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Biology and Fertility of Soils

Effects of urease and nitrification inhibitors on soil N, nitrifier abundance and activity in a sandy loam soil --Manuscript Draft--

Manuscript Number:	BFSO-D-19-00166R2	
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Abstract:	<p>Inhibitors of urease and ammonia monooxygenase can limit the rate of conversion of urea to ammonia and ammonia to nitrate, respectively; potentially improving N fertilizer use efficiency and reducing gaseous losses. Winter wheat grown on a sandy soil in the UK was treated with urea fertilizer with the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT), the nitrification inhibitor dicyandiamide (DCD) or a combination of both. The effects on soil microbial community diversity, the abundance of genes involved in nitrification, and crop yields and net N recovery were compared. The only significant effect on N-cycle genes was a transient reduction in bacterial ammonia monooxygenase abundance following DCD application. However, overall crop yields and net N recovery were significantly lower in the urea treatments compared to an equivalent application of ammonium nitrate fertilizer, and significantly less for urea with DCD than the other urea treatments.</p>	
Suggested Reviewers:	Davy Jones d.jones@bangor.ac.uk Soil and root biology expert	

Tim Daniell
tim.daniell@hutton.ac.uk
soil and plant molecular biologist

Dear Paolo,

We have endeavoured to answer all queries and to make changes and improvements as recommended by the reviewers. Where appropriate, we specify the line numbers in the manuscript where changes have been made. We hope that this now meets your approval.

With thanks for the constructive criticism from the reviewers,

Penny

Ref.: Ms. No. BFSO-D-19-00166

Effects of urease and nitrification inhibitors on soil N, nitrifier abundance and activity in a sandy loam soil
Biology and Fertility of Soils

Dear Penny,

Your manuscript titled "Effects of urease and nitrification inhibitors on soil N, nitrifier abundance and activity in a sandy loam soil" has been reviewed by one competent referee and by myself because the other contacted reviewers have not sent their comments despite my several soliciting messages. The manuscript is accepted for publication after revision according to the enclosed comments below.

After reading the manuscript myself I think that this is an interesting well-written contribution. However, the discussion of some data is inadequate. Therefore, I suggest the revision of the manuscript according to the following general comments.

1) Page 2 please delete the first 6 lines of the Abstract and begin with "Inhibitors" because the Abstract should only contain the main findings of the study and not general sentences

Done – section now deleted.

2) please report the innovative aspects of the research at the end of the Introduction;

Done (lines 60-66)

3) The policy of the journal is guided by editorial and opinion/position papers so as to avoid conceptual and technical problems. In the case of extraction and characterization of DNA from soil it is important to carry out negative controls because often kits and solutions are DNA-polluted, as reported by Vestergaard et al (2017) *Biol Fertil Soils* 53:479-484 and Scholer et al (2017) *Biol Fertil Soils* 53:485-489, It is also important evaluate if your analysis covered the microbial diversity of each sample

*We are very careful about our quality control for molecular approaches to soil metagenomics including qPCR and next generation sequencing and have worked on this for many years. We have checked taqs (from 12 manufacturers), soil extraction kits (from 4) and reagents for contamination and inhibitory effects and select only those that have no contamination or inhibition or at least the lowest possible when used without added template. All qPCR protocols include multiple positive and negative controls and a negative kit control either when a new batch is used or a blank well in 96 well formats. For this experiment all amplicon sequencing samples were at 10ng – 20 ng μl^{-1} per reaction, so from 2 - 4 times the amount suggested as requiring a negative DNA extraction control according to Vestergaard et al (2017) *Biol Fertil Soils* 53:479-484 and Scholer et al (2017) *Biol Fertil Soils* 53:485-489. Our previous experience has shown that negative kit controls give very different results from true samples and usually amplify only a few products that they are obvious outliers which we did not find in these results. We have added a few lines to the methods section to explain this and*

we now cite Vestergaard et al. (line 135) and our work, Delmont et al, 2011 Appl Env Microbiol (line 112) which found that bead-beating methods gave the highest diversity of soil microbes.

4) Page 13 last line, I suggest writing "extracellular and intracellular enzymes". Indeed, ureolytic microorganisms can hydrolyse urea and release ammonium very quickly. Finally the current enzyme assays do not distinguish intracellular from extracellular activity in soil (Nannipieri et al 2018 Biol Fertil Soils 54:11.18, opinion/position papers on the wrong concepts in soil enzymology).

Done, thanks for the insight. We refer to Nannipieri et al. (2018) in the Introduction lines 58-59 and have added "intracellular" on line 330 in the Conclusions.

These are my specific comments:

Please number the lines of the revised text

Done

There are three papers published in Biol Fertil Soils which the authors may read so as to improve the discussion of data. Please consider that this is a minor comment and thus I leave the relative decision to the authors. These are the papers: Stempfhuber et al (2017) 53:691-700 because it discusses the role of soil pH in affecting the co-occurrence of AOB and nitrifiers; Niu et al (2018) 54:645-658 and Zhang et al (2018) 54:697-706 because they deal with effects of nitrification inhibitors on N₂O evolution from soil

Thank you for the suggestions. We think that the Stempfhuber paper is not especially relevant as in concerns forest soils and the Niu paper is about the effects of biochar and, as with Stempfhuber, we did not think it added anything to understanding our results. However, the Zhang paper has some interesting observations on the abundance of AOB and nitrification inhibitors and we now cite it in the discussion (line 300).

Please delete commas in some of the citations; for example at page 3 ref "Amberger"; please check carefully throughout

Done

Please replace "&" with "and" in the citations; please check carefully throughout

Done

Please add "exchangeable" before "NH₄⁺" because you have determined it and not "fixed ammonium"; see page 8 last line; page 9 L. 5 and 7; page 12, 7 lines from the bottom of the page; page 13, L 10

Done – now "exchangeable NH₄⁺" on lines 176, 178, 183, 185, 289, 312 and in Table 2 and the legend for Fig. 1..

