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**Assessing the Efficacy, Acute Toxicity and Binding Modes of the  
Agricultural Nitrification Inhibitors 3,4-Dimethyl-1H-Pyrazole (DMP)  
and Dicyandiamide (DCD) With *Nitrosomonas europaea***

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## Abstract

Nitrification inhibitors have been co-formulated with nitrogen fertilizers since the 1970s to modulate the microbiological conversion of nitrogen in agricultural soils. 3,4-Dimethyl-1*H*-pyrazole (DMP) and dicyandiamide (DCD) are currently the most used commercial nitrification inhibitors, but their mode of action is not well understood. This work seeks to fill this void by assessing for the first time in detail their mechanism of inhibition, efficacy, and acute toxicity with pure cell cultures of *Nitrosomonas europaea*. Bacterial assays based on the quantification of the nitrite (NO<sub>2</sub><sup>-</sup>) production showed that both inhibitors reversibly target ammonia monooxygenase (AMO), which catalyzes the first step of the nitrification process. Michaelis-Menten kinetics suggest that both DMP and DCD act as uncompetitive inhibitors. Real-time measurements of the oxygen (O<sub>2</sub>) consumption confirmed the non-mechanistic mode of inhibition and showed that DMP reduced the O<sub>2</sub> uptake rate by AMO much more at considerably lower concentrations than DCD, in line with the lower inhibitory efficiency of the latter. Acute toxicity tests revealed that DCD has a 10% higher toxicity than DMP when comparing treatments at the same inhibition efficacy (*i.e.*, DMP at 10 ppm, DCD at 100 ppm), indicating that inhibition of the nitrification process cannot simply be achieved by increasing the inhibitor concentration. The methods presented in this study could assist the development of more reliable nitrification inhibitors in the future.

## Keywords

Ammonia monooxygenase; Bacterial assay; Dicyandiamide; 3,4-Dimethyl-1*H*-pyrazole; Inhibition mechanism; Nitrification inhibitor.

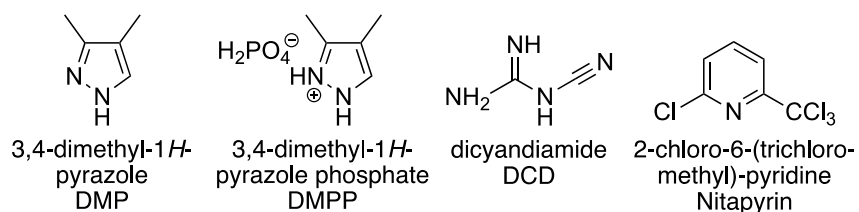
## Introduction

To meet the food demand of an ever-growing population, the Food and Agriculture Organization of the United Nations (FAO) has predicted that from 2016 – 2022 a 5% increase in nitrogen (N) fertilization from 106 Tg to 112 Tg is required.<sup>1</sup> However, since the 1980s, the N use efficiencies (NUEs) have remained at only around 50% globally.<sup>2,3</sup> The remaining 50% are lost from the soil through abiotic and biotic pathways, including volatilization of ammonia ( $\text{NH}_3$ ), which is a precursor of particulate matter ( $\text{PM}_{2.5}$ ), and nitrate ( $\text{NO}_3^-$ ) leaching that causes damaging surface water eutrophication and groundwater pollution.<sup>4-6</sup> In addition, microbiological denitrification reduces  $\text{NO}_3^-$  to nitrous oxide ( $\text{N}_2\text{O}$ ) and nitric oxide (NO).<sup>7</sup>  $\text{N}_2\text{O}$  has a 300 times higher global warming potential than  $\text{CO}_2$ , and mitigation of N losses in agriculture has become an important target for reducing the greenhouse gas (GHG) footprint.<sup>6</sup>

One strategy to improve N management in agricultural soils is to amend N fertilizers with nitrification inhibitors (NIs).<sup>8,9</sup> Nitrification is caused by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). NIs are small synthetic molecules that are designed to inhibit ammonia monooxygenase (AMO), a multimeric transmembrane enzyme, which is conserved in both AOB and AOA<sup>10-12</sup> and catalyzes the rate-limiting first oxidation step  $\text{NH}_3 \rightarrow$  hydroxylamine ( $\text{NH}_2\text{OH}$ ).<sup>13,14</sup> Subsequently,  $\text{NH}_2\text{OH}$  is converted to nitrite ( $\text{NO}_2^-$ ) by the enzyme hydroxylamine oxidoreductase (HAO),<sup>15</sup> followed by the rapid oxidation to  $\text{NO}_3^-$ , the end-product of the nitrification process, which is catalyzed by nitrite oxidase (NXR) present in, for example, *Nitrobacter* spp and *Nitrospira*. In fact, some strains of *Nitrospira* are capable of catalyzing the oxidation from  $\text{NH}_3$  to  $\text{NO}_3^-$  (complete ammonia oxidizers, comammox).<sup>16</sup> Furthermore, recent studies revealed that nitrification can also directly lead to formation of NO and  $\text{N}_2\text{O}$ .<sup>17-21</sup> Thus, by inhibiting AMO, the residence time of  $\text{NH}_3$  in soils should be increased, which in turn should reduce N losses through  $\text{NO}_3^-$  leaching and emission of gaseous N compounds produced through both nitrification and denitrification processes. While the crystal structure of AMO remains to be resolved, the evolutionally similar and recently crystallized methane monooxygenase (MMO) has provided some information on the active center in AMO, suggesting that a cupredoxin-like unit could be involved in the oxidation steps.<sup>10,22-24</sup>

Generally, NIs can be categorized into mechanism-based and non-mechanism-based enzyme inhibitors, such as chelators that coordinate to a metal center in the enzyme's active site without inducing a chemical change.<sup>13</sup> In contrast, mechanism-based inhibitors are converted in the active site to products that inactivate the enzyme, for example through the formation of covalent bonds. Consequently, recovery of activity of nitrifying bacteria requires *de novo* synthesis of AMO. One prominent example for a mechanism-based NI is the gas acetylene (C<sub>2</sub>H<sub>4</sub>),<sup>25,26</sup> but the high flammability and reactivity prohibits its use in agriculture.

Currently commercially available NIs are 3,4-dimethyl-1*H*-pyrazole (DMP), which is commonly applied in agricultural systems as the phosphate salt to reduce its volatility (DMPP or ENTEC®, BASF AG), dicyandiamide (DCD, AlzChem AG) and 2-chloro-6-(trichloromethyl)-pyridine (Nitrapyrin or N-Serve®, Dow Chemical Co.) (Figure 1).



**Figure 1.** Commercially available nitrification inhibitors (NIs).

Various *in vitro* studies have demonstrated that DMPP reduces nitrification rates; however, field studies revealed that its efficacy strongly depends on the agroecosystem.<sup>27-33</sup> DCD, which is widely applied in New Zealand, has been shown to reduce N<sub>2</sub>O emissions but is prone to leaching and has been detected in dairy products.<sup>34-36</sup> Nitrapyrin is highly volatile and is the most intensively studied NI regarding its mode of action.<sup>37,38</sup> It has been classified as a metal chelator,<sup>39</sup> although product analyses indicate that Nitrapyrin could also act as a mechanism-based enzyme inhibitor.<sup>37</sup>

Despite their widespread use, detailed mechanistic studies are unavailable for DMP (or DMPP, respectively) and DCD. Recently, the crystal structure of six DMP molecules coordinating to a Cu<sup>2+</sup> center was solved, demonstrating the ligand-binding ability of DMP.<sup>40</sup> DCD has also been

categorized as a metal-chelator<sup>12,41-43</sup> and been proposed to act as a competitive inhibitor for AMO.<sup>21</sup> However, because of the many remaining unknowns of the detailed mode of action of these two NIs, in this work we have performed a comparative study of DMP (as the active component in DMPP) and DCD using assays with pure bacterial cultures of *Nitrosomonas europaea* to determine for the first time important biochemical parameter in the absence of the complex matrix of soil experiments. This study provides crucial insight into the binding mode, efficacy, and acute toxicity of these two commercial NIs, which could help to understand their variable performance in the field and support the development of guidelines to aid the design of next-generation NIs with improved and more consistent performance.

