-chemical properties (Table 1) as well as climatic conditions (e.g.
446 rainfall amount and distribution), management factors (the UK is a grassland soil and
447 the ES is an arable soil), and plant species identity have all been shown to affect the
448 composition of nitrifying communities (Yao et al., 2011; Carey et al., 2016). For
449 instance, ammonium oxidizing archaea (AOA) dominate nitrification activity in acidic
450 soils (such as UK), while ammonium oxidizing bacteria may dominate in alkaline soils
451 (such as ES). The inhibition of the growth of AOB rather than that of AOA has been
452 proposed as the main mechanism for slowing nitrification activity from DCD and
453 DMPP (Ruser and Schulz, 2015, Shi et al., 2016). The specific microbial composition in
454 each soil could explain the complex soil×inhibitor and soil×temperature interactions
455 (Fig. 5). Regardless of the type of NI, the acid soil with the lowest CEC and clay
456 content (UK) was associated with significantly higher efficacies of DCD and DMPP
457 (Fig. 5 and Table S2). Even though there are few studies on the effectiveness of DMPP
458 compared to DCD in the UK (Misselbrook et al., 2014), our results suggest the potential
459 of DMPP to achieve higher nitrification inhibition rates than DCD in grassland soils
460 (acid pH and relatively low CEC) as the UK soil (Fig. 5 and Table S2).

461 **Conclusions**

462 Contrary to previous findings, higher mineralization of DMPP was observed for
463 both soils, in comparison to that of DCD, although the kinetics of mineralization of
464 these nitrification inhibitors was not necessarily linked with their overall effectiveness.
465 The effectiveness of both NIs was higher in the more acidic UK soil (pH 6.0). The
466 nitrification inhibition efficacy of DMPP was highly dependent on soil type (in
467 comparison to that of DCD), decreasing in the alkaline low-organic C content soil (pH
468 7.6). Comparing the behaviour of both NIs, higher amounts of $^{14}$C-DCD was sorbed to

469 the soil matrix and recovered in the soil extract, while the amount of NIs in the non-
470 measured (neither mineralized nor $K_2SO_4$ extracted) pool was much lower for DCD than
471 for DMPP. Temperature was a key factor influencing NIs efficacy (which was at a
472 minimum at 20 ºC and 30 ºC in the acidic UK and calcareous ES soils, respectively) and
473 mineralization (which increased with temperature). The cost-effective use of NIs
474 requires the evaluation of the interactions between the type of NI, soil properties and
475 regional temperature fluctuations. This laboratory experiment is a starting point to
476 analyse the drivers of the efficacy of DMPP and DCD, and contributes to the
477 understanding of the behaviour of both NIs in the soil in the short-term, as well as the
478 possible effects in the medium/long term, which should be confirmed and explored
479 under field conditions.

480 **Acknowledgements**

481 The authors are grateful to the Spanish Ministry of Economy and Innovation and
482 the Autonomous Community of Madrid for their economic support through Projects,
483 AGL2015-64582-C3-3-R (MINECO/FEDER) and the Agrisost Project (P2013/ABI-
484 2717). We also thank the technical support at the Environment Centre Wales (ECW)
485 and particularly to María Jesús García for her help with the soil analyses. This work was
486 done in the frame of the Moncloa Campus of International Excellence (UCM-UPM).

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## Figure captions

**Fig. 1** Mineralization of NIs (DCD or DMPP) expressed as a percentage of the total $^{14}$C-substrate added to two contrasting soils at the three different temperatures (10, 20 and 30 °C). The panels show DCD mineralization in **a)** UK soil and **b)** ES soil, and for DMPP in the **c)** UK soil and **d)** ES soil. Vertical bars indicate standard errors of the mean ($n$ = 3).

**Fig. 2** Recovery of $^{14}$C-labelled NIs (DCD or DMPP) with 0.5 M $K_2SO_4$ expressed as a percentage of the total $^{14}$C-substrate added to two contrasting soils at the three different temperatures (10, 20 and 30 °C). The panels show DCD mineralization in **a)** UK soil and **b)** ES soil, and for DMPP in the **c)** UK soil and **d)** ES soil. Vertical bars indicate standard errors of the mean ($n$ = 3).