Page 3 Please replace "nitrogen" with "N" at L. 5 and 11

Done – now lines 20, 26

5 lines from the bottom of the page, "N₂O and this process occurs in acid"

Done – now line 38

Page 5 Please add the reference of the method used to determine nitrite

This was also done on the Skalar but with less dilute extracts to detect the lower nitrite concentrations. Now clarified on line 96-7

Page 8 Please do not indent the line after the subheading "Soil pH..."

Done – line 166

4 lines from the bottom of the page, "mineral N and"

Done – line 175

Page 12 About the degradation of urease inhibitors in soil, please report some references. I have read some papers on this topic.

We have now read a paper from Engel et al. (2015) that reports degradation rates in acid soils which we discuss on line 261.

About the fate of urea under field conditions we (Nannipieri et al 1990 Soil Biol Biochem 22:549-553) have studied the short-term fate of labelled urea under field conditions; ureolysis occurred in 2 days;

Thank you – this relevant reference is now cited on lines 262 and 285.

Page 14 Please delete the paragraph in the Conclusion section

I have deleted the second paragraph so that it runs on from the first in the Conclusions section. I hope that this is what you intended, if not, please delete the offending text.

Please complete the list of authors: 1st ref page 15, 7th ref page 16, 5th ref page 17, 1st ref page 18; Page 16 ref Hink et al, "Environ Microbiol"; Table 1, please include the publication year between brackets

All done, plus extra references added.

Along with your revised manuscript, you will need to supply a separate file "author's response to the referees' comments" in which you list all the changes you have made to the manuscript and in which you detail your responses to all the comments passed by the referee(s). Should you disagree with any comment(s), please explain why. Please be sure to return the annotated copies of your manuscript.

Your revision is due by 10 October 2019.

To submit a revision, go to

<https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.editorialmanager.com%2Fbfso%2F&data=01%7C01%7Cpenny.hirsch%40rothamsted.ac.uk%7C275bd932b14646278c3e08d735d66d23%7Cb688362589414342b0e37b8cc8392f64%7C1&sdata=l%2BxlkNZQwzEVNGknvtpbhzeNK4jS8WCbilDBxAsoazw%3D&reserved=0> and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Please make sure to submit your editable source files (i. e. Word, TeX)

Yours sincerely

Paolo Nannipieri
Editor-in-Chief
Biology and Fertility of Soils

Reviewers' comments:

Reviewer #1: This is an interesting paper on the implication of nitrification and urease inhibitors on the overall soil microbial biodiversity and groups of organisms involved in nitrification, and yield. The paper is written very well and of interest to the wider scientific community.

Here are some main comments on the paper, followed by minor points.

The overall experimental design is good. The authors may like to explain their choice of sampling dates. The rainfall on day 0 implies wet soils and therefore reasonably fast hydrolysis of urea. As microbial processes respond to N application very fast, peak abundance and activity may have occurred before the first sampling date (day 2)?

This was a field experiment on a farm run on commercial lines, and we had no ability to control the weather on the day it was set up, and we could not get our sampling team onto the field until the second day. We discuss the problem on lines 278-281. This is the reality of conducting experiments on "real" farms. We do discuss the possibility that substantial ureolysis had occurred by day 2.

However, I am not an expert in molecular biology, and therefore cannot comment on the appropriateness of the molecular techniques used.

We have used techniques that are widely accepted and discuss them in context of the Journal's guidelines.

The Supplementary Figures 2 and 3 are important to this paper. Is it possible to move these figures into the main text?

We have moved supplementary Fig. 2 (now Fig. 1) and part of supplementary Fig. 3, grain and straw yields, become Fig. 2. The N offtake and net N recovery remain as supplementary Fig. 2, to avoid information overload in the main text.

The result and discussion sections are sound.

In the conclusion section you point out that the inhibitors did not increase the yield. But it looks like there was no significant yield loss either. Using inhibitors without yield increase is not economically viable to the farmer. However, even small reductions in NH₃ and N₂O losses can have a positive impact on air quality and reduced N₂O emissions. You may like to consider to add this to your conclusion/ discussion section?

Good point, now discussed in lines 298, -303.

Is great to see that your final sentence includes the important phrase 'with a caveat that our findings may not apply to other soils...'

Thanks – important that we looked only at our site

Minor comments:

Abstracts: Please include the full name of the inhibitors NBPT and DCD Page 4: What is the size of the treatment plots?

Page 4: Supplement Figure 2, shows large variability in pH after application of urea with/without NI and urease inhibitors. Was the pH prior treatment addition the same on all treatment plots?

Prior to establishing the different treatments, all plots were at similar pH 6.1. The pH had changed dramatically 2 days after treatments were applied. We have made this clearer in the text (line 170).

Page 4: Last sentence on p4: You write that soil temperature was measured at the surface and 20 cm depth, but the legend to supplement Figure 1 shows a temperature depth measurement at 10 cm.

Thank you for pointing the error: the temperature was measured at 10 cm depth and this has been corrected.

Page 5: 'For practical reasons only 4 out of 6 treatments ...were sampled for microbial analysis....' Please state that you used 2 additional plots for yield analysis. Say why you have chosen a larger replication rate for the yield.