## Materials and Methods

### 1. Chemicals

DMP (3,4-dimethyl-1*H*-pyrazole) was supplied by Incitec Pivot Ltd. Australia. DCD (dicyandiamide) and Griess reagent (modified) was purchased from Sigma Aldrich. Sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) was obtained from ChemSupply Australia. All aqueous solutions were prepared in Milli-Q water. *N. europaea* (ATCC19718) was purchased from the American Type Culture Collection.

### 2. Cell Preparation

#### 2.1 Growing *N. europaea* Protocol

AOB were grown for 3 – 5 d in Duran glass bottles containing 600 mL of mineral salts media (MSM, see below) at 100 rpm and 30°C in the dark. The slightly loose cap was sealed with an O<sub>2</sub> permeable membrane to ensure aeration (Breathe-Easy® sealing membrane, Sigma Aldrich). The MSM constituted the main bulk medium and consisted of dipotassium hydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>; 2.27 g L<sup>-1</sup>), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>; 0.95 g L<sup>-1</sup>) and ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.67 g L<sup>-1</sup>). The pH was adjusted to 7.0. To 1 L of the bulk medium 2 mL of a filter sterilized (0.2 µm millipore filter) solution of metals was added: disodium ethylenediamine

tetraacetate ( $\text{Na}_2\text{EDTA}$ ;  $6.37 \text{ g L}^{-1}$ ), zinc sulphate heptahydrate ( $\text{ZnSO}_4 \times 7 \text{ H}_2\text{O}$ ;  $1.0 \text{ g L}^{-1}$ ), calcium chloride dihydrate ( $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$ ;  $0.5 \text{ g L}^{-1}$ ), iron(II) sulphate heptahydrate ( $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$ ;  $2.5 \text{ g L}^{-1}$ ), sodium molybdate dihydrate ( $\text{NaMoO}_4 \times 2 \text{ H}_2\text{O}$ ;  $0.1 \text{ g L}^{-1}$ ), copper(II) sulphate pentahydrate ( $\text{CuSO}_4 \times 5 \text{ H}_2\text{O}$ ;  $0.1 \text{ g L}^{-1}$ ), cobalt(II) chloride hexahydrate ( $\text{CoCl}_2 \times 6 \text{ H}_2\text{O}$ ;  $0.2 \text{ g L}^{-1}$ ), manganese(II) sulphate monohydrate ( $\text{MnSO}_4 \times \text{H}_2\text{O}$ ;  $0.52 \text{ g L}^{-1}$ ) and magnesium sulphate heptahydrate ( $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$ ;  $60.0 \text{ g L}^{-1}$ ). To this media solution 1v/v% of aqueous sodium carbonate ( $\text{Na}_2\text{CO}_3$ ;  $50 \text{ g L}^{-1}$ ) was added aseptically as a carbon source.

## 2.2 Harvesting Cells Protocol

After 3 - 5 d of incubation, the turbid cultures were harvested at an  $\text{OD}_{600}$  of approximately 0.1, which represented the mid-exponential growth phase and an  $\text{NO}_2^-$  production of approximately  $800 \mu\text{M}$  (determined by Griess assay), cells were harvested by filtration onto  $0.2 \mu\text{m}$  membrane filters (Rowe Scientific, mixed cellulose esters (MCE)). The cells were washed with sodium phosphate buffer (NaPB,  $\text{pH} = 7.5$ ,  $0.1 \text{ M}$ ,  $2 \times 100 \text{ mL}$ ) containing  $\text{MgSO}_4$  ( $0.2 \text{ mM}$ ). The filter paper with the cells was transferred into a sterile  $50 \text{ mL}$  tube, and the cells were washed off by resuspending in NaPB ( $15 \text{ mL}$ ), followed by 5 s of vortexing (Ratek, Australia) and 3 s of sonication (Vevor, Australia). The initial inoculum  $\text{OD}_{600}$ , which was between  $0.9 - 1.2$ , was adjusted to a final  $\text{OD}_{600}$  of  $0.03$  and stored at  $4^\circ\text{C}$  until used for the assay. Cells could be stored for up to 24 h without losing activity.

## 3. Nitrification Assay and Analysis

### 3.1 Standard Assay Protocol

In a deep 96-well plate ( $2 \text{ mL}$  capacity),  $980 \mu\text{L}$  of the bacterial inoculum ( $\text{OD}_{600} = 0.03$  in NaPB at  $\text{pH} 7.5$ ) was added to the inhibitor ( $10 \mu\text{L}$  of a  $30 \text{ mmol L}^{-1}$  or  $3 \text{ mmol L}^{-1}$  stock solution, respectively), the solutions were mixed thoroughly and pre-incubated in the dark for 5 min at  $30^\circ\text{C}$  and  $100 \text{ rpm}$  (Ratek, Australia).  $(\text{NH}_4)_2\text{SO}_4$  ( $10 \mu\text{L}$ ,  $150 \text{ mM}$ , from a sterile solution containing  $19.8 \text{ g L}^{-1}$  of  $(\text{NH}_4)_2\text{SO}_4$  in Milli-Q water) was then added. In experiments in which the  $\text{NH}_2\text{OH}$ -

dependent activity was measured,  $(\text{NH}_4)_2\text{SO}_4$  was replaced by equimolar amounts of  $\text{NH}_2\text{OH}$ . The plate was covered with an  $\text{O}_2$  permeable membrane to ensure aeration (Breathe-Easy® sealing membrane, Sigma Aldrich) and incubated in the dark for 30, 60 or 90 min at  $30^\circ\text{C}$  and 100 rpm. The nitrification process was stopped by adding an excess of DMP ( $10 \mu\text{mol L}^{-1}$ , 30 mM; the final concentration of DMP in the solution was 0.27 mM, which was considerably higher than the  $\text{IC}_{50\text{abs}}$  value of  $6.6 \mu\text{M}$  determined previously).<sup>44</sup> An aliquot of the reaction solution ( $50 \mu\text{L}$ ) was transferred to a 96-well spectrophotometric plate (Greiner Cellstar®, polystyrene) to which  $50 \mu\text{L}$  of Griess reagent was added. The color was allowed to develop for 15 min at room temperature, and the absorbance was measured at 540 nm (Clariostar® BMG Labtech, Australia). Each assay was accompanied by control treatments to determine the 0% and 100%  $\text{NO}_2^-$  signal. The percentage inhibition was calculated according to eqn. (1) from the  $\text{NO}_2^-$  production of the cells in NaPB (i) without additive ("untreated cells"; 0% signal), (ii) with  $[\text{NH}_4^+] = 3 \text{ mM}$  ("uninhibited cells"; 100% signal) and (iii) with  $[\text{NH}_4^+] = 3 \text{ mM}$  and  $[\text{inhibitor}] = 0.3 \text{ mM}$  (10 mol% of  $[\text{NH}_4^+]$ ; "inhibited cells").

