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## Structure–activity relationship study of substituted 1,2,3-thiadiazoles as novel nitrification inhibitors for agriculture

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Nitrification inhibitors are used in agricultural soils to maintain ammonium ( $\text{NH}_4^+$ ) available for crops for longer periods while reducing leaching of nitrate ( $\text{NO}_3^-$ ) and emission of the potent greenhouse gas nitrous oxide ( $\text{N}_2\text{O}$ ). Unfortunately, and for reasons not well understood, the current commercial inhibitors have shown inconsistencies in their performance across various agroecosystems, underscoring the need for the development of new nitrification inhibitor compounds to increase agriculture's environmental sustainability. In this work, we have performed structure–activity relationship (SAR) studies to explore the potential of 12 mono- and disubstituted 1,2,3-thiadiazoles as nitrification inhibitors through laboratory soil incubations. 1,2,3-Thiadiazoles substituted with one or two methyl groups as well as those with a fused cyclopentyl ring showed the most promising inhibitory activities, which can outperform the commercial nitrification inhibitor 3,4-dimethylpyrazole (DMP). Larger alkyl substituents as well as substituents with polar functional groups showed poorer or no inhibitory activity. These data align with our previous findings for substituted 1,2,3-triazoles that short, non-polar alkyl substituents on the heteroaromatic framework are beneficial for nitrification inhibitory properties.

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### Introduction

By 2050, the worldwide usage of nitrogen (N) fertilisers is predicted to increase by 70–100% to enable provision of food for the constantly growing population.<sup>1</sup> Unfortunately, a large fraction of N fertilisers is lost from agricultural systems through ammonia ( $\text{NH}_3$ ) volatilisation and nitrate ( $\text{NO}_3^-$ ) leaching.<sup>2</sup> In addition, soil microbial processes lead to emission of the gases nitrous oxide ( $\text{N}_2\text{O}$ ), the free radical nitric oxide ( $\text{NO}^\bullet$ ) and nitrogen ( $\text{N}_2$ ).<sup>3</sup> These losses are responsible for a low N use efficiency (NUE) in agricultural systems, which has hovered just around 50% globally for several decades.<sup>2a,4</sup> The environmental impact is substantial:  $\text{NH}_3$  is a precursor for particulate matter ( $\text{PM}_{2.5}$ ),<sup>5</sup>  $\text{NO}_3^-$  leaching causes surface water eutrophication and groundwater pollution, and  $\text{N}_2\text{O}$  has not only 300 times higher global warming potential than carbon dioxide but also contributes to stratospheric ozone destruction.<sup>6</sup> To increase agriculture's sustainability, reduction of N losses is therefore of utmost importance.<sup>2c</sup>

Ammonium ( $\text{NH}_4^+$ ) and  $\text{NO}_3^-$  are the main mineral-N nutrients for crops.<sup>7</sup> However,  $\text{NH}_4^+$  uptake by plants competes with its enzymatic oxidation (nitrification) carried out by ammonia-oxidising bacteria (AOB) and archaea (AOA). To

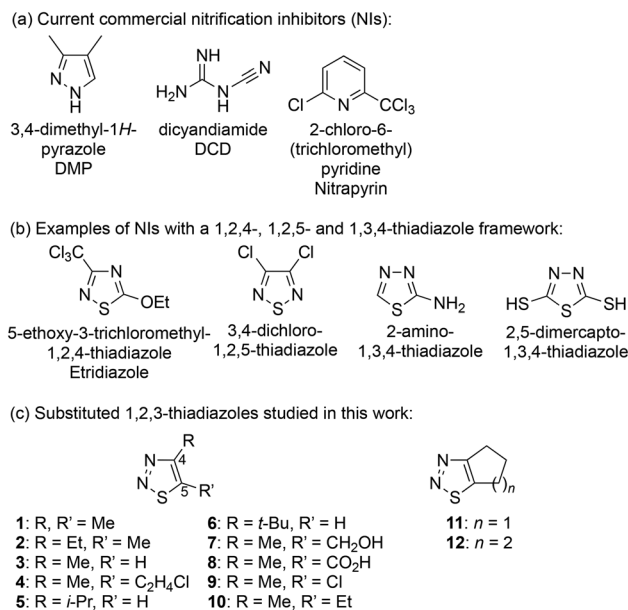
improve N-management in agricultural soils, one strategy is to amend N fertilisers with nitrification inhibitors (NIs).<sup>2c,8</sup> NIs are designed to target ammonia monooxygenase (AMO),<sup>9</sup> which catalyses the rate-limiting first oxidation step of  $\text{NH}_3$  to hydroxylamine ( $\text{NH}_2\text{OH}$ ).<sup>10</sup> Hydroxylamine oxidoreductase (HAO) subsequently converts  $\text{NH}_2\text{OH}$  *via*  $\text{NO}^\bullet$  to nitrite ( $\text{NO}_2^-$ ), followed by rapid oxidation to  $\text{NO}_3^-$ , the end product of nitrification catalysed by nitrite oxidase.<sup>11</sup> Some strains of *Nitrospira* (complete ammonia oxidisers or comammox) can catalyse the entire oxidation from  $\text{NH}_3$  to  $\text{NO}_3^-$ , where the initial step is also mediated by AMO.<sup>12</sup> Microbial denitrification leads to the formation of  $\text{N}_2\text{O}$ , for example through the reduction of  $\text{NO}_3^-$ . Thus, by inhibiting AMO, the residence time of  $\text{NH}_4^+$  in soil could be increased, and N losses through  $\text{NO}_3^-$  leaching and  $\text{N}_2\text{O}$  emissions could be reduced.

While many compounds with nitrification-inhibitory properties are known,<sup>10</sup> only three NIs are currently available on the market: 3,4-dimethyl-1*H*-pyrazole (DMP), which is commonly used as the salt of phosphoric acid (DMPP or ENTEC®; BASF AG)<sup>13</sup> or glycolic acid (DMPG or eNpower®; Incitec Pivot Fertilisers), and as the adduct with succinate (DMPSA-K2, BASF AG);<sup>14</sup> dicyandiamide (DCD; AlzChem AG); and 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin or N-Serve®; Dow Chemical Co.) (Fig. 1a).