*Now that the yield data is reported in **Fig. 2**, we reanalysed just the 4 replicates sampled for DNA and soil chemistry and present that data (n = 4). However, in the supplementary data we include all 6 reps and the ammonium nitrate treatment for comparison as the net N recovery is relevant and is more representative with the higher number of replicates **supplementary Fig. 2**).*

Page 6: Please provide a reason why you replaced the flatbed vortex with a bead beading step
*Like many labs, we have always used a Fastprep machine for soil extractions and the reason for the manufacturer's protocol specifying a flatbed vortex is due to the need to avoid infringing the patent of an earlier competitor's product. We consider the Fastprep more efficient for larger DNA extractions but it is difficult to engage in a long explanation in print: we think other molecular labs understand this and we have not previously had problems using this form of words to describe our protocol but we have made a small alteration to the text that we hope is acceptable (**line 106**).*

Page 6: 'DNase max kit': is the 'a' supposed to be a small rather than capital letter?

*Thank you for pointing out this typo, now corrected to DNase throughout (**lines 107-110**).*

Page 6: 'NMDS' and 'OUT': include the full names here instead of page 8

*Done (**lines 114, 115**)*

Page 11/12: 'insufficiently acid for spontaneous decomposition to hydroxylamine' add a reference (Heil et al?)

*Heil et al. added (**line 260**)*

Page 12: 2nd paragraph: What does PPDA stand for?

*PPDA now defined as phenylphosphoryldiamidate (**line 269**)*

Page 12: Urea hydrolysis happens very fast, does this mean the first measurement should have been carried out earlier? Perhaps the authors could make some recommendations here.

*This is now suggested on **lines 278-281**, along with an observation of the practical restrictions on sampling (i.e. we had to wait until day 2 for access to the field).*

Page 12-13: 'The rate of nitrification(Booth et al..) This sentence is not very clear. Do the values reported refer to the nitrification rates or urea hydrolysis rates, or both?

*Now clarified as "nitrification rate" on **line 295**.*

Page 13: Why do you use a subscript for NBPT but not for DCD? For completeness include the soil information (sandy loam) here.

*I can't see a subscript NBPT – it could be a text glitch on your system or else I don't understand the comment. "Sandy loam" added on **line 328**.*

Supplement Fig 2: Provide more detail: T1, T2, T3 stands for?

*This is now **Fig1** in the main text, following your recommendation and the horizontal axes are now adjusted to specify day 2, 8, 15 as in Fig 3 and 4.*

Add mineral N = NH₄ + NO₃.

*Done in **Fig 1 legend** but too long to fit on the axis.*

Supplement Figure 3: please indicate what the different letters stand for and the level of significance.

*Done – now **Suppl. Fig 2 legend**.*

Supplementary Table 1 should be below Supplementary Figure 6

Done – re-ordered

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<https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.editorialmanager.com%2Fbfso%2Flogin.asp%3Fa%3Dr&data=01%7C01%7Cpenny.hirsch%40rothamsted.ac.uk%7C275bd932b14646278c3e08d735d66d23%7Cb688362589414342b0e37b8cc8392f64%7C1&data=7RTXqnKZP6WJdEn9%2Bn%2FDSQIFxP%2Btm%2BXHuZHlk6bKWk%3D&reserved=0>). Please contact the publication office if you have any questions.

[Click here to view linked References](#)

Effects of urease and nitrification inhibitors on soil N, nitrifier abundance and activity in a sandy loam soil

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1 **Abstract**

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4 2 Inhibitors of urease and ammonia monooxygenase can limit the rate of conversion of urea to
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6 3 ammonia and ammonia to nitrate, respectively; potentially improving N fertilizer use efficiency and
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8 4 reducing gaseous losses. Winter wheat grown on a sandy soil in the UK was treated with urea fertilizer
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10 5 with the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT), the nitrification inhibitor
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12 6 dicyandiamide (DCD) or a combination of both. The effects on soil microbial community diversity, the
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14 7 abundance of genes involved in nitrification, and crop yields and net N recovery were compared. The
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16 8 only significant effect on N-cycle genes was a transient reduction in bacterial ammonia
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18 9 monooxygenase abundance following DCD application. However, overall crop yields and net N
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20 10 recovery were significantly lower in the urea treatments compared to an equivalent application of
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22 11 ammonium nitrate fertilizer, and significantly less for urea with DCD than the other urea treatments.
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31 13 **Key Words** urea fertilizer, urease inhibitor, nitrification inhibitor, arable soil, soil microbial
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33 14 diversity, nitrification genes
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16 Introduction

17 Nitrogen fertilizer is required for arable crop production but nitrous oxide (N₂O) losses due to both
18 microbial activity and abiotic processes are a major environmental concern and a challenge for
19 sustainable agriculture. Fertilizers that do not contain nitrate (the substrate for denitrification) are
20 rapidly converted to nitrate in soil. Urea, globally the most commonly used N fertilizer, is subject to
21 hydrolysis by the action of microbial urease to generate ammonia which can be lost by volatilization or
22 oxidized to nitrate by microbial nitrifiers. Various chemical compounds have been assessed for their
23 effectiveness in reducing ammonia emissions from urea fertilizer through their inhibition of the urea
24 hydrolysis process (e.g. Silva et al. 2017), in reducing N₂O emissions from urea and ammonia-based
25 fertilizers through their inhibition of the nitrification process (e.g. Akiyama et al. 2010; Gilsanz et al.
26 2016), and the consequent impacts on crop yield and N use efficiency (e.g. Abalos et al. 2014; Rose et
27 al. 2018). The urease inhibitor (UI), N-(n-butyl) thiophosphoric triamide (NBPT) occupies the active
28 sites in urease and is the basis of commercial products that are applied together with urea fertilizers
29 (Sigurdarson et al. 2018). NBPT is reported to delay the hydrolysis of urea fertilizer by 7 to 10 days
30 (Zaman et al. 2008), resulting in a smaller pH increase around the urea granule than for urea alone,
31 and hence lower ammonia volatilization losses. Dicyandiamide (DCD) is a nitrification inhibitor (NI)
32 that slows oxidation of ammonia-N to nitrate⁻-N by deactivating the bacterial ammonia
33 monooxygenase, AMO (Amberger 2008). AMO-containing ammonia oxidizing bacteria (AOB) and
34 archaea (AOA) convert ammonia to hydroxylamine, which is further oxidised to nitrite (Prosser and
35 Nicol 2012). Although denitrifying bacteria are thought to be the main source of N₂O in arable soil,
36 losses are also directly attributed to both AOA and AOB, which generate N₂O by “nitrifier
37 denitrification” (Wrage-Mönnig et al. 2018). Also, hydroxylamine can decompose spontaneously to
38 generate N₂O and this process occurs in acid soils <pH 5.0 (Heil et al. 2016). The final step in
39 nitrification is the conversion of nitrite to nitrate by nitrite oxidizing bacteria (NOB) containing nitrite
40 oxidoreductase (NXR), which includes the genera *Nitrobacter* and *Nitrospira*. The *Nitrospira* include a
41 recently discovered group of “comammox” bacteria that contain AMO and can undertake complete