$$\% \text{ Inhibition} = \left[ 1 - \frac{[\text{NO}_2^-]_{\text{inhibited cells}} - [\text{NO}_2^-]_{\text{untreated cells}}}{[\text{NO}_2^-]_{\text{uninhibited cells}} - [\text{NO}_2^-]_{\text{untreated cells}}} \right] \times 100 \quad (\text{eqn. 1})$$

### 3.2 $\text{O}_2$ Consumption Measurements

$\text{O}_2$  consumption rates of cell suspensions of *N. europaea* were measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) mounted in a water-jacketed electrode chamber (3 mL capacity) that was connected to a recirculating cooler (Lauda, Austria). The data were recorded using a Data-trax™ (World Precision Instruments, UK) sensor data collection system. All measurements were taken at  $20^\circ\text{C}$  and 1 mL final reaction solution volume (the lower temperature, compared to the other experiments in this study that were performed at  $30^\circ\text{C}$ , was required here to increase signal stability). The polarizing voltage was set to 0.6 V. To calibrate the oxygen signal, an excess (approximately 50 mg) of  $\text{Na}_2\text{S}_2\text{O}_4$  was added to 1 mL of Milli-Q water to chemically remove dissolved  $\text{O}_2$ . Additional  $\text{O}_2$  flux was prevented by applying a stopper, and the residual voltage was referred to as "0%  $\text{O}_2$ ". The voltage at saturated  $\text{O}_2$  concentration ("100%

O<sub>2</sub>) was determined by measuring the voltage of the equilibrated aerated reaction system consisting of 1 mL Milli-Q water. Sample measurements were taken as follows: The 1 mL reaction mixture, composed of 980 µL *N. europaea* cell solution in NaPB (OD<sub>600</sub> = 0.8; corresponding to approximately 468 µg L<sup>-1</sup> protein) was equilibrated for 5 min in the chamber until the voltage reading was stable. The reaction was then initiated by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (10 µL of an aqueous 150 mM stock solution, the final concentration in the reaction solution was 3 mM) and the chamber immediately sealed with a stopper. After 5 min of oxygen consumption (a linear rate coefficient of approximately 186 ± 63 nmol O<sub>2</sub> L<sup>-1</sup> s<sup>-1</sup> was determined), 10 µL of the inhibitor stock solution of DMP (0.012 mM, 0.12 mM, 0.6 mM, 1.2 mM) or DCD (1.2 mM, 2.0 mM, 5.0 mM, 10.0 mM) were added via a 10 µL Eppendorf pipette through a capillary opening, ensuring the emergence of the pipette tip in the solution. The voltage was recorded over a period of 5 min in intervals of 5 s. The trace describing the O<sub>2</sub> concentration after addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> against the time (initial 15-300 s) was used as the baseline O<sub>2</sub> consumption for the uninhibited cells, whereas the trace describing the consumption in the presence of the inhibitor (300-590 s time window) was used to determine the rate of O<sub>2</sub> consumption in the presence of inhibitor. All experiments were conducted in triplicate at 20°C under constant stirring. The voltage was converted to [O<sub>2</sub>] according to equation 2:<sup>45</sup>

$$[O_2]_t = \frac{\text{voltage (sample)}_t - \text{voltage (0\% O}_2)}{\text{voltage (100\% O}_2) - \text{voltage (0\% O}_2)} \times 280 \mu\text{M} \quad (\text{eqn. 2})$$

### 3.3 Activity Recovery Assay

Cells were harvested using the 'Harvesting Cells Protocol'. The bacteria solution was adjusted to OD<sub>600</sub> = 0.8 (~ 468 µg L<sup>-1</sup> protein), and 980 µL aliquots were transferred to 1.5 mL centrifuge tubes (Eppendorf®, polypropylene). The inhibitor (10 µL of a stock solution of 150 mM in Milli-Q water) was added, and after equilibrating for 5 min (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (10 µL of a stock solution of 150 mM in Milli-Q water) was added using a multichannel pipette to ensure simultaneous addition in each tube. The tubes were incubated in a temperature-regulated rotary incubator (Ratek, Australia) for 30 min at 30°C and 100 rpm in the dark. A 50 µL aliquot was then transferred to a 96-well plate (Greiner Cellstar®, polystyrene) to which 50 µL of Griess reagent was added and the mixture

incubated for 15 min. The absorbance was measured at 540 nm (Clariostar® BMG Labtech, Australia). The remaining cells were subsequently washed (3x) by alternating centrifuging (Boeco, Germany; 10,000 rpm, 10 min) and resuspending the cell pellet in NaPB (1 mL). After the final centrifuging step, the pellet was resuspended in NaPB (990 µL) and re-incubated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (10 µL of an aqueous 150 mM stock solution, see above), and the NO<sub>2</sub><sup>-</sup> concentration was measured under the previously described conditions. Each assay was accompanied by control treatments to determine the 0% and 100% NO<sub>2</sub><sup>-</sup> signal. The %activity was determined according to equation 3, where "untreated cells" (0% signal) is the NO<sub>2</sub><sup>-</sup> production of the cells without additive, "uninhibited cells" (100% signal) the NO<sub>2</sub><sup>-</sup> production of cells treated with [NH<sub>4</sub><sup>+</sup>] = 3 mM, and "inhibited" cells" the NO<sub>2</sub><sup>-</sup> production of cells treated with [NH<sub>4</sub><sup>+</sup>] = 3 mM and [inhibitor] = 1.5 mM. All experiments were performed in triplicate.

$$\% \text{ Activity} = 1 - \frac{[\text{NO}_2^-]_{\text{inhibited cells}} - [\text{NO}_2^-]_{\text{untreated cells}}}{[\text{NO}_2^-]_{\text{uninhibited cells}} - [\text{NO}_2^-]_{\text{untreated cells}}} \quad (\text{eqn. 3})$$

### 3.4 Michaelis-Menten Kinetics

In a deep 96-well plate (2 mL capacity), 980 µL of the bacterial inoculum (OD<sub>600</sub> = 0.03, ~ 18 µg L<sup>-1</sup> protein in NaPB at pH 7.5) was added to DMP (10 µL from 0.75 mM and 1.5 mM stock solutions, respectively) or DCD (10 µL from 15 mM and 30 mM stock solutions, respectively). The solutions were mixed thoroughly and pre-incubated in the dark for 5 min at 100 rpm and at 30°C (Ratek, Australia). 10 µL of the respective (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> stock solution (150 mM, 75 mM, 5 mM, 2.5 mM, 1.5 mM, 0.15 mM) was added to each well (final [NH<sub>4</sub><sup>+</sup>] in well: 3.0 mM, 1.5 mM, 0.1 mM, 0.05 mM, 0.03 mM, 0.003 mM). The plate was covered with an O<sub>2</sub> permeable membrane to ensure aeration (Breathe-Easy® sealing membrane, Sigma Aldrich) and incubated in the dark for 60 min at 30°C and 100 rpm. Termination of the nitrification process and determination of the NO<sub>2</sub><sup>-</sup> production was performed as described in 'Standard Assay Protocol'. Data analysis was performed with the GraphPad Prism software, using nonlinear regression (curve fit) for Michaelis Menten Kinetics.<sup>46</sup> The results used the best fit values with 95% likelihood.