**Fig. 3** Soil $NH_4^+$ (left) and $NO_3^-$ (right) contents at 10 °C (**a**, **b**), 20 °C (**c**, **d**) and 30 °C (**e**, **f**) for DCD, DMPP, $NH_4Cl$ without nitrification inhibitors (no NI) and control (C) in the ES soil (see Table 1). Vertical bars indicate standard errors of the mean ($n$ = 3).

**Fig. 4** Soil $NH_4^+$ (left) and $NO_3^-$ (right) contents at 10 °C (**a**, **b**), 20 °C (**c**, **d**) and 30 °C (**e**, **f**) for DCD, DMPP, $NH_4Cl$ without nitrification inhibitors (no NI) and control (C) in the UK soil (see Table 1). Vertical bars indicate standard errors of the mean ($n$ = 3).

**Fig. 5** Increase in half-lives (days) of nitrification for the inhibitors (DCD and DMPP) in the two soils (ES and UK, see Table 1) and for the three temperatures tested (10, 20 and 30 °C) with respect to no application of nitrification inhibitors. Vertical bars indicate standard errors of the mean ($n$ = *3*).

**Fig. 6** Sorption isotherms for $^{14}$C-DCD in the **a)** UK soil **b)** ES soil, and for $^{14}$C-DMPP in the **c)** UK soil and **d)** ES soil. Bi-directional error bars represent the standard errors of the mean for sorption and equilibrium solution concentrations ($n$ = 3).

**Table**

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**Table 1** Properties of soils (0–10 cm) used in the experiment.

| Parameter | Soil | |
|---|---|---|
| | ES (Sandy clay loam) | UK (Loam) |
| Sand (%) | 55 | 49 |
| Silt (%) | 17 | 31 |
| Clay (%) | 28 | 20 |
| Bulk density (g $cm^{-3}$) | 1.4 | 1.1 |
| Cation exchange capacity (meq 100 $g^{-1}$) | 25.7 | 14 |
| pH | 7.6 | 6.0 |
| $CaCO_3$ (g $kg^{-1}$) | 13.2 | <0.1 |
| Total organic C (%) | 0.8 | 3.1 |
| Extractable $NO_3^-$ (mg N $kg^{-1}$) | 1.33±0.1 b | 0.88±0.1 a |
| Extractable $NH_4^+$ (mg N $kg^{-1}$) | 0.02±0.0 a | 0.24±0.0 b |
| Total N (%) | 0.13±0.0 a | 0.24±0.0 b |
| Total C (%) | 1.46±0.0 a | 2.64±0.1 b |
| C:N ratio | 11.45±0.1 | 10.93±0.3 |
| Cu (mg $kg^{-1}$) | 15.3±0.1 a | 23.3±0.3 b |
| Microbial C (g $kg^{-1}$) | 0.22±0.03 a | 0.28±0.03 b |
| Microbial N (mg $kg^{-1}$) | 3.64±0.55 a | 22.08±1.08 b |

Different letters within rows indicate significant differences by applying the Tukey's honest significance test at $P < 0.05$. Values represent means ± standard error of the mean (when included).

**Table**

Click here to download Table: Table 2.docx

**Table 2** Proportion of $^{14}C$-labelled nitrification inhibitors mineralized, recoverable by 0.5 M $K_2SO_4$ or unrecoverable for ES and UK soils (see Table 1) incubated at different temperatures (10, 20 and 30 °C) for either 1 or 2 months.