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nitrification, converting ammonia to nitrate (Daims et al. 2016). Two clades have been identified but only the AMO gene of comammox clade B was detected in soil (Pjevac et al. 2017).

The objective of this study was to evaluate the effects of NBPT and DCD used singly or in combination on soil mineral N dynamics and the functional genes involved in urea hydrolysis (*ureC*) and nitrification (*amoA*, *nxrA*). Genes for urease are relatively common in soil, produced by 17 – 30% of soil microorganisms (Lloyd and Scheaffe 1973). Reportedly, up to 50% of soil urease is extracellular (Klose and Tabataba 1999; Qin et al. 2010) thus readily accessible to inhibitors. In contrast, AMO is membrane bound in both bacteria and archaea (Prosser and Nicol 2012) and the AOA and AOB together were found to comprise fewer than 1% of prokaryotes in an arable soil (Hirsch et al. 2017). The abundance of NOB and comammox bacteria in soil is uncertain but ammonia oxidation is usually considered to be the rate-limiting step in nitrification (Kowalchuk and Stephen 2001). In this study, we test the hypothesis that a combination of a UI and NI together with urea fertilizer applied to an arable crop is more efficient at delaying nitrification than either inhibitor alone. We measured soil N during the experiment, crop yields at the end of the season, and we monitored the responses of different soil microbial groups to the changes in soil mineral N, using qPCR with 16S rRNA gene diagnostic primers for Bacteria and Archaea, ITS sequence primers for Fungi as well for functional nitrification genes. Measuring gene abundance and activity in conjunction with N-cycling *in situ* in the field should advance understanding of enzyme-mediated soil processes (Nannipieri et al. 2018).

Although there have been many studies on the combined effects of urease and nitrification inhibitors in the field, very few have attempted to relate this the abundance and activity of the relevant microbial genes. This is the first report of how a combination of the commercially important inhibitors DCD and NBPT together influence gene abundance and expression in the soil nitrifier community. This includes bacterial and archaeal ammonia oxidizers and nitrite-oxidizing bacteria in an arable soil after application of urea or ammonium nitrate fertilizer with different combinations of DCD and NBPT.

67

68 **Material and Methods**

69 **Experimental site**

70 The experiment was conducted in 2017 with winter wheat at Horsepool field, Woburn in Bedfordshire
71 UK, on a sandy loam soil classified as Cambic Arenosol (FAO 1990), pH 6.2, total N 1.84 g kg⁻¹, total C
72 18.9 g kg⁻¹, average annual rainfall 640 mm and soil temperature 10.4 °C (Johnston et al. 2017).
73 Rainfall and soil temperature (surface and 10 cm depth) are monitored daily at Woburn. The field had
74 previously been in an arable rotation; subsoiled after harvest in September 2013; with crops of spring
75 barley in 2014 and 2015. Winter wheat var. Siskin was drilled in 2016.

76 **Experimental design**

77 The field experiment consisted of six treatments: nil (zero N control); ammonium nitrate fertilizer
78 (AN); urea fertilizer (urea); urea with 6500 mg kg⁻¹ DCD incorporated (urea + NI); urea with 660 mg kg⁻¹
79 NBPT coating (urea + UI); urea with DCD incorporated and NBPT coating (urea + NI + UI). There were
80 six replicates of each treatment in a completely randomized design. The total fertiliser application
81 rate to all plots apart from the zero-N control was 200 kg N ha⁻¹, considered the optimum rate for this
82 site and wheat variety. This was applied as a split dose: 50 kg N ha⁻¹ on March 6th, 100 kg N ha⁻¹ on
83 April 4th and 50 kg N ha⁻¹ on May 3rd. The management of different treatments was identical apart
84 from the different fertilizer / inhibitor combinations. Soil monitoring commenced on April 6th, 2 days
85 after the highest dose was applied and > 4 weeks after the initial lower dose. For practical reasons, to
86 keep numbers manageable, only four of the six replicate plots were sampled for mineral and
87 microbiological analysis, and the AN treatment was not included although total N offtake and recovery
88 in wheat grain and straw was calculated for all replicates and treatments.

89 Soil cores (5 cm diameter and 0 - 20 cm depth) were collected on April 6th, 12th and 19th (2, 8
90 and 15 days after urea application). Cores were processed straight away in the field and flash-frozen

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91 in liquid N within 3 min of collection. Processing of field samples included removal of stones, plant
92 roots, fauna and debris, followed by sieving <2 mm then placing in liquid N. Samples were stored at -
93 80 °C for subsequent molecular analysis. Subsamples were extracted in 2M KCl (5 mL g⁻¹ dw soil) by
94 vigorous shaking (120 rpm) for 2 h then left to stand for 45 min before filtering through Whatman no 1
95 paper. Nitrate (NO₃⁻) and ammonium (NH₄⁺) in the filtrate were analysed simultaneously using a Skalar
96 SAN^{PLUS} System continuous flow analyser; nitrite (NO₂⁻) was measured in a separate Skalar run using
97 less dilute soil extracts.