### 3.5 Acute Toxicity Test

Cells were harvested according to 'Harvesting Cells Protocol'. The bacteria solution was adjusted to  $OD_{600} = 0.8$  ( $\sim 468 \mu\text{g L}^{-1}$  protein) and divided into 1 mL aliquots. To each well of a 24-well tissue culture plate (Greiner Cellstar®, polystyrene Tissue Culture treated) was added 980  $\mu\text{L}$  of bacterial solution, 10  $\mu\text{L}$  of a 150 mM aqueous  $(\text{NH}_4)_2\text{SO}_4$  solution and 10  $\mu\text{L}$  of either a DMP or a DCD solution with a final concentration in the well of 10 ppm (0.015 mM), 100 ppm (0.15 mM) and 1000 ppm (1.5 mM), respectively. The well plate was sealed with an  $\text{O}_2$  permeable membrane to ensure aeration (Breathe-Easy® sealing membrane, Sigma Aldrich) and incubated in the dark for 4 h at  $30^\circ\text{C}$  and 100 rpm. Cells were then transferred into a centrifuge tube and sedimented at 10,000 rpm for 10 minutes. The supernatant was separated, and the cells were re-suspended in NaPB (pH = 7.5, 1 mL). A 5  $\mu\text{L}$  aliquot was transferred into a 96-well plate and the bacterial stain (LIVE/DEAD™ BacLight™ Bacterial Viability Kit for microscopy, ThermoFisher Scientific) was added following the manufacturer's instructions. 10  $\mu\text{L}$  of the solution was transferred onto a microscopic slide (Fisher Scientific, Australia, microscope slides 7.6 cm x 2.5 cm (L x W), thickness 1 - 1.2 mm). Seven images were taken per treatment with a fluorescent microscope (Leica DM6000, Germany) using the red channel to detect dead cells (excitation: 575/30 nm (dichromatic) DC: 600; emission: 635/40 nm) and the green channel to detect live cells (excitation: 500/20 nm DC: 515; emission: 535/30 nm). The percentage of live and dead cells was calculated via equations 4 and 5:

$$\% \text{ Live} = \frac{\text{Live cell count}}{\text{total cell count}} \times 100 \quad (\text{eqn. 4})$$

$$\% \text{ Dead} = \frac{\text{Dead cell count}}{\text{total cell count}} \times 100 \quad (\text{eqn. 5})$$

Prior to the microscopic imaging, 50  $\mu\text{L}$  of supernatant was reacted with 50  $\mu\text{L}$  Griess reagent to determine  $[\text{NO}_2^-]$  following the 'Standard Assay Protocol'.

## 4. Statistics

Statistical analysis was performed with the GraphPad Prism software<sup>46</sup> using student's t-test  $P < 0.05$  as the level of statistical significance. All results are reported as mean  $\pm$  standard error of the mean. In addition, significances among three treatments were compared by the least significant differences  $P < 0.05$  level using one-way ANOVA.

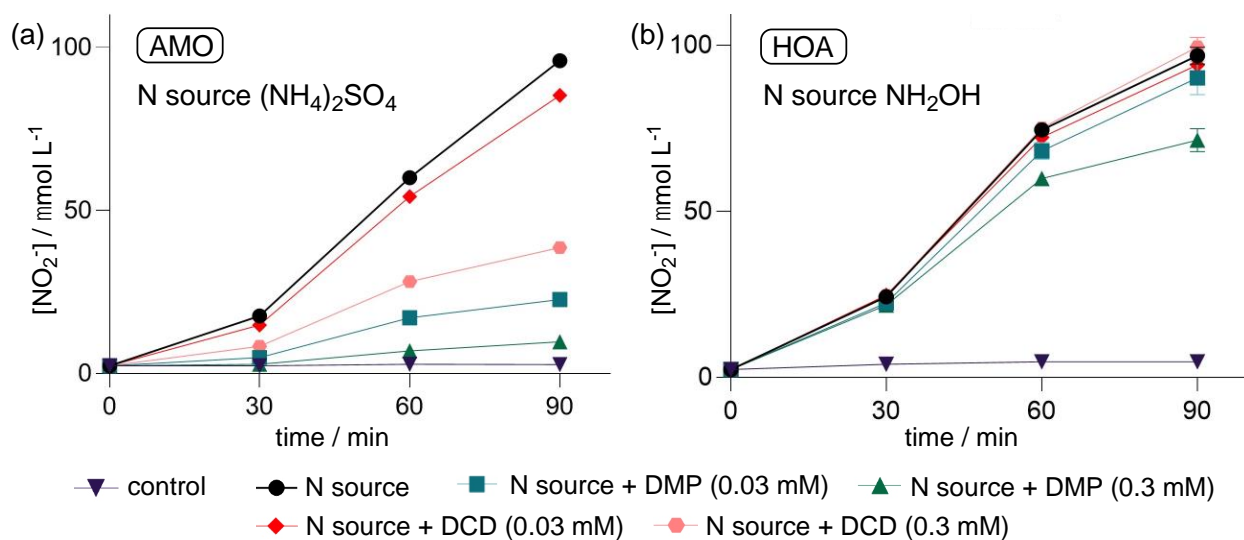
## Results and Discussion

We first identified the enzyme targeted by DMP and DCD and their mode of binding using *N. europaea* as model AOB. The analysis was performed by measuring the amount of  $\text{NO}_2^-$  produced in the absence and presence of the NI, which was determined using a recently developed assay based on the Griess reaction.<sup>44</sup> We then explored the mode of inhibition by measuring the Michaelis-Menten kinetics and the inhibitory efficacy by establishing the rate of  $\text{NH}_3$  oxidation by *N. europaea*, which was obtained from  $\text{O}_2$  consumption measurements. Analysis of the acute toxicity of both inhibitors was performed to investigate whether the lower production of  $\text{NO}_2^-$  in the presence of DMP and DCD could be due to a potential toxicity of the inhibitors for the bacteria.

### *Identification of the enzyme targeted by DMP and DCD*

As outlined in the Introduction, oxidation of  $\text{NH}_3$  to  $\text{NO}_3^-$  occurs in several steps, where AOB are responsible for the first two steps, *i.e.*,  $\text{NH}_3 \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_2^-$ , which are catalyzed by the enzymes AMO and HAO, respectively. While DMP and DCD are believed to target AMO, to our knowledge, no study is currently available that unequivocally confirms this hypothesis. Thus, to explore, which of these two enzymes is inhibited by DMP and DCD, we performed assays where pure cell cultures of *N. europaea* were treated in sodium phosphate buffer (NaPB) at pH 7.5 and 30°C separately with either  $\text{NH}_4^+$  (as substrate for AMO) or  $\text{NH}_2\text{OH}$  (as substrate for HAO) and measured the cumulative  $\text{NO}_2^-$  production over 90 min in the presence and absence of the NI. Inhibition of the enzyme should result in a lower  $\text{NO}_2^-$  production, compared to the non-inhibited cell culture. The concentration of the N source (provided as  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{NH}_2\text{OH}$ , respectively)

was 3 mM, and the inhibitors were supplied at two different concentrations (*i.e.*, 1 mol% and 10 mol% of applied N). Figure 2 shows the production of  $\text{NO}_2^-$  over 90 min under the different conditions. Control experiments, which were performed with cells in NaPB without N source and inhibitor, did not reveal a notable production of  $\text{NO}_2^-$ , confirming that  $\text{NO}_2^-$  resulted from oxidation of  $\text{NH}_4^+$  or  $\text{NH}_2\text{OH}$ , respectively. The detailed data are provided in Tables S1 and S2 in the Supporting Information (SI).