| Factor | Microbial mineralization (%) | | Recovery by $K_2SO_4$ extract (%) | | Non-recoverable (%) | |
|---|---|---|---|---|---|---|
| | 28 days | 63 days | 28 days | 63 days | 28 days | 63 days |
| Inhibitor | | | | | | |
| DCD | 6.3 a | 10.7 a | 67.0 b | 58.7 b | 26.6 a | 31.9 a |
| DMPP | 11.8 b | 15.3 b | 21.6 a | 17.3 a | 66.6 b | 67.3 b |
| S.E. | 0.2 | 0.4 | 0.35 | 1.4 | 0.36 | 1.6 |
| Soil | | | | | | |
| UK | 7.7 a | 10.5 a | 39.2 a | 31.4 a | 53.1 b | 58.1 b |
| ES | 10.3 b | 15.5 b | 49.4 b | 44.7 b | 40.2 a | 41.2 a |
| S.E. | 0.2 | 0.4 | 0.35 | 1.4 | 0.43 | 1.6 |
| Temperature (°C) | | | | | | |
| 10 | 6.0 a | 9.4 a | 46.3 c | 39.4 | 47.7 b | 51.2 b |
| 20 | 8.1 b | 11.3 b | 44.6 b | 38.2 | 47.3 b | 50.5 ab |
| 30 | 12.9 c | 18.4 c | 42.1 a | 36.5 | 45.0 a | 47.2 a |
| S.E. | 0.20 | 0.4 | 0.43 | 1.7 | 0.43 | 1.4 |
| Inhibitor by Soil | | | | | | |
| DCD- UK | 3.9 a | 6.2 a | 62.0 | 51.8 | 34.0 b | 42.0 |
| DCD-ES | 8.6 b | 15.2 b | 72.0 | 65.7 | 19.2 a | 21.9 |
| DMPP-UK | 11.5 c | 14.9 c | 16.4 | 11.0 | 72.1 d | 74.1 |
| DMPP-ES | 11.9 d | 15.8 c | 26.8 | 23.7 | 61.2 c | 60.5 |
| S.E. | 0.2 | 0.5 | 0.49 | 2.0 | 0.5 | 1.6 |
| Soil by Temperature | | | | | | |
| UK-10 | 5.3 a | 7.8 a | 43.7 c | 31.9 | 51.0 d | 60.3 |
| UK-20 | 7.6 c | 10.8 b | 40.0 b | 31.8 | 52.4 e | 57.3 |
| UK- 30 | 10.3 e | 13.0 c | 33.9 a | 30.4 | 55.8 f | 56.6 |
| ES-10 | 6.8 b | 11.0 b | 48.9 d | 47.0 | 44.4 c | 42.0 |
| ES-20 | 8.7 d | 11.7 c | 49.1 d | 44.6 | 42.2 b | 43.7 |
| ES-30 | 15.5 f | 23.9 d | 50.0 d | 42.5 | 34.1 a | 37.8 |
| S.E. | 0.3 | 0.6 | 0.6 | 2.5 | 0.62 | 2.0 |
| Inhibitor by Temperature | | | | | | |
| DCD-10 | 3.4 | 5.5 a | 71.1 d | 62.8 | 25.4 a | 31.7 |
| DCD-20 | 5.0 | 8.0 b | 67.5 c | 57.7 | 27.5 b | 34.3 |
| DCD-30 | 10.4 | 18.7 e | 62.5 b | 55.7 | 26.9 b | 29.8 |
| DMPP-10 | 8.7 | 13.3 c | 21.5 a | 16.1 | 69.9 f | 70.6 |
| DMPP-20 | 11.2 | 14.6 d | 21.7 a | 18.7 | 67.0 e | 66.7 |
| DMPP-30 | 15.3 | 18.2 e | 21.7 a | 17.2 | 63.0 d | 64.6 |
| S.E. | 0.3 | 0.6 | 0.6 | 2.5 | 0.62 | 2.0 |

Different letters within columns indicate significant differences by applying the Tukey's honest significance test at $P < 0.05$. Standard Error (S.E.) is given for each effect ($n = 3$).

**Table 3** Half-life of each nitrification inhibitor (DCD and DMPP) in the two soils (ES and UK) at three different temperatures (10, 20 and 30 °C). The $R^2$ coefficient indicates the degree and significance of correlation with a first-order kinetic model (the P value was < 0.01 for all treatments). S.E. = Standard Error.

| Treatment | Half-life (days) | $R^2$ |
|---|---|---|
| UK 10 DCD | 1222 | 0.87 |
| UK 20 DCD | 786 | 0.95 |
| UK 30 DCD | 520 | 0.93 |
| ES 10 DCD | 654 | 0.96 |
| ES 20 DCD | 444 | 0.93 |
| ES 30 DCD | 186 | 0.98 |
| UK 10 DMPP | 360 | 0.90 |
| UK 20 DMPP | 273 | 0.82 |
| UK 30 DMPP | 260 | 0.68 |
| ES 10 DMPP | 245 | 0.96 |
| ES 20 DMPP | 288 | 0.79 |
| ES 30 DMPP | 211 | 0.75 |
| S.E. | 88 | |