98 **Grain and straw yields**

99 Plots were harvested on August 16th using a small plot harvester. Harvest weights of grain and straw
100 per plot were recorded and subsamples of each taken for analyses of dry matter (DM) content by
101 drying at 100 °C to constant weight and total N content using a Dumas combustion analyser (LECO).

102 **Nucleic acid extraction and 16S rRNA amplicon sequencing**

103 DNA and RNA were co-extracted from the same 2 g frozen soil sample using the RNA PowerSoil®
104 isolation kit and RNA PowerSoil® DNA Elution Accessory Kit (MO BIO Laboratories, Inc) following a
105 modification to the manufacturer's instructions, whereby the 15-min shaking on a flatbed vortex was
106 replaced by an alternative strategy, a 2 x 30-s bead beating step (5.5 m s⁻¹, Fastprep). RNA samples
107 were DNase treated to remove DNA contamination using the DNase Max Kit (Qiagen, Manchester,
108 UK), following the manufacturer's protocol. Direct PCRs were carried out on DNase treated RNA to
109 confirm all contaminating DNA had been removed. The quantity and quality of extracted DNA and
110 DNase-treated RNA were analysed by fluorometer Qubit® 2.0 dsDNA and RNA BR Assay Kits and
111 Nanodrop microvolume spectrophotometer (Thermo Fisher Scientific). Previously, we found bead-
112 beating methods to reveal the greatest diversity in soil metagenomic DNA (Delmont et al. 2011.).

113 Soil bacterial diversity was assessed at the first sampling point by next generation sequencing
114 of the V4-V5 region of 16S rRNA genes, assigning to operational taxonomic units (OTU) and performing

115 non-metric multidimensional scaling (NMDS) as described previously (Hirsch et al. 2017) with the
116 following modifications. The 16SrRNA gene high throughput amplicon sequencing was performed at
117 Novogene (HK) Co. Ltd (Hong Kong, China) using an Illumina HiSeq platform with a paired-end read
118 length of 250bp and primers 515F (GTGYCAGCMGCCGCGGTAA, Parada et al. 2016) and 926R
119 (CCGYCAATTYMTTTRAGTTT, Quince et al. 2011; Parada et al. 2016) as used by the Earth Microbiome
120 project (<http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>). Sequence data
121 were analysed using QIIME 2 version 2018.11.0. Raw reads were quality checked, trimmed (removing
122 primers, adapters, and the last 10 bp), merged using VSEARCH, quality filtered, denoised, dereplicated
123 and assigned to amplicon sequence variants by Deblur.

124 **Quantitative real-time PCR and reverse transcription PCR (RT-qPCR)**

125 Gene abundance and expression (bacterial and archaeal 16S rRNA genes, fungal ITS, bacterial *ureC*,
126 bacterial and archaeal *amoA*, *nxrA* from *Nitrobacter* and *Nitrospira* and *amoA* from comammox clade
127 B) was estimated using quantitative real-time PCR (qPCR) and reverse transcriptase qPCR (RT-qPCR),
128 respectively. Primer details are given in Table 1.

129 Amplifications were performed in 10 μ l volumes containing 5 μ l of QuantiFast SYBR Green PCR
130 Master Mix for DNA and QuantiFast SYBR Green RT-PCR Master Mix for RNA (Qiagen, Manchester,
131 UK), 0.1 μ l of each primer (1 μ M), 0.1 μ l of QuantiFast RT Mix for RT-qPCR, 2 μ l of template DNA at 5
132 ng μ l⁻¹ or 2-4 μ l of RNA at 10 ng μ l⁻¹ and nuclease-free water (Severn Biotech, Kidderminster, UK) up to
133 10 μ l, using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK). The
134 amount of soil-extracted DNA or RNA added to each PCR reaction, at least 10 ng DNA or 20 ng RNA, is
135 well above the 5 ng minimum recommended to avoid spurious results (Vestergaard et al. 2017).

136 The standards for each molecular target were obtained using a 10-fold serial dilution of PCR
137 products amplified from an environmental reference DNA and purified by gel extraction using the
138 Wizard® SV Gel and PCR Clean Up System (Promega, Southampton, UK) following the manufacturer's
139 instruction then quantified by fluorometer Qubit® 2.0 dsDNA BR Assay Kit (Thermo Fisher Scientific).

140 Standard curve template DNA and the negative/positive controls were amplified in triplicate.
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2 141 Amplification conditions for all qPCR assays consisted of an initial denaturation at 95°C for 5 min
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4 142 followed by 40 (two step) cycles; 95°C for 10s and 60 °C for 30s. The RT-qPCR program had an initial
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7 143 reverse-transcription step at 50 °C for 10 min.
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10 144 The conditions for comammox *amoA* clade B communities was adapted from Pvejac et al.
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12 145 (2017) to fit the constraints of the qPCR kit used but still matched the original conditions: initial
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14 146 denaturation at 95 °C for 5 min followed by 45 (three step) cycles; 95 °C for 30s, 52 °C for 45s and 72
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16 147 °C for 1min. Each amplification was followed by melt curve analysis (60 °C to 95 °C, with incremental
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18 148 readings every 0.5 °C) to assess the specificity of each assay. Results are expressed as gene copies g⁻¹
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25 150 **Statistical analysis**