**Figure 2.** (a) Effect of DMP and DCD at different concentrations on the cumulative  $\text{NO}_2^-$  production after 30 min, 60 min and 90 min of inoculation with (a)  $[\text{NH}_4^+] = 3 \text{ mM}$  and (b)  $[\text{NH}_2\text{OH}] = 3 \text{ mM}$  as N source, respectively. The inoculations were performed in NaPB (pH = 7.5) at 30°C and 100 rpm in the dark. Standard errors were calculated from three biological replicates, each performed with three technical replicates.

In the incubations targeting AMO (Figure 2(a)) uninhibited cells generated a total  $[\text{NO}_2^-]$  of 17.7  $\mu\text{M}$  after 30 min, corresponding to an  $\text{NO}_2^-$  production rate of about 0.5  $\mu\text{M min}^{-1}$ . Over the next 60 min the activity of AMO increased to an average  $\text{NO}_2^-$  production rate of 1.3  $\mu\text{M min}^{-1}$ . Treatment with both DMP and DCD reduced the  $\text{NO}_2^-$  production when compared to the uninhibited cells, however, the inhibiting effect was generally more pronounced with DMP than with DCD at the same concentration, in agreement with literature data.<sup>47,48</sup> Thus, in the first 30

min, cells exposed to 0.03 and 0.3 mM of DMP produced  $[\text{NO}_2^-]$  of 4.9 and 2.9  $\mu\text{M}$ , respectively. Compared to the uninhibited cells, the %inhibition (calculated according to equation (1)) was 83% for 0.03 mM DMP and 93% for the ten-fold higher concentration. Over the following 60 mins the %inhibition with  $[\text{DMP}] = 0.03 \text{ mM}$  decreased to 74%, whereas with  $[\text{DMP}] = 0.3 \text{ mM}$  the %inhibition remained unchanged at 94%. In contrast, the inhibitory performance of DCD depended much stronger on its concentration. After 90 mins, the %inhibition with  $[\text{DCD}] = 0.03 \text{ mM}$  was only 11%, whereas with  $[\text{DCD}] = 0.3 \text{ mM}$  the %inhibition was 61%.

Providing  $\text{NH}_2\text{OH}$  to the cell culture enabled to study the HAO catalysed transformation, *i.e.*,  $\text{NH}_2\text{OH} \rightarrow \text{NO}_2^-$ , under exclusion of AMO. Figure 2(b) shows that uninhibited cells produced around the same amount of  $\text{NO}_2^-$  after 90 min as those treated with  $\text{NH}_4^+$ . Treatment of the cells with DMP at the lower concentration and with DCD at both concentrations had practically no impact on the  $\text{NO}_2^-$  production. Only when the cells were exposed to 0.3 mM of DMP, a noticeable reduction of  $[\text{NO}_2^-]$  occurred, which amounted to a %inhibition of about 27% after 90 min. However, we do not believe that the lower  $\text{NO}_2^-$  production at higher  $[\text{DMP}]$  indicates HOA inhibition but is likely an indirect effect of the strong AMO inhibition. As AMO accepts two electrons from HOA that are produced during the oxidation of  $\text{NH}_2\text{OH}$ , AMO inhibition could disrupt the electron transfer chain between these two enzymes, thereby reducing HOA activity. With regards to the 'fate' of both NIs in AMO, it is unlikely that they undergo conversion in the enzyme to produce a more active form, as no increase in %inhibition over time was observed. This finding suggests that both DMP and DCD are chelators (*i.e.*, non-mechanism based enzyme inhibitors) that could coordinate to the  $\text{Cu}^{2+}$  centers in AMO through their N atoms *via* a vacant electron pair, as has been found in crystal structures of the  $\text{DMP-Cu}^{2+}$  and the  $\text{DCD-Cu}^{2+}$  complexes.<sup>43,49</sup> However, it cannot be excluded that the inhibitory effect of DMP and DCD could, at least in part, be due to a potential toxicity for AMO. We will explore this factor below.

### *Determining the inhibition mechanism*

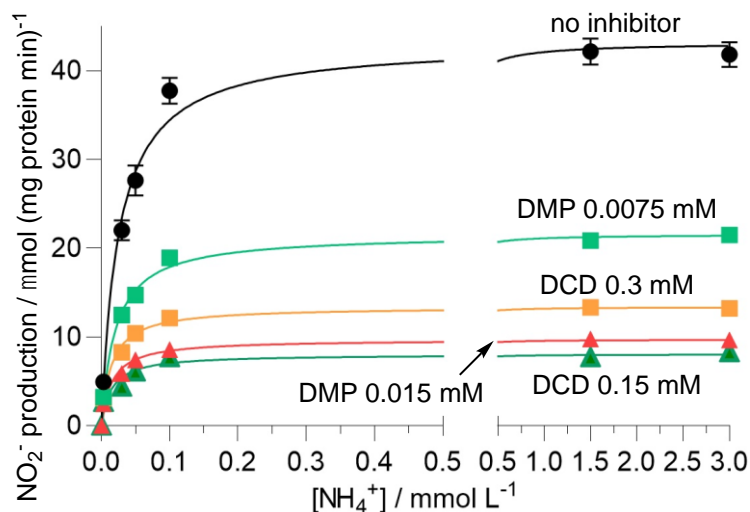
To gain mechanistic insight how DMP and DCD are inhibiting AMO, we studied the Michaelis-Menten kinetics by measuring the production of  $\text{NO}_2^-$  at different  $[\text{NH}_4^+]$  and constant  $[\text{NI}]$ . The Michaelis constant,  $K_m$ , is the substrate concentration,  $[\text{S}]$ , at which the reaction rate,  $v$ , is 50%

of the maximal rate  $V_{\max}$  and can be regarded as an inverse measure of the enzyme-substrate affinity (see equation 6).

$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad (\text{eqn. 6})$$

Thus, non-competitive inhibition is characterized by a decrease of  $V_{\max}$  that is independent of  $[S]$ , as substrate and inhibitor are not competing for the same site in the enzyme ( $K_m$  unchanged). In contrast to this, uncompetitive inhibition shows both a decreased  $K_m$  and  $V_{\max}$ , whereas competitive inhibition shows an increased  $K_m$  and an unchanged  $V_{\max}$ .<sup>50</sup>

[DMP] and [DCD] were chosen close to their  $IC_{50\text{abs}}$  values (DMP: 6.6  $\mu\text{M}$  and DCD: 0.1 mM)<sup>44</sup> to achieve partial inhibition (50 – 80%) of the  $\text{NO}_2^-$  production. Figure 3 shows that formation of  $\text{NO}_2^-$  followed Michaelis-Menten-type saturation kinetics for both inhibitors, clearly revealing that not only less  $\text{NO}_2^-$  was produced in the presence of the inhibitor but that increasing  $[\text{NH}_4^+]$  beyond 1.5  $\text{mmol L}^{-1}$  did not have any impact on  $[\text{NO}_2^-]$ .



**Figure 3.** Effect of DMP and DCD on the  $\text{NO}_2^-$  production in dependence of  $[\text{NH}_4^+]$  after 60 min. The inoculations were performed with  $[\text{NH}_4^+] = 0.003 \text{ mM}, 0.03 \text{ mM}, 0.05 \text{ mM}, 0.1 \text{ mM}, 1.5 \text{ mM}$  and  $3.0 \text{ mM}$  in NaPB ( $\text{pH} = 7.5$ ) at  $30^\circ\text{C}$  and  $100 \text{ rpm}$  in the dark. Note the different axis scale to include the data at higher  $[\text{NH}_4^+]$ . Standard errors were calculated from three biological replicates.