However, none of these NIs exhibit reliable efficacy irrespective of the agricultural setting. Thus, the effect of DMP in improving crop yields has been found to vary quite

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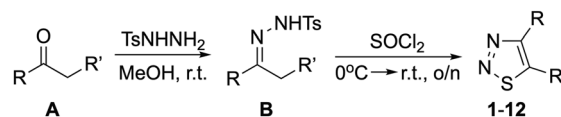




**Fig. 1** Commercial nitrification inhibitors and inhibitor compounds possessing a thiadiazole framework.

substantially in neutral soils.<sup>15</sup> In acidic soils and dry climates, the nitrification inhibitory effect was found to decline with increasing temperature from a modest 45% (10 °C) to just 23% (25 °C).<sup>16</sup> Field studies in the hot-dry climates of Australia revealed essentially no inhibitory effect of DMP.<sup>16a,17</sup> DCD is ten times less effective than DMP,<sup>18</sup> with its inhibitory activity decreasing at higher temperatures,<sup>19</sup> and can leach into groundwater. This inhibitor has been detected in dairy products in New Zealand, prompting the government to halt the sale of contaminated milk products and ultimately the voluntary suspension of DCD use in agriculture.<sup>20</sup> On the other hand, Nitrapyrin is poorly water soluble and volatile, which not only limits its applications<sup>21</sup> but also contributes to air pollution.<sup>22</sup> In addition, Nitrapyrin has shown bactericidal properties and considerable acute and chronic aquatic toxicity and is therefore banned in some countries.<sup>22</sup> In light of this, it is notable that no new inhibitor compounds with enhanced efficiency and reliability have been introduced to the market since the launch of DMP over two decades ago.<sup>13</sup>

AMO exhibits pronounced instability *ex vivo*, and its structure within the native membranes of *Nitrosomonas europaea* has been elucidated only recently *via* cryogenic electron microscopy, revealing the presence of three discrete copper centres.<sup>23</sup> However, the exact active site still remains to be identified, challenging the development of novel nitrification inhibitors. We have recently presented 1,2,3-triazoles as a promising new class of NIs,<sup>24</sup> which, depending on the substitution pattern, can inhibit AMO through a reversible (*i.e.*, likely through coordination to a Cu centre)<sup>25</sup> or an irreversible mechanism (*i.e.*, through the (additional) formation of a covalent bond).<sup>26</sup> While we are currently exploring the performance of triazoles in field studies, we aim to identify mul-



**Scheme 1** General synthesis of substituted 1,2,3-thiadiazoles through the Hurd–Mori procedure.<sup>30</sup>

multiple compound classes as potential NIs in parallel, as some may not meet the manufacturing, cost, or ecotoxicological criteria required for use in agricultural settings.

Like nitrogen, sulphur is a known chelator of Cu. Thus, we hypothesised that 1,2,3-thiadiazoles, which result from the formal replacement of one nitrogen atom in 1,2,3-triazoles by sulphur, should also inhibit AMO through coordination to a Cu centre in the active site. In fact, thiadiazoles are present in numerous biologically active compounds,<sup>27</sup> and derivatives of 1,2,4-, 1,2,5- and 1,3,4-thiadiazoles have been previously proposed as potential NIs (Fig. 1b).<sup>28</sup> For example, 5-ethoxy-3-trichloromethyl-1,2,4-thiadiazole (Etridiazole, Dwell™), a fungicide and pesticide, has shown inhibitory properties, whereas 3,4-dichloro-1,2,5-thiadiazole was found to be as effective as Etridiazole in inhibiting nitrification in soil.<sup>28a,29</sup> To our knowledge, 1,2,3-thiadiazoles with substituents at positions 4 and 5 have not been explored as NIs so far.

In this work, we investigated the nitrification inhibitory properties of a series of mono- and disubstituted 1,2,3-thiadiazoles (Fig. 1c) through structure–activity relationship (SAR) studies in two Australian soils. This heterocyclic framework is readily available in only two steps using the Hurd–Mori procedure through the reaction of a ketone **A** with tosylhydrazine, followed by cyclisation of the resulting hydrazone **B** with thionyl chloride (Scheme 1).<sup>30</sup>

By monitoring the mineral-N transformations over four weeks, the data show that short, non-polar alkyl substituents are beneficial for inhibitory efficacy that can outperform the current commercial ‘gold standard’ DMP.

## Results and discussion

Laboratory incubations in agricultural soils serve as a crucial first step in evaluating the effectiveness of new inhibitor compounds. These controlled experiments allow assessment of the compounds’ impact on mineral-N transformations under standardised conditions and help identify promising candidates with desirable inhibitory effects for further, more resource-intensive evaluation, while minimising external variability.

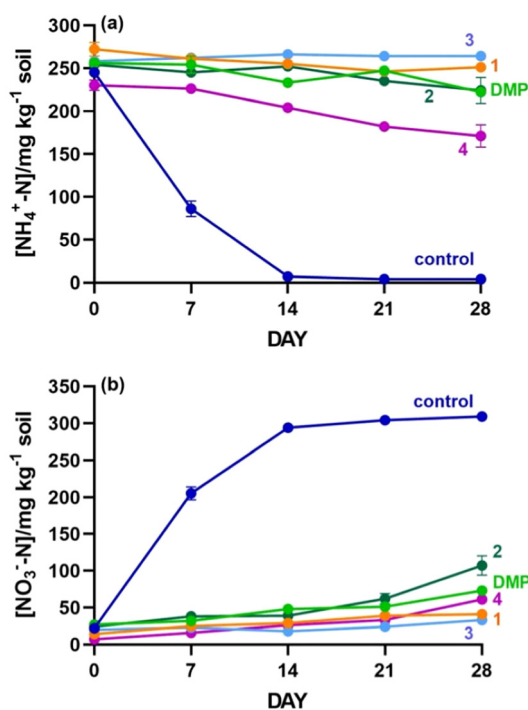
The synthesis of compounds 1–12 and their spectroscopic data are provided in the SI. The inhibitor compounds were usually applied at a rate of 5 mol% (based on their molecular mass) of the applied fertiliser-N, after converting them into their phosphate salts to reduce volatility and increase solubility, except for compounds 4 and 8 (see Table S1). These application rates, which were higher than those typically used in agricultural settings, were chosen to enable conclusive per-