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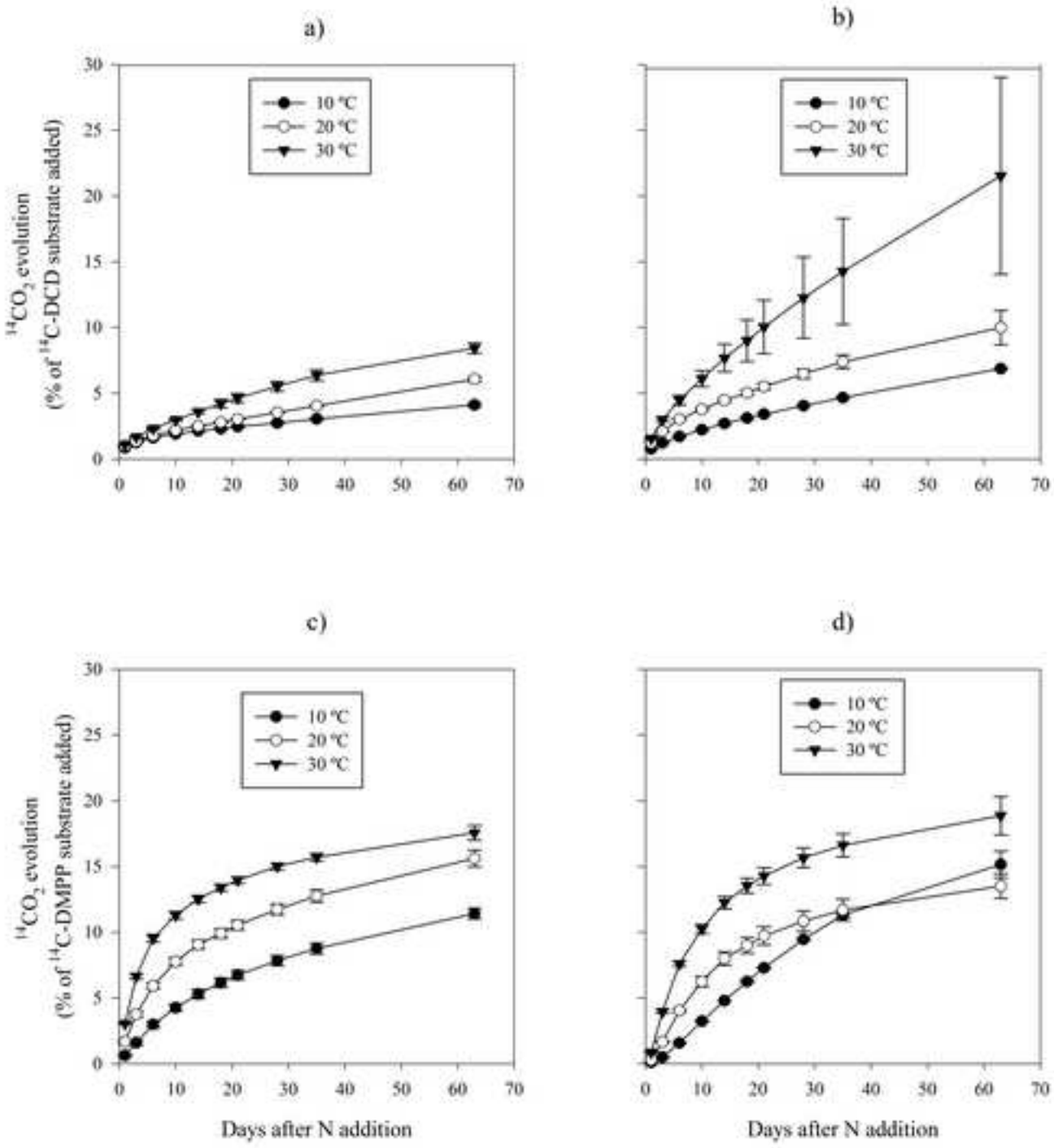