Apparent half-saturation constants for ( $K_{m(\text{app})}$ ) and maximum velocities ( $V_{\text{max}(\text{app})}$ ) in the presence/absence of DMP and DCD were calculated using the hyperbolic regression analysis (see section 3.4 in Materials and Methods). Note that the prefix 'app' (apparent) was used as these studies were performed with bacterial cells and not with the pure enzyme. The data are shown in Table 1.

**Table 1.** Michaelis Menten kinetics parameter of the  $[\text{NH}_4^+]$  dependent production of  $\text{NO}_2^-$  by *N. europaea* in the absence and presence of DMP or DCD, respectively, at different concentrations.<sup>a</sup>

Inhibitor	Concentration / mM	$V_{\text{max}(\text{app})} / \mu\text{mol (mg protein min)}^{-1}$	$K_{m(\text{app})} / \mu\text{M}$
none	--	$43.2 \pm 1.2$	$25.4 \pm 1.8$
DMP	0.0075	$21.6 \pm 0.2$	$20.5 \pm 0.2$
	0.015	$9.7 \pm 0.3$	$15.1 \pm 1.9$
DCD	0.15	$13.4 \pm 0.3$	$14.4 \pm 0.7$
	0.3	$8.0 \pm 0.3$	$14.4 \pm 4.7$

<sup>a</sup>[NI] were selected to achieve partial inhibition of the  $\text{NO}_2^-$  production and amended according to their efficacy (see text). Standard errors were calculated from three biological replicates, each performed with three technical replicates.

Compared to uninhibited cells, DMP and DCD reduce  $V_{\text{max}(\text{app})}$  by about 50 - 85%. Furthermore,  $K_{m(\text{app})}$  for both inhibitors is significantly lower ( $P < 0.05$ ) than for the uninhibited cells. These findings suggest that both DMP and DCD inhibit pure bacterial cultures of *N. europaea* through uncompetitive inhibition.

Although the crystal structure of AMO, particularly its active site, is unknown, simulations of bacterial AMO revealed that the enzyme contains multiple  $\text{Cu}^{2+}$  containing subunits.<sup>12,51</sup> DMP and DCD could principally bind to any of these sites, depending on accessibility. However, as

uncompetitive inhibitors bind to the enzyme-substrate complex to show an inhibitory effect, it is highly likely that the inhibitor binding site is near the substrate binding site.

#### *Exploring the reversibility of the inhibition*

Next, we explored whether the inhibition of AMO by DMP or DCD is reversible or irreversible. For these experiments, *N. europaea* cells were incubated for 30 min with 3 mM of  $\text{NH}_4^+$  and a high concentration of DMP or DCD, respectively ( $[\text{NI}] = 1.5 \text{ mM}$ ), to ensure considerable inhibition, and the  $\text{NO}_2^-$  production was measured ("inhibited cells"). The cells were then washed several times with NaPB with the intention to remove both unbound and enzyme-bound inhibitor. After the final washing cycle,  $\text{NH}_4^+$  was added to the cells, and the  $\text{NO}_2^-$  production was measured again after 30 min of incubation. The %activity was determined according to equation 3 (details are provided in section 3.3 in Materials and Methods). The data are shown in Table 2.

**Table 2.** Determination of the activity of inhibited AMO, using pure cell cultures of *N. europaea*, and after removal of the NI through repeated washing with NaPB.<sup>a</sup>

Inhibitor	%Activity	
	inhibited cells	after washing
DMP	4.2 ± 2.6	66.6 ± 3.3
DCD	32.6 ± 7.3	54.6 ± 13.0

<sup>a</sup>The inoculations were performed with  $[\text{NH}_4^+] = 3.0 \text{ mM}$  and  $[\text{DMP}]$  or  $[\text{DCD}] = 1.5 \text{ mM}$  in NaPB (pH = 7.5) at 30°C and 100 rpm in the dark. Standard errors were calculated from three biological replicates, each performed with three technical replicates.

In the first step of the experiment, cells exposed to DMP showed an activity of about 4% (nearly quantitative inhibition due to the high  $[\text{DMP}]$  used), whereas in the case of DCD the nitrification activity of the cells was reduced to about one-third, re-confirming that DMP is a more efficient inhibitor than DCD. After washing the cells and re-incubation with  $\text{NH}_4^+$ , the activity increased to

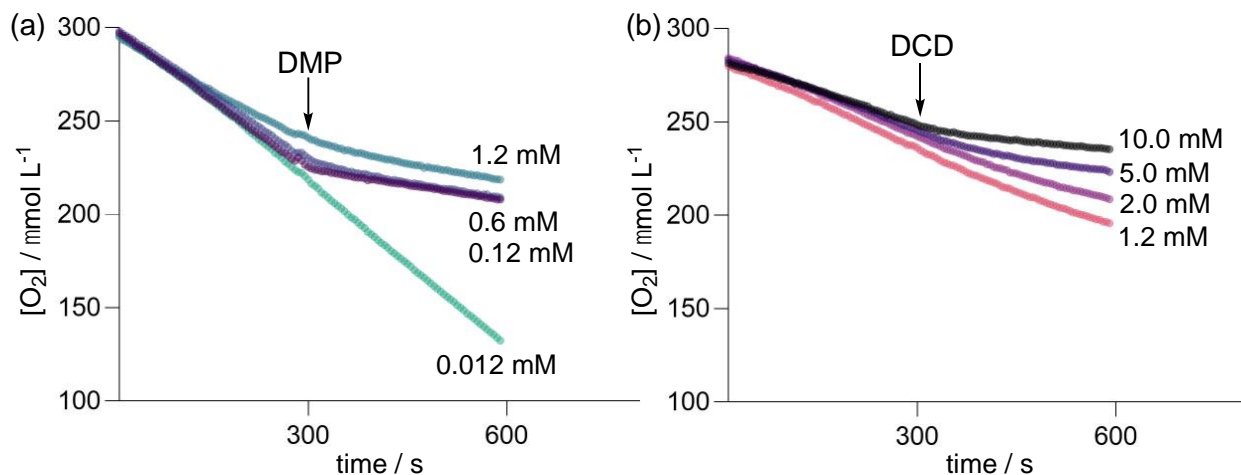
67% for the cells previously treated with DMP and to 55% for the DCD treated cells. The considerable recovery of enzyme activity after washing the cells, particularly in the case of DMP, suggests reversible inhibition, whereas irreversible inhibitors form covalent bonds with the enzyme, requiring *de novo* synthesis of AMO to regain activity. For example, nitrifying cells exposed to the irreversible inhibitors acetylene and 1-octyne recovered their activity only after four hours.<sup>52</sup> Binding studies performed with Nitapyrin showed that the release of the inhibitor required washing of the cells with buffer containing  $\text{Cu}^{2+}$  to 'force' de-complexation of the NI from AMO.<sup>38</sup> In the case of DMP and DCD, however, washing the cells with buffer solution alone was sufficient to remove the inhibitors from the enzyme, suggesting that their coordination to the relevant Cu center(s) in AMO is not very strong, which could provide a rationale for the variable performance of these NIs in the field. The finding that no full recovery of activity was observed is not surprising, given the high inhibitor concentration used in this experiment, which could lead to the partial loss of cells (see below).