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27 151 GenStat 17th Edition (VSN International Ltd, Hemel Hempstead, UK) was used to perform One-way
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29 152 and General ANOVA to compare values obtained from soil analyses, grain and straw yield and N
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31 153 offtake and from qPCR estimations of gene and transcript copy numbers. To check that each set of
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33 154 measured values met the assumptions of ANOVA and were normally distributed, residuals were
34
35 155 plotted. If they did not show normal distribution, data was log-transformed and again checked for
36
37 156 normal distribution of residuals. Where ANOVA results were significantly different ($P < 0.05$), means
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39 157 were further tested using Tukey's post-hoc method in the GenStat multiple comparison menu with
40
41 158 95% confidence; significantly different means are considered to have $\alpha = 0.05$ and are referred to as
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43 159 "significant" throughout the text. Where appropriate, the standard error of difference of means
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45 160 (s.e.d.) is indicated. Results with no significant differences are referred to as NSD.
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51 161 The statistics package PAST v. 3.16 (Hammer 2001) was used to perform NMDS with OTU data
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53 162 and Spearman's rank correlation for soil properties and gene and transcript abundances at all
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55 163 sampling times.
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165 Results

166 Soil pH, soil temperature, soil moisture and rainfall

167 During the 16 d monitoring period, soil temperature at 10 cm was relatively stable, ranging from 7.5 to
168 10 °C (mean 9 °C). Rainfall of less than 2 mm was recorded on 5 days, making loss of urea or nitrate by
169 leaching unlikely (supplementary Fig. 1). Prior to applying treatments, the field soil (previously
170 reported to be 6.2) was measured at pH 6.1 in all designated plots and the nil plot soil remained at pH
171 6.1 throughout the monitoring period; the urea + NI and urea + NI + UI treatments were not
172 significantly different but the plots with urea or urea + UI showed significantly lower pH at 2 and 8
173 days after application (Fig. 1 a). ANOVA indicated that treatment, but not time since urea application,
174 had a significant effect on pH (Table 2).

175 Soil mineral N and crop yields

176 Total soil mineral N levels (exchangeable NH_4^+ + NO_3^- + NO_2^-) in the nil plots were significantly lower
177 than those where urea was applied, with or without inhibitors but there was NSD between these plots,
178 with similar results for exchangeable NH_4^+ (Fig. 1 b, c). The majority of mineral N in soil at 2 d was
179 exchangeable NH_4^+ , indicating rapid hydrolysis of urea that was not significantly affected by the
180 presence of UI. However, on average, the NH_4^+ levels where urea was applied had declined 61% at 15
181 d after application, indicating active nitrification. Levels of NO_3^- increased slightly 8 d after urea
182 application but had decreased 40% at 15 d, with significantly less soil NO_3^- observed where NI was
183 applied with urea (Fig. 1 d). ANOVA comparison of all samples for total mineral N, exchangeable
184 NH_4^+ and NO_3^- showed that both sampling time and treatment effects were significant, and interaction
185 between these factors was significant for exchangeable NH_4^+ (Table 2). The total mineral N, NO_3^- and
186 NH_4^+ levels were strongly correlated ($r_s = 0.62$ and 0.99 respectively, $P < 0.001$ – supplementary Table
187 3). The levels of NO_2^- were too low and variable to infer statistical significance and are not reported.

188 All plots with fertilizer addition yielded significantly higher than the nil plots for both grain and
189 straw (Fig. 2 a, b). There was a small but significant yield decrease in both grain and straw for urea + NI
190 compared with urea, but not for the urea + UI or urea + NI + UI treatments. Results for grain and
191 straw N offtake followed the same pattern (supplementary Fig. 2 a,b). Significantly more fertilizer N
192 was recovered in straw and grain from ammonium nitrate fertilizer applied at the same N rate as the
193 urea fertilizer treatments and compared to these, net N recovery from urea + NI was significantly less
194 (supplementary Fig. 2 c).

195 **Abundance and activity of soil microorganisms at kingdom level**

196 A survey of the total bacterial community diversity in the different plots based on 16S rRNA amplicon
197 sequencing showed no clear differences according to either sampling time or treatment
198 (supplementary Fig. 3). The number of bacterial 16S rRNA genes was > 10-fold higher than the fungal
199 ITS around 100 x more than the archaeal 16S rRNA. Since there are thought to be on average 4 – 5
200 copies of the 16S rRNA gene in soil bacteria, 1 – 2 copies in soil archaea and an unknown number of
201 ITS repeats, it is difficult to infer the actual cell numbers in each group. In contrast, the functional
202 genes (*amoA*, *nxr*, *ureC*) generally have a single copy per genome. However, within-group
203 comparisons show that the abundance of all genes had fallen 15 d after urea application, most of
204 them significantly (supplementary Fig. 4; Table 3). The treatment, however, did not have a significant
205 effect on abundance except for bacterial *amoA* (Table 3). The number of transcripts also showed a
206 significant response to time but not to treatment and indicated that bacteria were 5-fold more active
207 than archaea and 50-fold more active than fungi (supplementary Fig. 5) and that activity increased
208 over the 15d period. ANOVA confirmed that sampling time, but not treatment, had a significant effect
209 on all these measurements, $P \leq 0.05$ (Table 3). Although the variability in efficiency of different PCR
210 primers means that abundance estimates are not absolute but relative, there was no indication of PCR
211 inhibitors in the DNA and RNA preparations as their amplification profiles matched those of the
212 standard curves (supplementary Table 1, 2).