formance assessment within four weeks of soil incubation. Chlorinated thiadiazoles (*i.e.*, 4 and 9) were included in this study, given the presence of chlorine in the commercial inhibitor Nitrapyrin as well as in isomeric thiadiazoles that have shown inhibitory activity (Fig. 1b).

The following figures present the results from different soil incubations as not all compounds could be tested simultaneously. The experiments were conducted using soil maintained at 60% water-filled pore space (WFPS), which is the midpoint of the recommended 50–70% range for microbial activity.<sup>31</sup> Details of the incubations are provided in the Experimental section. DMP was included as a benchmark in all incubations (used as the phosphate salt, DMPP). To monitor potential interference by other soil processes, for example, N mineralisation, *i.e.*, conversion of organic N into mineral N, the concentration–time profiles for both  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N over the duration of the incubation were measured following extraction of the soil. Inhibitory potential was evaluated based on the persistence of  $\text{NH}_4^+$  in the soil and the corresponding rate of  $\text{NO}_3^-$  formation.

Fig. 2 shows the development of the  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N concentrations during the 28-day incubation period in the



**Fig. 2** Change in  $\text{NH}_4^+$ -N (a) and  $\text{NO}_3^-$ -N (b) concentrations over an incubation period of 28 days at 25 °C and 60% WFPS in the Wimmera soil in the absence and presence of inhibitors DMP and compounds 1–4. The application rate of  $(\text{NH}_4)_2\text{SO}_4$  was 100 mg N  $\text{kg}^{-1}$  dry soil. Inhibitor treatments were 5 mol% of applied fertiliser-N, *i.e.* (in mg  $\text{kg}^{-1}$  dry soil) DMP: 35.01, 1: 40.70, 2: 45.71, 3: 35.70, and 4: 57.83. DMP and compounds 1–3 were used as phosphate salts. Each concentration profile was obtained from three replicates, and the given error is the standard error of the mean (some errors are too small to decipher). See Table S2 for detailed statistical data.

absence (control) and presence of DMP and compounds 1–4. This experiment was performed in a clay cropping soil from the Wimmera region in Victoria, Australia, which had a pH value (1 : 5  $\text{CaCl}_2$ ) of 7.9 (Table 3 in the Experimental section shows selected soil properties). The incubation temperature was maintained at 25 °C.

The control experiment in the absence of an inhibitor revealed complete consumption of  $\text{NH}_4^+$  after two weeks of incubation (Fig. 2a), by which time the  $\text{NO}_3^-$  concentration had also reached its maximum value (Fig. 2b). Inhibitors 1–4 and DMP considerably slowed down  $\text{NH}_4^+$  conversion. In fact, the mono- and bis-methylated compounds 3 and 1, respectively, seemed to perform better than DMP at the same application rate by essentially preventing  $\text{NH}_4^+$  oxidation over the duration of the incubation ( $\text{NH}_4^+$  loss on day 28 compared to the fertiliser control:  $P < 0.0001$  for DMP and 1–3;  $P < 0.05$  for 4). These inhibitors are structurally comparable to DMP; however, the incorporation of an additional sulphur atom may enhance their binding affinity to the metal centre of AMO, thereby improving their inhibitory efficacy. Thiadiazole 2, which had an ethyl and methyl substituent, was slightly less effective than DMP with regard to  $\text{NO}_3^-$  formation ( $\text{NO}_3^-$  production on day 28 compared to the fertiliser control:  $P < 0.0001$  for DMP and 1–3;  $P < 0.001$  for 4), although the  $\text{NH}_4^+$  loss was comparable to that using DMP. On the other hand, the chlorinated compound 4 was less efficient than DMP in suppressing  $\text{NH}_4^+$  loss.

To obtain a more quantitative picture, we calculated the percentage loss of  $\text{NH}_4^+$ -N and production of  $\text{NO}_3^-$ -N on day 28 of the incubation period compared to day 0 for each treatment using eqn (1) and (2) (see Experimental). The data in Table 1 underscore the findings from Fig. 2 that, among this series of thiadiazoles, the monomethyl-substituted compound 3 was the best-performing NI with regard to both  $\text{NH}_4^+$  retention and suppression of  $\text{NO}_3^-$  formation, and was also more effective than DMP. Based on the percentage change, the inhibitory performance of the dimethyl-substituted thiadiazole 1 was similar to that of DMP.