#### *Determination of the rate of $\text{NH}_3$ oxidation by AMO*

The rate of the  $\text{NH}_3$  oxidation by AMO and whether NIs directly target this process can be determined through  $\text{O}_2$  respiration measurements. As  $\text{NH}_3$  oxidation requires equimolar  $\text{O}_2$ , the  $\text{O}_2$  consumption (or  $\text{O}_2$  uptake by the enzyme) is directly proportional to the  $\text{NH}_3$  oxidation rate.<sup>53</sup> We measured the  $\text{O}_2$  consumption by cell suspensions of *N. europaea* at 20°C using a Clark-type oxygen electrode by first monitoring the decrease of  $[\text{O}_2]$  in the presence of  $\text{NH}_4^+$  (3 mM) for 5 min. The inhibitor was then added at different concentrations, the system was allowed to equilibrate for 15 s, and the  $\text{O}_2$  decay was subsequently monitored for a further 5 min. In all measurements,  $\text{NH}_4^+$  and  $\text{O}_2$  were both present in excess so that the rate was only determined by the enzyme concentration (the total protein concentration was  $\sim 468 \mu\text{g L}^{-1}$ , determined via a BCA assay kit). Under these conditions, the  $\text{O}_2$  consumption should follow zero-order kinetics, and the rate coefficient,  $k$ , can be determined from the slope of the plot of  $[\text{O}_2]$  over time. In the presence of inhibitor,  $\text{NH}_3$  oxidation should slow down, resulting in a slower rate of  $\text{O}_2$  consumption. It should be noted that the inhibitor concentrations were chosen such that the  $\text{O}_2$

consumption was not completely stopped. Thus, to compensate for the lower efficacy of DCD, the latter required higher concentrations than DMP.

Figure 4(a) shows the  $O_2$  consumption of cells treated with DMP, and Figure 4(b) that for DCD treated cells.



**Figure 4.** Measurements of the  $O_2$  consumption by *N. europaea* in the absence and presence of (a) DMP and (b) DCD at different concentrations. The first 300 s is the rate in the absence of inhibitor. At the 300 s timepoint the inhibitor was added (indicated with an arrow). The cells were treated with  $[\text{NH}_4^+] = 3.0 \text{ mM}$  at  $\text{pH} = 7.5$  at  $20^\circ\text{C}$  under constant stirring in the dark. The plot shows the mean of three measurements for each concentration; standard errors are omitted for clarity.

In the initial stage of the experiment in the absence of inhibitor, the measured linear dependence of the  $O_2$  consumption over time confirmed zero-order kinetics, revealing a rate coefficient of  $186 \pm 63 \text{ nmol L}^{-1} \text{ s}^{-1}$  (see Table S3 and Figure S1). The rate of  $O_2$  consumption slowed down after addition of the inhibitors, as would be expected for a reduced enzyme activity, but remained zero order (see Figures S2 and S3). Table 3 compiles the rate coefficients for the  $O_2$  consumption in the absence and presence of the NIs. The data for each replicate measurement are compiled in Table S4.

**Table 3.** Rate coefficient,  $k$ , for the O<sub>2</sub> consumption by *N. europaea* in the absence and presence of DMP and DCD, respectively, and the %inhibition at different [NI].<sup>a</sup>

Inhibitor	Concentration / mM	$k$ / nmol L <sup>-1</sup> s <sup>-1</sup>	%Inhibition
none	--	186 ± 63	--
DMP	0.012	199 ± 117	no inhibition
	0.12	31 ± 13	75 ± 6
	0.6	64 ± 9	65 ± 5
	1.2	56 ± 1	70 ± 1
DCD	1.2	118 ± 20	28 ± 6
	2.0	112 ± 9	40 ± 7
	5.0	68 ± 9	63 ± 5
	10.0	39 ± 9	79 ± 5

<sup>a</sup>Cells were treated with [NH<sub>4</sub><sup>+</sup>] = 3.0 mM in NaPB (pH = 7.5) at 20°C under constant stirring in the dark. All errors are standard errors calculated from three measurements of biological replicates. %Inhibition determined according to: 100 - [rate (with inhibitor) / rate (without inhibitor) x 100].

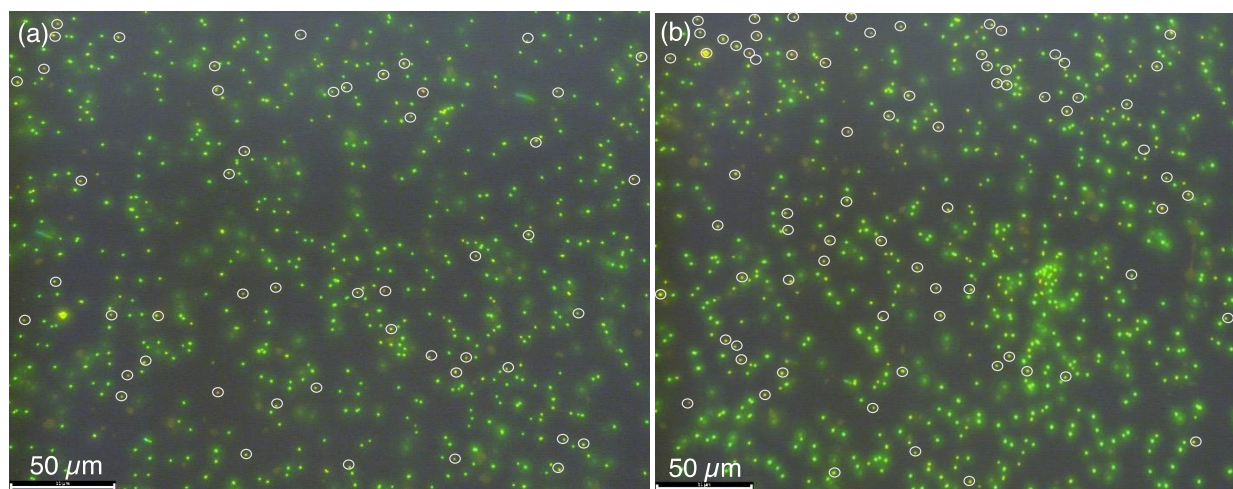
The zero-order behavior in the presence of DMP and DCD supports our findings from the previous sections that both NIs are chelating agents that reversibly bind to the enzyme. In contrast, mechanism-based inhibitors, such as acetylene and phenylacetylene, bind irreversibly to the enzyme, which reduces the enzyme's 'active' concentration over time and results in a first-order consumption of O<sub>2</sub> by the enzyme.<sup>54,55</sup>

Overall, cells treated with DCD consumed more O<sub>2</sub> than cells treated with DMP, although [DCD] was considerably higher than [DMP], clearly confirming that both inhibitors, although with different efficiency, directly impact on the ability of AMO to promote NH<sub>3</sub> oxidation. The %inhibition at the different [NI] could be obtained from the ratio of the rate coefficients in the presence and absence of the inhibitor, which are included in Table 3. Thus, the %inhibition by DMP was relatively constant in the concentration range 0.12 - 1.2 mM, averaging to 70 ± 4%. In contrast, the %inhibition by DCD was considerably more concentration dependent, dropping from 79% for 10 mM to just 28% for 1.2 mM.

### Determination of short-term toxic effects of DMP and DCD

A bacterial viability stain can provide insights of toxic effects of chemical compounds for *N. europaea*. While this method has been previously used to explore the toxicity of silver nanoparticles for *N. europaea*,<sup>56</sup> no such evaluation has been done for NIs.

To assess the acute toxicity of DMP and DCD for *N. europaea*, cells were incubated at 30°C in NaPB (pH = 7.5) with  $[\text{NH}_4^+] = 3 \text{ mM}$  for 12 hours with three different concentrations of DMP and DCD (10 ppm, 100 ppm and 1000 ppm). Figure 5 shows two exemplary microscopic images for *N. europaea* bacterial cells without inhibitor and with  $[\text{DMP}] = 10 \text{ ppm}$ . It should be noted that the incubations in the absence of inhibitor were also performed with  $[\text{NH}_4^+] = 3 \text{ mM}$  to avoid cell death caused by starvation. Standard errors were determined from seven microscopic images. The numbers of life/dead cells for each image are provided in Table S5.