213 The results indicate that the three kingdoms (Bacteria, Archaea, Fungi) increased
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2 214 transcriptional activity over the monitoring period whilst declining in abundance. The abundance of all
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4 215 three groups was strongly correlated, indicating similar responses to changes in soil conditions
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7 216 (supplementary Table 4).
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10 217 **Abundance and activity of microorganisms involved in N-cycling**

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13 218 The genes involved with N-cycling, apart from bacterial *amoA*, showed a similar pattern to those at
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15 219 kingdom-level, with no significant treatment response but a significant decline over the 15 d
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18 220 monitoring period and moderate to strong correlation in all samples (supplementary Fig. 4,
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20 221 supplementary Table 3). The order of abundance of N-cycling genes was bacterial *ureC* > bacterial
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22 222 *amoA* > *Nitrospira nxr* > archaeal *amoA* > *Nitrobacter nxr* (supplementary Fig. 4). The proportion of
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24 223 AOB appeared to be relatively high compared with previous reports, at >1 % of total Bacteria; the AOA
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26 224 were > 20% of total Archaea. The under-representation of *ureC* compared to previous reports may be
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28 225 due to suboptimal primers for soil communities, although assuming it is a single copy gene and there
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30 226 are 5 copies of 16S rRNA genes per bacterial genome, it is present in c. 10% of soil bacteria and there
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32 227 is a strong correlation in abundance of 16S rRNA genes and *ureC* ($r_s = 0.69$, $P < 0.001$, supplementary
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34 228 Table 3). The PCR product from the comammox *amoA* clade B primers gave the wrong melting
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36 229 temperature (T_m) and a double peak in the melting curves. These products gave a smear of multiple
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38 230 bands when run on a gel indicating that there was not a single specific product, in contrast to the
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40 231 other PCR assays. Because it was unclear which genes the primers were amplifying, the comammox
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42 232 results were disregarded. RNA extraction from the soils gave insufficient yields to detect transcription
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44 233 of the genes, apart from *amoA*, where the archaeal version increased over time whereas there was a
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46 234 drop in bacterial *amoA* expression (supplementary Fig. 5). ANOVA showed sampling time to be
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48 235 significant ($P \leq 0.05$) for most genes but not for the Archaea, AOA *amoA*, or *Nitrospira nxr*, and
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50 236 treatment effects were significant only for AOB *amoA* (Table 3). The abundance of archaeal 16S RNA
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52 237 was strongly correlated with that of AOA *amoA* ($r_s = 0.91$, $P \ll 0.001$) whereas bacterial 16S RNA
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238 showed only a weak negative correlation with AOB *amoA* RNA ($r_s = -0.29$, $P = 0.03$, supplementary
239 Table 3).

240 **Responses of bacterial AOB to urea and inhibitors**

241 AOB abundance dropped over the sampling period in all treatments. For the urea and urea + UI
242 treatment, AOB numbers remained significantly higher than those receiving NI at 2 and 8 d after urea
243 application (Fig. 3). The transcript numbers were low and variable, and no statistical significance could
244 be inferred but gene transcription was noticeably higher in the urea and urea + UI treatments (Fig. 4).

246 **Discussion**

247 At the end of the experiment, grain and straw yields from the nil plots were less than half of those
248 where N fertilizer was applied and the plots receiving ammonium nitrate yielded significantly more
249 grain than plots receiving the same rate of N as urea (Fig. 2, supplementary Fig. 2), most likely because
250 of a higher ammonia volatilization loss from the urea (Chambers and Dampney 2009). However, no
251 yield enhancement was observed for any of the urea + inhibitor treatments, and the urea + NI
252 treatment was associated with a small but significant reduction in N offtake and net N recovery in
253 straw and grain when compared with urea (Fig. 2, supplementary Fig. 2). The reasons for this are
254 unclear but may be related to the lower soil nitrate content in the period following application (Fig. 1).
255 It is possible that delayed ammonia oxidation meant that when the bulk of the urea fertilizer was
256 converted to hydroxylamine and nitrite, soil conditions were more conducive to biotic reduction to
257 N₂O resulting in net N loss from the system, compared to the other treatments but we could not
258 confirm this as we were unable to measure gaseous losses during these experiments. The soils ranged
259 between pH 5.5 – 6.1, insufficiently acid for spontaneous decomposition of hydroxylamine to play a
260 major role (Heil et al. 2016). It is possible that some degradation of the UI occurred: NBPT is reported
261 to have a half-life of 1.6 d in soils at pH 5, 9.8 d at pH 5.5 and 42 d at pH 6 (Engel et al. 2015).
262 However, most ureolysis is likely to have occurred with 2 d (Nannipieri et al., 1990), before significant

263 decomposition of the UI. Surprisingly, yields and net N uptake were not significantly diminished when
264 NI was combined with UI but remained similar to urea alone or urea with just UI. While the meta-
265 analysis of Abalos et al (2014) showed predominantly positive impacts of NI use on crop yields, yield
266 suppression has also been observed (e.g. Bell et al. 2015), which may be related to the timing of
267 availability of different forms of soil N in relation to plant uptake.

268 There are conflicting reports in the literature on UI influence on crop yield (Sigurdarson et al.
269 2018). For example, the UI phenylphosphoryldiamidate (PPDA) was found to have no effect on wheat
270 yields in Syria (Monem et al. 2010) but in tropical soils, NBPT was reported to reduce urea hydrolysis
271 by 35 % and in conjunction with a NI, increase maize yields significantly (Martins et al. 2017). In a
272 large UK study, Chambers and Dampney (2009) reported a mean ammonia emission reduction of 70%
273 (range 25-100 %) from the use of NBPT with urea, and on average the use of NBPT increased crop N
274 recovery compared with urea alone. However, differences at an individual site were not always
275 significant and this may indicate lack of effectiveness of the UI due to rapid breakdown in soil under
276 certain conditions.