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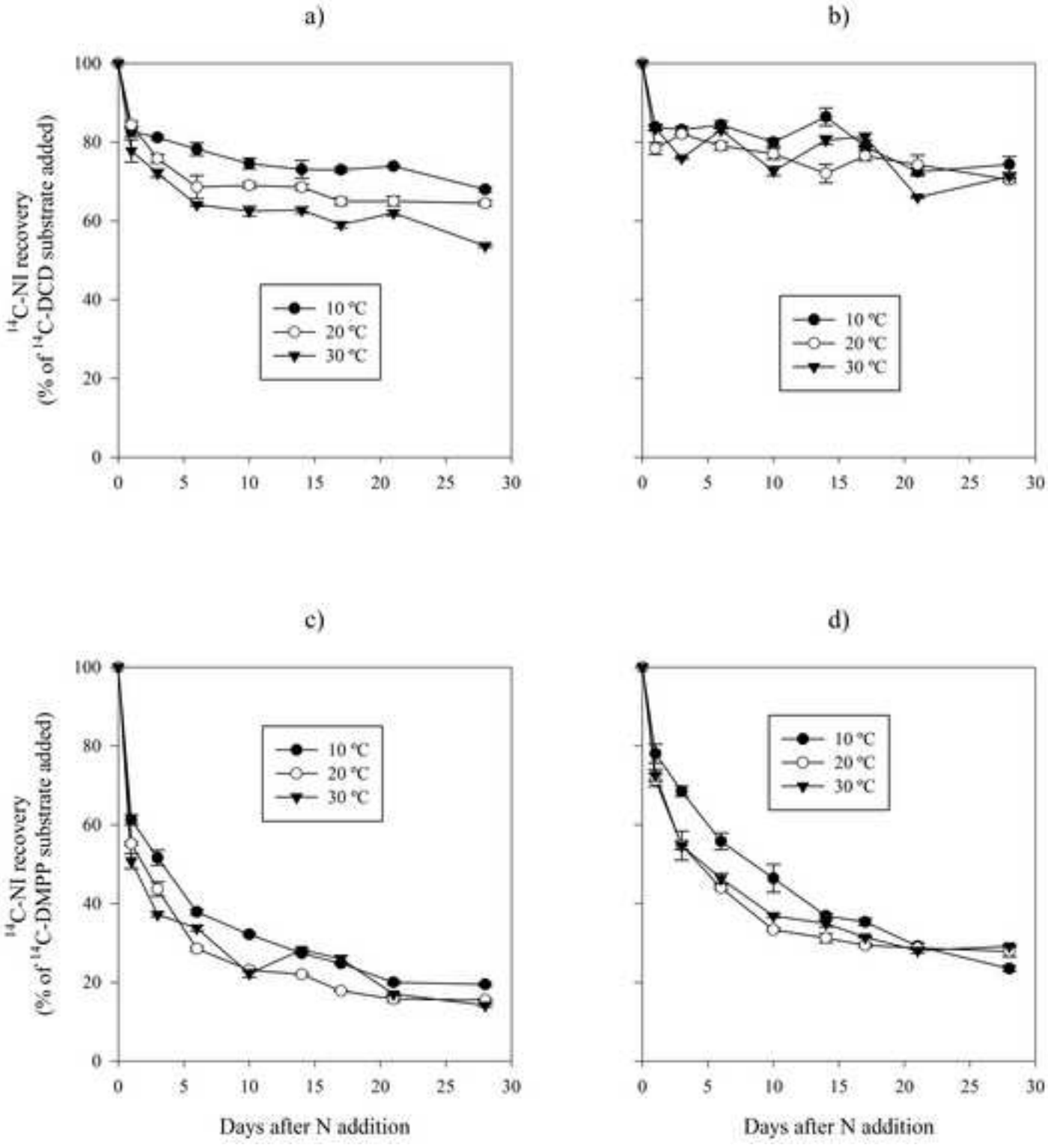