**Figure 5.** Microscopic images of *N. europaea* in the (a) absence of DMP and (b) presence of 10 ppm DMP. The cells were visualized with the BaCLight™ viability stain. Channels were overlaid to show 'alive' cells (green) and 'dead' cells (red) in a single image. Note that the dead cells (which are in the minority) appear orange in these images, which have been circled for clarity.

Table 4 shows the percentage of live and dead cells (calculated using eqn. 5 and 6) at the different concentrations of the NI.

**Table 4.** Acute toxicity of DMP and DCD at different concentrations for cells of *N. europaea* determined with a bacterial viability stain and determination of the  $\text{NO}_2^-$  production.<sup>a</sup>

Inhibitor	Concentration / ppm	% Live	% Dead	$[\text{NO}_2^-]$ / $\mu\text{M}$
none	--	96 ± 3	5 ± 3	454
DMP	10	87 ± 9	7 ± 7	338
	100*	70 ± 8	29 ± 5	112
	1000*	67 ± 5	34 ± 5	46
DCD	10	89 ± 4	10 ± 4	430
	100*	77 ± 15	22 ± 15	444
	1000*	74 ± 11	25 ± 11	445

<sup>a</sup>The incubations were performed in NaPB at pH = 7.5 and 30°C with  $[\text{NH}_4^+] = 3 \text{ mM}$ ,  $[\text{inhibitor}] = 10 \text{ ppm}$  (0.015 mM), 100 ppm (0.15 mM) and 1000 ppm (1.5 mM) at 100 rpm for 12 h in the dark. Standard errors were determined from seven microscopic images; \* indicates significant difference in comparison to uninhibited cells ( $P < 0.05$ ).

At the lowest concentration (10 ppm), cell death caused by both NIs was non-significant compared to the control treatment. However, both inhibitors decreased the %live cells when their concentration was increased to 100 ppm, *i.e.*, 70% for DMP and 77% for DCD, respectively. With  $[\text{inhibitor}] = 1000 \text{ ppm}$ , 74% of cells treated with DCD remained alive, whereas the toxicity of DMP was higher, with just 65% of the cells still alive. Parallel to the viability stain, the  $\text{NO}_2^-$  production was also measured, which showed that the higher cell death rate with increasing [DMP] correlated with a reduction of  $[\text{NO}_2^-]$ . In contrast, in the case of DCD the  $\text{NO}_2^-$  production remained largely unchanged at all three concentrations (even in comparison with the control treatment), which further confirms the much lower inhibitory activity of this inhibitor.

However, when assessing inhibitor efficiency and toxicity, it needs to be considered that an at least ten times higher application rate of DCD would be required to achieve a nitrification inhibition comparable with DMP (see Figure 1). For example, while with [DMP] = 10 ppm no significant cell death occurs, the cell death is much higher for [DCD] = 100 ppm. Therefore, increasing the application rate of DCD is not a useful approach to increase nitrification inhibition, as it would lead to increased cell death.

In conclusion, using pure cell cultures of *N. europaea* we have shown that DMP and DCD target the first step of nitrification by inhibiting AMO. Although at higher concentrations of DMP the  $\text{NO}_2^-$  production through  $\text{NH}_2\text{OH}$  oxidation mediated by HOA decreased too, we suggest that this reduction is an indirect effect of the strong AMO inhibition. Both inhibitors can be removed from the enzyme by washing with buffer, indicating that they act as chelating agents, which reversibly bind to the enzyme. These findings were confirmed by measuring the kinetics of the  $\text{O}_2$  consumption by *N. europaea* in the presence of DMP and DCD, respectively. DMP reduced the  $\text{O}_2$  uptake rate considerably more at much lower concentrations than DCD, in line with the lower inhibitory efficiency of the latter. However, the binding between enzyme and NIs is not very strong, which may, at least to some extent, explain the highly variable performance of both NIs in different soils,<sup>27-33</sup> and could provide a potential pathway to develop more efficient and consistent NIs in the future. Furthermore, Michalis-Menten kinetics revealed that both DMP and DCD act as uncompetitive inhibitors, whereas studies of the acute toxicity suggest that increasing the application rate of the poorer performing DCD to increase inhibition of AMO cannot be recommended as it is associated with increased cell death. On a final note, it has been shown that temperature is also a crucial factor affecting inhibitory efficiency, as more rapid degradation of the nitrification inhibitors could occur in soils at higher temperatures.<sup>21,57,58</sup> In future work, we will therefore apply the bacterial assay used here to explore in detail the role of temperature on the inhibitory activity through kinetic studies.

## Abbreviations Used

AMO – ammonia monooxygenase

ANOVA – Analysis Of Variance

AOA – ammonia oxidizing archaea

AOB – ammonia oxidizing bacteria

ATCC – American Type Culture Collection

BCA – bicinchoninic acid

DCD – dicyandiamide

DMP – 3,4-dimethyl-1*H*-pyrazole

DMPP – 3,4-dimethyl-1*H*-pyrazole phosphate

HAO – hydroxylamine oxidoreductase

FAO – Food and Agriculture Organization of the United Nations

GHG – greenhouse gas

IC<sub>50abs</sub> – concentration of inhibitor to decrease response to 50%

K<sub>m</sub> – Michaelis-Menten constant

MSM – mineral salts media

NaPB – sodium phosphate buffer

NI – nitrification inhibitor

*N. europaea* – *Nitrosomonas europaea*

NUE – nitrogen use efficiency

OD – optical density

OD<sub>600</sub> – optical density at 600 nm

PM<sub>2.5</sub> – particulate (diameter ≤ 2.5 μm)

ppm – parts per million

rpm – rotations per minute

SI – supporting information

UV/vis – ultraviolet visible region

$V_{\max}$  – maximal rate

### **Supporting Information**

Supplementary Table S1 (Cumulative  $\text{NO}_2^-$  production by *N. europaea* with ammonium ( $\text{NH}_4^+$ ) in the absence and presence of DMP and DCD over three consecutive 30-min intervals); Table S2 (Cumulative  $\text{NO}_2^-$  production by *N. europaea* with hydroxylamine ( $\text{NH}_2\text{OH}$ ) in the absence and presence of DMP and DCD over three consecutive 30-min intervals); Table S3 (Rate coefficients,  $k$ , for the time-dependent  $\text{O}_2$  consumption by *N. europaea* in the absence of NI); Table S4 (Rate coefficients,  $k$ , for the time-dependent  $\text{O}_2$  consumption by *N. europaea* in the presence of DMP and DCD at different concentrations); Table S5 (Number of alive and dead cells of *N. europaea* in the absence and presence of DMP and DCD at different concentrations determined with a bacterial viability stain), Supplementary Figure S1 ( $\text{O}_2$  consumption by *N. europaea* as a function of time in the absence of NI); Figure S2 ( $\text{O}_2$  consumption by *N. europaea* as a function of time after the addition of DMP at different concentrations); Figure S3 ( $\text{O}_2$  consumption by *N. europaea* as a function of time after the addition of DCD at different concentrations).

### **Author Contributions**

S. C. Y performed the experiments, processed the experimental data and performed the analysis. R. W., U. R. and U. W. designed, planned and supervised the work. All authors aided in interpreting the results. S. C. Y. and U. W. wrote the manuscript and designed the figures with input of all authors.

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