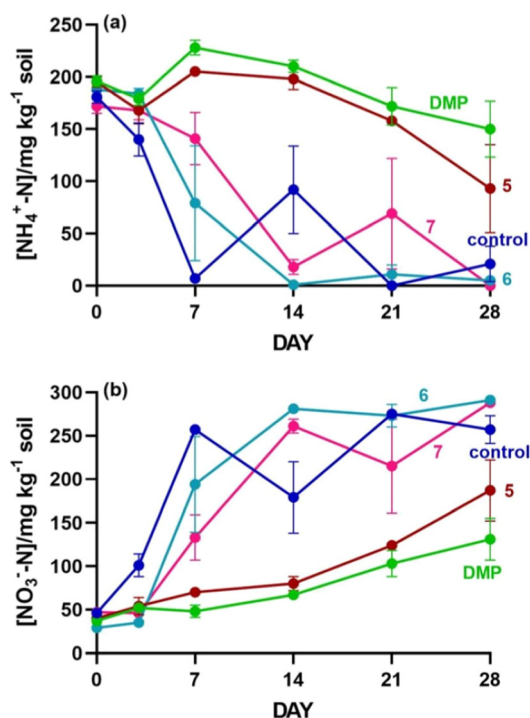
Next, we explored thiadiazoles with larger alkyl substituents as well as a polar hydroxyl group under otherwise similar con-

**Table 1** Percent  $\text{NH}_4^+$ -N loss and  $\text{NO}_3^-$ -N production on day 28 of the incubation using DMP and thiadiazoles 1–4<sup>a</sup>

Inhibitor	$\text{NH}_4^+$ -N loss/%	$\text{NO}_3^-$ -N production/%
– (Control)	99 ± 2	1300 ± 77
DMP	13 ± 1	171 ± 8
1	8 ± 3	183 ± 37
2	12 ± 6	346 ± 59
3	–2 ± 1	64 ± 7
4	26 ± 6	768 ± 76

<sup>a</sup> Wimmera soil at 25 °C and 60% WFPS.  $(\text{NH}_4)_2\text{SO}_4$  was applied at a rate of 100 mg N  $\text{kg}^{-1}$  dry soil. Inhibitors were applied at a rate of 5 mol% of applied fertiliser-N, *i.e.* (in mg  $\text{kg}^{-1}$  dry soil) DMP: 35.01, 1: 40.70, 2: 45.71, 3: 35.70, and 4: 57.83. Data are presented as mean value with standard errors, calculated from three replicates. The data for all incubation timepoints are provided in Table S3.





**Fig. 3** Change in  $\text{NH}_4^+\text{-N}$  (a) and  $\text{NO}_3^-\text{-N}$  (b) concentrations over an incubation period of 28 days at 25 °C and 60% WFPS in the Wimmera soil in the absence and presence of inhibitors DMP and compounds 5–7. The application rate of  $(\text{NH}_4)_2\text{SO}_4$  was 100 mg N  $\text{kg}^{-1}$  dry soil. Inhibitor treatments were 5 mol% of applied fertiliser-N, i.e. (in mg  $\text{kg}^{-1}$  dry soil) DMP: 35.01, 5: 45.71, 6: 50.71, and 7: 46.41. DMP and compounds 5–7 were used as phosphate salts. Each concentration profile was obtained from three replicates, and the given error is the standard error of the mean. See Table S4 for detailed statistical data.

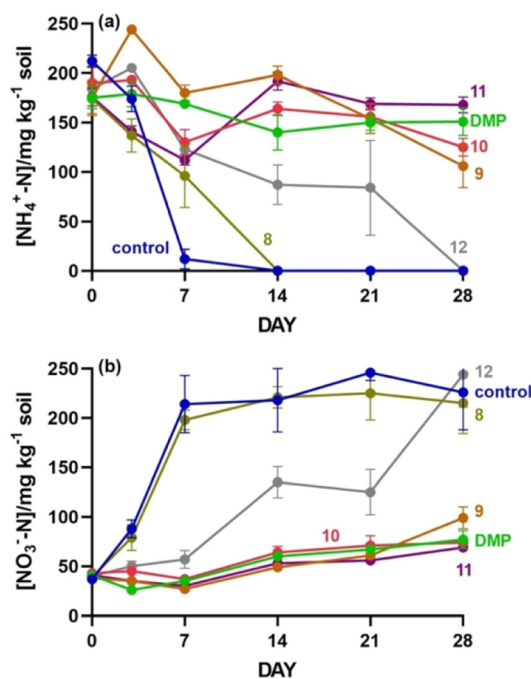
ditions (Fig. 3). Although some data points exhibited larger error margins, likely due to the variability in soil samples (such as micro-scale heterogeneity in nitrogen content, despite thorough mixing), there was a clear trend in inhibitory performance.

The incubations in the presence of DMP and the isopropyl-substituted thiadiazole 5 showed an increase in  $\text{NH}_4^+\text{-N}$  up to day 7 (Fig. 3a), which could suggest N mineralisation. The control experiment in the absence of an inhibitor revealed a rapid decline of  $\text{NH}_4^+$  within the first seven days, which was paralleled by a rise in  $\text{NO}_3^-$ . Of the three thiadiazoles tested in this incubation experiment, none outperformed DMP ( $P < 0.01$  for both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  compared to the control). Comparison of the percentage  $\text{NH}_4^+\text{-N}$  loss and  $\text{NO}_3^-\text{-N}$  production on day 28 of the various treatments (Table S5) revealed essentially complete consumption of  $\text{NH}_4^+$  in the presence of thiadiazoles 6 and 7. Compound 5 retained about 50% of  $\text{NH}_4^+$ , compared to ca. 75% by DMP in this incubation experiment.

Structurally, thiadiazole 5 differed from thiadiazole 3, the best-performing inhibitor in the first incubation (see Fig. 2), in that the methyl group in 3 was replaced by an isopropyl group. This increase in substituent size appeared to be detrimental to

inhibitory efficiency, which was supported by the finding that the presence of an even bulkier, more lipophilic *tert*-butyl group in thiadiazole 6 eradicated any inhibitory activity. On the other hand, thiadiazole 7, which carries a polar hydroxy-methyl substituent, retained  $\text{NH}_4^+$  up to day 7 ( $P < 0.01$  compared to the control) but showed minimal inhibition already by day 14.