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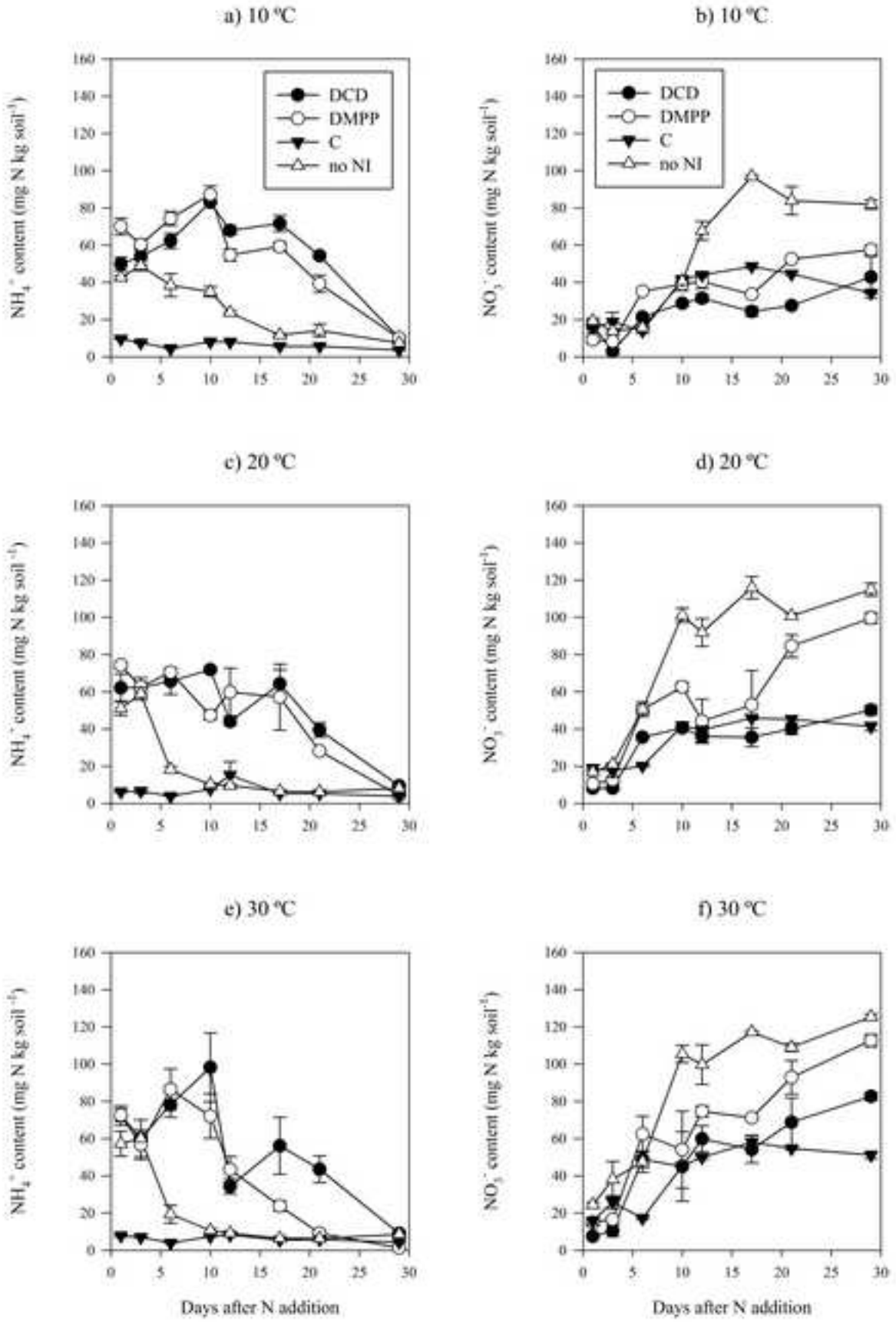