We then assessed 4-methyl-substituted thiadiazoles containing a carboxyl group (8) or a chlorine atom (9) directly attached to the heterocycle at position 5, along with the bicyclic compounds 11 and 12. Inhibitor 10 was distinguished from compound 2 by swapping the methyl and ethyl substituents on the thiadiazole ring. Interestingly, inspection of the  $\text{NH}_4^+\text{-N}$  loss and  $\text{NO}_3^-\text{-N}$  production data in Fig. 4 revealed that the presence of the carboxyl group in 8 essentially abolished any inhibitory activity after the first week. This finding was confirmed by the percentage changes of  $\text{NH}_4^+\text{-N}$  loss on day 28, i.e., 8: (100 ± 12)%; DMP: (14 ± 10)% and  $\text{NO}_3^-\text{-N}$  production, i.e., 8: (457 ± 82)%; DMP: (90 ± 23)%, respectively (Table S6). Given the slightly alkaline nature of the soil, this compound was likely present as its carboxylate, implying that a negative charge could be detrimental for inhibitory activity (see below).



**Fig. 4** Change in  $\text{NH}_4^+\text{-N}$  (a) and  $\text{NO}_3^-\text{-N}$  (b) concentrations over an incubation period of 28 days at 25 °C and 60% WFPS in the Wimmera soil in the absence and presence of inhibitors DMP and compounds 8–12. The application rate of  $(\text{NH}_4)_2\text{SO}_4$  was 100 mg N  $\text{kg}^{-1}$  dry soil. Inhibitor treatments were 5 mol% of applied fertiliser-N, except 9 which was applied at 1 mol%, i.e. (in mg  $\text{kg}^{-1}$  dry soil) DMP: 35.01, 8: 51.40, 9: 9.56, 10: 45.71, 11: 44.99, and 12: 49.99. DMP and compounds 9–12 were used as phosphate salts. Each concentration profile was obtained from three replicates, and the given error is the standard error of the mean. See Table S6 for detailed statistical data.



The chlorinated thiadiazole **9** was obtained as a minor by-product in the synthesis of inhibitor **3** and could only be tested at 1 mol% of applied fertiliser-N. Despite this lower application rate, at the 28-day mark, thiadiazole **9** exhibited a remarkable performance compared to DMP in inhibiting  $\text{NH}_4^+$  loss and  $\text{NO}_3^-$  formation ( $P < 0.001$  compared to the control), clearly warranting further exploration in the future. Conversely, comparison of the data for the isomeric compounds **2** and **10** suggests that the positioning of the ethyl substituent at either C-4 or C-5 on the thiadiazole ring had no considerable impact on inhibitory activity. Thus, concerning the percentage change for  $\text{NH}_4^+$ -N (about 34%), compound **10** demonstrated slightly lower efficacy than DMP (about 10%), whereas regarding the percentage change for  $\text{NO}_3^-$ -N, both inhibitors exhibited a similar performance within error (**10**:  $73 \pm 12\%$ ; DMP:  $90 \pm 23\%$ ; see Table S7).

Compound **11**, featuring a fused cyclopentyl ring, surpassed DMP in both  $\text{NH}_4^+$  retention and  $\text{NO}_3^-$  formation ( $P < 0.001$  compared to the control). Conversely, compound **12**, with a larger cyclohexyl fused ring, performed better than the control until day 21 ( $P < 0.001$  for  $\text{NO}_3^-$ -N) but lost all inhibitory activities by day 28. The effective inhibition exhibited by thiadiazole **11** is noteworthy (percentage  $\text{NH}_4^+$ -N loss:  $5 \pm 7\%$ ; percentage  $\text{NO}_3^-$ -N production:  $67 \pm 9\%$ ; see Table S7), as this inhibitor had the same number of carbon atoms as the ethyl- and methyl-substituted compounds **2** and **10**. Whether the heightened inhibitory activity of **11**, compared to **2** and **10**, could be attributed to its constrained conformation caused by the two fused five-membered rings will be further investigated by us in future work.

To explore the inhibitory performance at higher soil temperatures, which will become a more frequent scenario with progressing climate change,<sup>32</sup> we studied selected thiadiazoles in the Wimmera soil at 35 °C (Fig. 5).

The three tested 1,2,3-thiadiazoles **1**, **2** and **5** were all effective in slowing down the conversion of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  at 35 °C compared to the control experiment in the absence of an inhibitor ( $P < 0.05$ – $0.01$  for **1** and **2** on day 28,  $P < 0.001$  for **5** on day 21). With regard to the percentage change of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N on day 28, the 1,2,3-thiadiazoles **1** and **2** appeared to be most effective at retaining  $\text{NH}_4^+$  (**1**:  $36 \pm 4\%$  and **2**:  $38 \pm 3\%$ ), compared to  $42 \pm 9\%$  for DMP; Table S9), whereas the isopropyl-substituted thiadiazole **5** inhibited slightly worse than the other compounds, confirming the findings from the incubations at 25 °C. With regard to inhibiting  $\text{NO}_3^-$  formation, all inhibitor compounds performed similarly within experimental error.

Worldwide, soils are more acidic than alkaline due to various natural and anthropogenic factors. We therefore also performed an incubation in an acidic cropping soil “Red Brown Earth” (pH (1 : 5 CaCl<sub>2</sub>) = 4.7; Table 3) at 25 °C to investigate the inhibitory efficacy of thiadiazoles **1**–**5**. This soil was low in organic carbon content, which can be considered as a proxy for microbiological activity. Thus, both  $\text{NH}_4^+$ -N loss and  $\text{NO}_3^-$ -N production occurred at a considerably slower rate than in the Wimmera soil (Fig. 6).

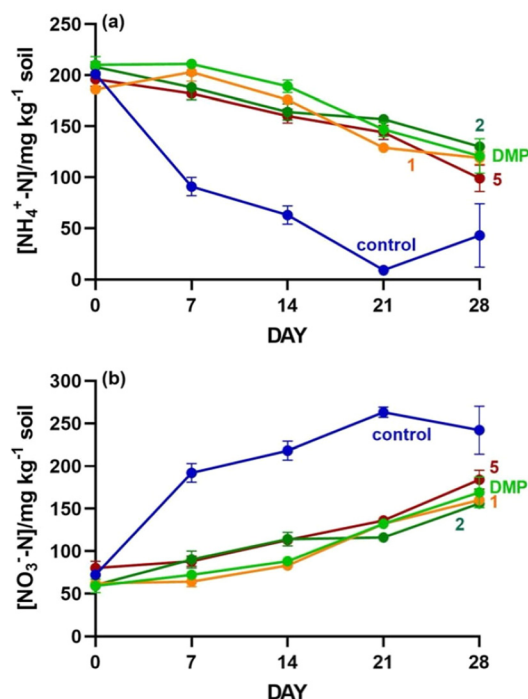
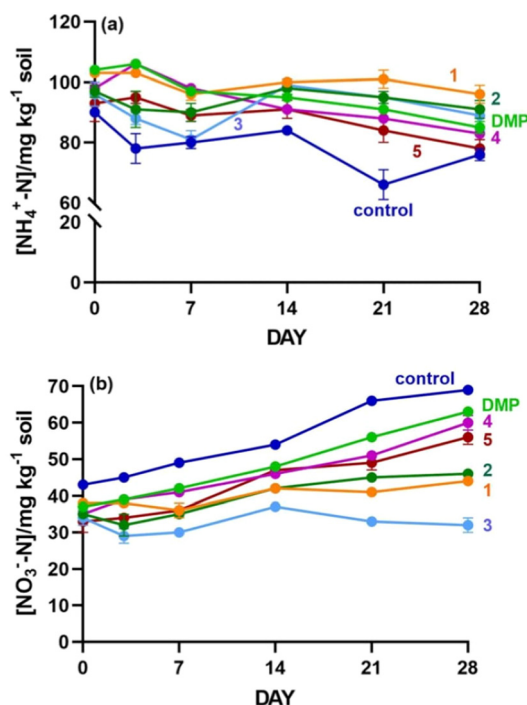


Fig. 5 Change in  $\text{NH}_4^+$ -N (a) and  $\text{NO}_3^-$ -N (b) concentrations over an incubation period of 28 days at 35 °C and 60% WFPS in the Wimmera soil in the absence and presence of inhibitors DMP and compounds **1**, **2** and **5**. The application rate of  $(\text{NH}_4)_2\text{SO}_4$  was  $100 \text{ mg N kg}^{-1}$  dry soil. Inhibitor treatments were 5 mol% of applied fertiliser-N, i.e. (in  $\text{mg kg}^{-1}$  dry soil) DMP: 35.01, **1**: 40.70, **2**: 45.71, and **5**: 45.71. DMP and compounds **1**, **2** and **5** were used as phosphate salts. Each concentration profile was obtained from three replicates, and the given error is the standard error of the mean. See Table S8 for detailed statistical data.

All inhibitor treatments appeared to retain  $\text{NH}_4^+$  better than the control with  $P < 0.01$ – $0.001$  on day 21 and  $P < 0.01$  for compound **1** on day 28. The formation of  $\text{NO}_3^-$  was significantly suppressed ( $P < 0.01$ – $0.001$ ) on day 28 for inhibitors **1**–**3**, compared to the control treatment. With regard to the percentage change in  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N on day 28 compared to day 0 for the various treatments, thiadiazoles **1**–**3** retained  $\text{NH}_4^+$  by about 94% ( $\text{NH}_4^+$ -N loss: 6–7% vs.  $19 \pm 2\%$  for DMP and  $15 \pm 3\%$  without an inhibitor; Table S11) and increased  $\text{NO}_3^-$ -N production in the range of 0% (compound **3**) up to 30% (compound **2**). On the other hand, compared to the control, DMP, the chlorinated thiadiazole **4** and the isopropyl-substituted compound **5** had essentially no impact on the N-transformations in this soil (Table S11).

What factors influence inhibitory activity? Since analysing enzyme–inhibitor complexes is challenging due to the structural and functional loss of the membrane-bound AMO upon isolation, we assessed the molecular weights and selected physicochemical parameters of the compounds used in these soil incubations (calculated data)<sup>33</sup> to tentatively explore their contribution to inhibitory efficacy. As can be seen from Table 2, all compounds have a low molecular weight (not exceeding  $163 \text{ g mol}^{-1}$ ) and differences in their performance are likely caused by other factors.





**Fig. 6** Change in  $\text{NH}_4^+\text{-N}$  (a) and  $\text{NO}_3^-\text{-N}$  (b) concentrations over an incubation period of 28 days at 25 °C and 60% WFPS in the Red Brown Earth soil in the absence and presence of inhibitors DMP and compounds 1–5. The application rate of  $(\text{NH}_4)_2\text{SO}_4$  was 100 mg N  $\text{kg}^{-1}$  dry soil. Inhibitor treatments were 5 mol% of applied fertiliser-N, *i.e.* (in mg N  $\text{kg}^{-1}$  dry soil) DMP: 35.01, 1: 40.70, 2: 45.71, 3: 35.70, 4: 57.83, and 5: 45.71. DMP and compounds 1, 2, 3 and 5 were used as phosphate salts. Each concentration profile was obtained from three replicates, and the given error is the standard error of the mean. See Table S10 for detailed statistical data.

The soil organic carbon–water partition coefficient ( $K_{oc}$ ) is a measure to describe how a chemical compound partitions between the organic carbon portion of soil and the aqueous phase in soil. Values between 50 and 150 indicate a high mobility (weak absorption on organic matter), whereas values in the range 150–500 represent a moderate soil mobility. Correlating the  $K_{oc}$  values for the various compounds with their inhibitory performance suggests that the very high mobility of the most soluble compounds 7 and 8 is detrimental to inhibitory efficiency, as they only poorly absorb on the soil organic matter that contains AMO. In fact, in the slightly alkaline Wimmera soil, the carboxylic acid group in compound 8 is expected to be deprotonated, resulting in electrostatic repulsion from the negatively charged soil particles. In contrast, the moderate or poor inhibitory performance of the more lipophilic compounds 4, 6 and 12 could indicate that they ‘get stuck’ on the soil organic matter and cannot reach the active site in AMO. On the other hand, the apparent lack of a correlation between efficacy and the  $\text{p}K_a$  value of the respective conjugate acids of the compounds tested indicates that this parameter is likely not important.