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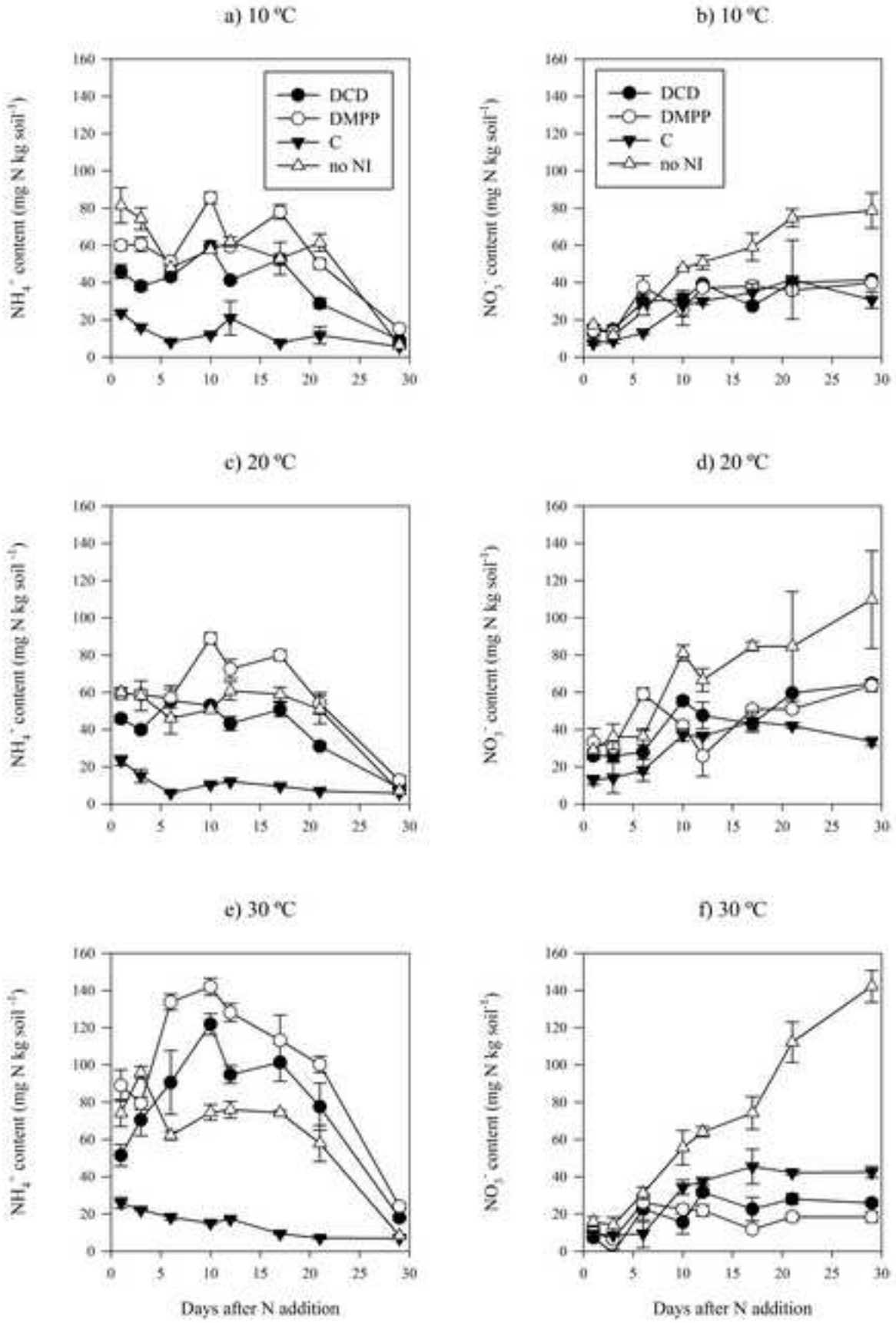

**Figure**

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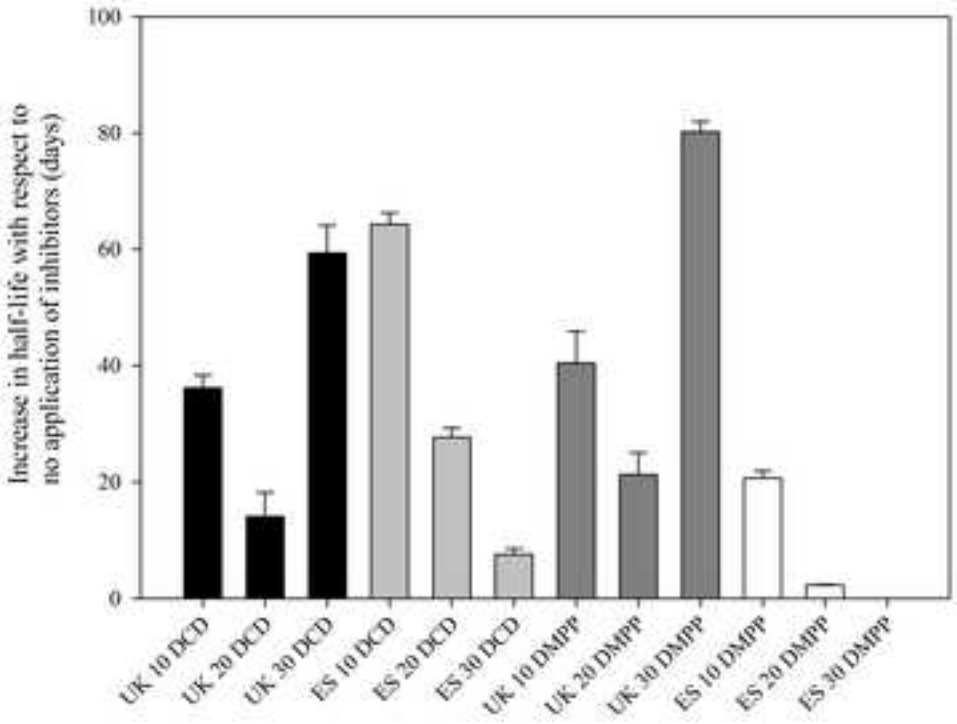