Overall, these findings suggest a ‘sweet spot’ in lipophilicity where inhibitory activity is maximised: compounds with moderate lipophilicity are best able to partition between the soil water and soil organic matter, maintaining sufficient mobility and bioavailability without becoming sequestered by organic matter. These observations align with our prior data for di-substituted 1,2,3-triazoles, where those with shorter linear alkyl substituents showed improved inhibitory performance over those with branched, bulkier alkyl substituents or substituents carrying polar functional groups such as alcohol, amine and ester moieties.<sup>24</sup>

## Experimental

### Chemicals

Thiadiazoles 1–12 were synthesised according to the literature.<sup>30</sup> All inhibitors were tested as phosphate salts, except compounds 4 and 8. The experimental procedures and spectroscopic data are provided in the SI.

### Soil incubations

Two different cropping soils were used in this study: a clay soil, “Wimmera”, and a sandy loam soil, “Red Brown Earth”, which were collected from different locations in Victoria, Australia. Table 3 provides a selection of physical and chemical properties of these soils.

The soils were air-dried, crushed, and sieved (2 mm) to remove plant debris and stones and dried in an oven until constant weight. The required amount of water to achieve the desired WFPS was determined based on soil volume, soil water content and soil bulk density.

Each soil sample contained 20 g of dry-weight soil and was incubated in a 250 mL polypropylene container (Sarstedt, Germany). About half the volume of water (Milli-Q) required to

**Table 2** Molecular weights and selected calculated physicochemical parameters for the inhibitor compounds tested in this study<sup>a</sup>

Inhibitor	Molecular weight/g	$\text{p}K_a^b$	$K_{oc}^b$	Solubility <sup>b</sup> /mol $\text{L}^{-1}$	Inhibitory performance
DMP	96.1	4.06 15.38 <sup>c</sup>	106	0.02	Good
1	114.2	−0.08	115	0.15	Good
2	128.2	−0.12	182	0.06	Good
3	100.1	−0.37	65	0.32	Good
4	162.6	−0.47	299	0.04	Moderate
5	128.2	−0.48	152	0.06	Moderate
6	142.2	−0.59	282	0.03	Poor
7	130.2	−0.86 13.08 <sup>c</sup>	35	1.65 <sup>d</sup>	Poor
8	144.2	−1.33	1	6.94	Poor
9	134.6	−2.65	170	0.06	Good
10	128.2	−0.02	182	0.06	Good
11	126.2	−0.13	191	0.05	Good
12	140.2	−0.02	323	0.02	Poor

<sup>a</sup> Data for  $\text{p}K_a$ ,  $K_{oc}$  and solubility were taken from ref. 33. Data were calculated for 25 °C at pH 7;  $K_{oc}$ : soil organic carbon–water partition coefficient. <sup>b</sup> Conjugate acid. <sup>c</sup> NH (DMP) or OH (7) group. <sup>d</sup> At pH 6.5.



**Table 3** Selected physical and chemical properties of the soils used

Analyte	Wimmera	Red brown Earth
Colour	Grey	Yellow-brown
Texture	Clay	Sandy loam
pH (1 : 5 CaCl <sub>2</sub> )	7.9	4.7
Organic carbon/%	2.23	0.72
[NO <sub>3</sub> <sup>-</sup> -N]/mg kg <sup>-1</sup>	1.1	40
[NH <sub>4</sub> <sup>+</sup> -N]/mg kg <sup>-1</sup>	170	8.1

meet the desired WFPS (60%) was added to reactivate microorganisms in the soils, followed by pre-incubation at 25 °C (or 35 °C) for seven days after which the remaining volume of water to reach a WFPS of 60% was applied as one of the treatment solutions: (i) ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) only (fertiliser control) and (ii) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and one NI. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was applied at a rate of 100 mg N kg<sup>-1</sup> of dry soil, whereas inhibitor treatments were 5 mol% of applied fertiliser-N, except for compound **9**, which was applied at 1 mol%. All treatment solutions were prepared with ultrapure (Milli-Q) water. Each treatment (fertiliser control and compound treatment) was performed in triplicate at each time point (for example, an incubation experiment with six different treatments and sampling at five time points had 3 × 6 × 5 = 90 containers). The treated soil samples were incubated at 25 °C (or 35 °C) for up to 28 days without any direct UV or visible light exposure. During the incubation period, the lids of the polypropylene containers were loosely placed for air exchange to keep the soil samples aerated, and soil water content levels were maintained by the addition of water every three to four days based on weight loss.

### Soil extraction and analysis

After each desired incubation period (*i.e.*, on days 0, 7, 14, 21 and 28, respectively), the soil samples were destructively sampled by adding 100 mL of aqueous potassium chloride solution (KCl, 2 M) and shaking for one hour. The soil/KCl solutions were filtered (Whatman filter paper No. 42). The extracts from each time point were stored at -16 °C until the end of the experiment where the concentrations of NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N for all samples were measured by Segmented Flow Analysis (San++, Skalar, Breda, The Netherlands) or Flow Injection Analysis (FIAlyzer-1000, FIALab Instruments, Inc.). To ensure consistent analytical accuracy and enable correction for any minor variability introduced by solvent loss during analysis, standard and drift solutions were run at the beginning and end of each session, and additional calibration checks were inserted after every 20 samples. The results are reported as the mean value of the three replicates, and errors are reported as standard errors of the mean. Errors associated with the raw data were calculated by using the standard error propagation protocol.