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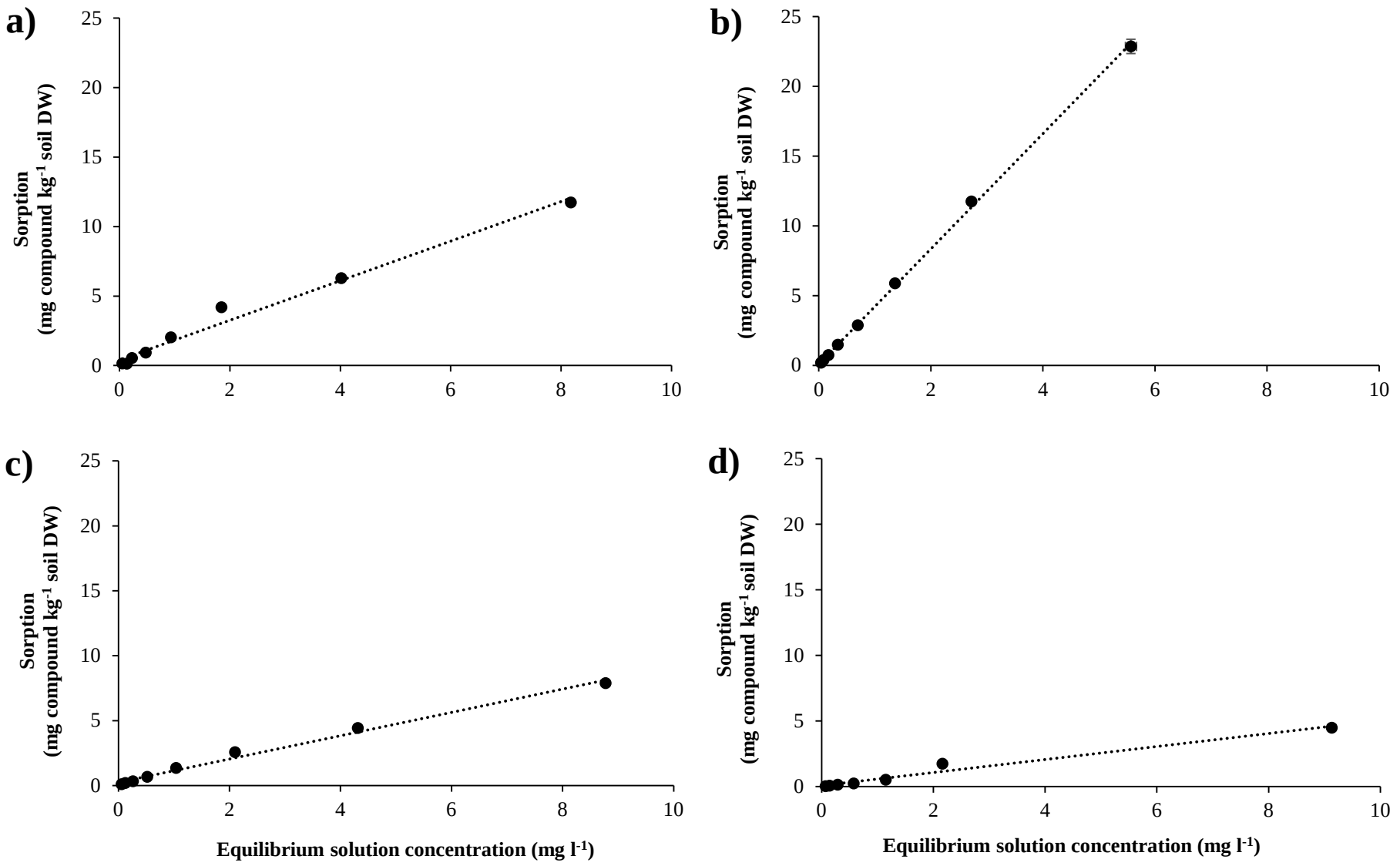

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