Nitrification was assessed based on the rate of NH<sub>4</sub><sup>+</sup>-N loss and rate of NO<sub>3</sub><sup>-</sup>-N accumulation over the incubation period (note that NO<sub>3</sub><sup>-</sup> was reduced and detected as NO<sub>2</sub><sup>-</sup> during the analysis, and NO<sub>3</sub><sup>-</sup>-N represents the combined concentration

of NO<sub>2</sub><sup>-</sup>-N and NO<sub>3</sub><sup>-</sup>-N in the soil). For each treatment, the loss of NH<sub>4</sub><sup>+</sup>-N and production of NO<sub>3</sub><sup>-</sup>-N were calculated as percentages, according to eqn (1) and (2), respectively:

$$\text{NH}_4^+\text{-N loss (\%)} = \frac{[\text{NH}_4^+\text{-N}]_{t=0} - [\text{NH}_4^+\text{-N}]_t}{[\text{NH}_4^+\text{-N}]_{t=0}} \times 100 \quad (1)$$

where [NH<sub>4</sub><sup>+</sup>-N]<sub>t=0</sub> is the concentration of NH<sub>4</sub><sup>+</sup>-N (mg N kg<sup>-1</sup> soil) on day 0 and [NH<sub>4</sub><sup>+</sup>-N]<sub>t</sub> is the concentration of NH<sub>4</sub><sup>+</sup>-N (mg N kg<sup>-1</sup> soil) on a given day, *t*, and

$$\text{NO}_3^-\text{-N production (\%)} = \frac{[\text{NO}_3^-\text{-N}]_t - [\text{NO}_3^-\text{-N}]_{t=0}}{[\text{NO}_3^-\text{-N}]_{t=0}} \times 100 \quad (2)$$

where [NO<sub>3</sub><sup>-</sup>-N]<sub>t=0</sub> is the concentration of NO<sub>3</sub><sup>-</sup>-N (mg N kg<sup>-1</sup> soil) on day 0 and [NO<sub>3</sub><sup>-</sup>-N]<sub>t</sub> is the concentration of NO<sub>3</sub><sup>-</sup>-N (mg N kg<sup>-1</sup> soil) on a given day, *t*.

It should be noted that the NH<sub>4</sub><sup>+</sup>-N loss and NO<sub>3</sub><sup>-</sup>-N production data for DMP were different in all soil incubations performed in this study, reflecting the highly variable nature of soil experiments. Because of this, the performance of thiadiazoles compared to DMP should only be assessed within the same incubation experiment.

In some treatments, higher concentrations of NH<sub>4</sub><sup>+</sup>-N were measured at a given time point compared to day 0, which may be due to N-mineralisation, resulting in negative values for the percentage changes. These also caused the differences between nitrification inhibition based on the percent loss of NH<sub>4</sub><sup>+</sup>-N and the percent production of NO<sub>3</sub><sup>-</sup>-N.

### Statistical analysis

Statistical analyses were performed on raw NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N data in R (version 3.5.2),<sup>34</sup> using the statistical package *emmeans*.<sup>35</sup> The statistical significance (*P* < 0.05) of the data and the impact of two factors, “Day” and “Treatment”, were assessed *via* two-way analysis of variance (ANOVA). Pair-wise comparisons between different treatments at each time point were performed using a Tukey HSD *post-hoc* adjustment.

## Conclusions

A guiding principle in the design and development of novel nitrification inhibitors for agricultural applications is the synthetic accessibility of these compounds (*i.e.*, short synthesis, availability of starting materials, *etc.*) as they would need to be manufactured on a large scale. In this work, we have assessed for the first time the performance of 1,2,3-thiadiazoles as novel NIs, which are readily available with a variety of substitution patterns through the Hurd–Mori process. By conducting SAR studies, we showed that compounds carrying short alkyl substituents at position 4 (and 5) were the most promising inhibitors, which performed equal to or better than the current commercial ‘gold standard’ DMP, even at the higher temperature of 35 °C and in an acidic soil. Increasing the size of the alkyl substituents appeared to be detrimental to the inhibitory efficacy. Interestingly, fusing a cyclopentyl ring to



the thiadiazole and thereby reducing the conformational degree of freedom (compound **11**) were found to be beneficial for inhibitory activity, whereas the larger homologue with a fused cyclohexyl ring (compound **12**) prevented inhibition after day 21. Analysis of the  $K_{oc}$  values suggests that a too high lipophilicity caused by larger alkyl substituents may lead to immobilisation on the soil organic matter, preventing the inhibitor from reaching the active site in AMO. In contrast, compounds with intermediate lipophilicity strike the right balance—allowing them to effectively partition between the aqueous and organic phases in soil. This balance is likely key to achieving strong and consistent inhibitory performance. With regard to functional groups, our experiments revealed that 1,2,3-thiadiazoles substituted with polar alcohol or carboxylic acid moieties were unsuitable as NIs, likely due to a too high mobility in the soil water and poor absorption on soil organic matter.

To conclude, our laboratory soil incubations are a crucial step towards the systematic development of new and effective nitrification inhibitors to increase NUE in agriculture. Subsequent efforts will focus on evaluating the most promising compounds through more resource-intensive glasshouse and field trials, microbiological and degradation studies in soil as well as ecotoxicological assessments and elucidation of the mechanism of inhibition using bacterial assays.<sup>26</sup> This tiered approach ensures efficient use of time and resources by narrowing the focus to the most promising inhibitors early in the development process.

## Author contributions

Parvinder K. Sidhu: writing – review & editing, investigation, formal analysis, methodology, and visualisation (equal). Yue Ming: writing – review & editing, investigation, formal analysis, and visualisation (equal). Uta Wille: writing – original draft, review & editing, conceptualisation, visualisation (equal), funding acquisition, and supervision.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the SI: synthesis of the inhibitor compounds; mineral N transformation studies; SI tables showing the actual concentrations of the inhibitors in the phosphate salts (Table S1) and  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N concentrations and percent  $\text{NH}_4^+$ -N loss and  $\text{NO}_3^-$ -N production over 28 days of incubation (Tables S2–S11). See DOI: <https://doi.org/10.1039/d5ob00930h>.

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