277 The Woburn soil is slightly acid and well-drained, and the experiment took place in a period of low
278 rainfall although it rained on the day that treatments were applied. Sampling of the field was
279 constrained by practical considerations: the farm is run on commercial lines which dictate timing of
280 treatments and access for sampling. In retrospect it would have been informative to sample on the
281 day following application. The soil pH was slightly lower 2 d after application, where urea and urea +
282 UI were applied, compared to nil plots and those with NI. This indicates a very rapid hydrolysis of urea
283 to NH_4^+ and subsequent nitrification. The finding is supported by reports of an experiment where ^{15}N -
284 labelled urea was applied to a grass and legume sward under Mediterranean conditions: ureolysis
285 occurred within 2 days and $^{15}\text{N-NH}_4^+$ peaked at 2 days (Nannipieri et al. 1990). Urea can increase soil
286 pH as hydrolysis to NH_4^+ releases one OH^- but subsequent nitrification to NO_3^- releases two H^+ ,
287 resulting in net soil acidification. The presence of UI did not retard acidification, but pH in soil where
288 NI was added was similar to the nil control, indicating less acidification due to delayed ammonia

289 oxidation. The mineral N and exchangeable NH_4^+ concentrations in soil were similar during the
290 experiment confirming that most urea was already hydrolysed by the first sampling and the drop in pH
291 was a residual effect of urea hydrolysis followed by nitrification. The mineral N concentrations also
292 were higher in plots with urea alone, compared to the various inhibitor combinations, but this was not
293 statistically significant and only the nil plot had significantly less N. However, the presence of NI
294 resulted in significantly lower NO_3^- , indicating an effect over 2 weeks. The rate of nitrification in soil is
295 reported to be less rapid than urea hydrolysis: a meta-analysis reported nitrification rates of 1.4 – 2 μg
296 $\text{NH}_4^+\text{-N g soil}^{-1} \text{ d}^{-1}$ (Booth et al. 2005). This compares with rates of 5 μg – 6 mg urea-N $\text{kg soil}^{-1} \text{ d}^{-1}$ for
297 urea measured in a range of moist soil (Reynolds et al. 1985). With lower NO_3^- concentrations in soil,
298 less N_2O will be emitted due to denitrification. We did not measure gaseous losses in the field but
299 experiments with the NI 3,4-dimethylpyrazole phosphate (DMPP) indicated that it resulted in lower
300 N_2O emissions (Zhang et al. 2018). It would be interesting to determine whether the inhibitors have
301 any beneficial environmental effects in the Woburn soil by decreasing NH_3 or N_2O emissions.
302 Otherwise, the lack of any significant yield increases with NI and UI (singly or in combination) negates
303 any economic case for their use in this situation.

304 The abundance of soil microorganisms at kingdom level (bacteria, archaea, fungi) fell during
305 the monitoring period, and gene expression increased, presumably a response to an earlier
306 stimulation due to temperatures, rainfall and plant growth. The bacterial urease, archaeal AMO and
307 NXR genes showed the same pattern of a drop-in abundance and increase in activity, indicating a
308 common trend in the soil community responding to environmental factors but not to the different
309 treatments which were NSD.

310 In arable soils receiving N fertilizer, AOB have been reported to be more active than AOA (Hink
311 et al. 2017). In our experiment, the AOB increased in both abundance and activity in response to urea
312 or urea + UI applications despite insignificant differences in the levels of exchangeable NH_4^+ ; the
313 presence of NI reduced this effect. This indicates that inhibition of AMO affected AOB growth even
314 when differences in the substrate NH_4^+ were not discernible. A drop in the abundance of AOB *amoA*

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315 has been reported in Australian sugarcane soils treated with the NI DMPP (Zhang et al. 2018). Since
316 AOB numbers were already significantly higher 2 d after urea application, it is likely that the NH_4^+
317 levels in soil resulting from urea hydrolysis had increased very rapidly after application and were
318 falling due to AOB activity at this first sampling date. The larger AOB community in the urea and urea
319 + UI plots appeared to result in more NO_3^- as well as lower soil pH as mentioned above. Although the
320 NOB must have been actively oxidizing NO_2^- to NO_3^- , no effects on their abundance were detected.
321 The AOB *amoA* primers are not expected to amplify comammox *amoA* (Pjevac et al. 2017) and it is
322 unlikely that the comammox bacteria were major contributors to nitrification in the soil as they are
323 only a sub-population of the *Nitrospira*, in turn 70% less abundant than the AOB. To monitor
324 comammox in these soils it will be necessary to develop new primers for PCR with improved *amoA*
325 specificity.

327 **Conclusions**

328 For the winter wheat crop on sandy loam at Woburn in 2017, the addition of the UI NBPT and the NI
329 DCD had only transient effects on soil N dynamics and did not result in increased crop yields. It is likely
330 that urea hydrolysis by extracellular and intracellular enzymes was very rapid, followed by nitrification
331 due to AOB and NOB activity. There were no discernible effects on soil microbial community
332 dynamics, whether bacteria, archaea or fungi, nor on urease gene frequency, ammonia oxidizing
333 archaea or nitrite-oxidizing bacteria. However, ammonia oxidizing bacteria numbers increased in
334 response to urea and urea + UI, less so when NI was present, indicating that the UI had only a short-
335 lived effect within the first two days on the supply of the NH_4^+ substrate for AOB. The lack of response
336 from AOA and NOB implies that services provided by these groups are largely unaffected by soil
337 treatments and furthermore, that growth of AOA in soil is not inhibited by DCD. In conclusion, with a
338 caveat that our findings may not apply to other soils, crops and climates, the UI NBPT and the NI DCD
339 had only minor effects on soil pH, N dynamics and AOB with no discernible influence on other soil
340 microorganisms and no positive effects on crop yields.

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516 **Figure legends**

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3 517 **Fig. 1.** Soil edaphic factors measured at each sampling point (n = 4). All points were subjected to

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5 518 Tukey's pot-hoc test on ANOVA, significant results reported where $\alpha = 0.05$. (a) - soil pH, significantly

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7 519 lower in plots with urea or urea + UI; (b) mineral N (exchangeable $\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$); (c) -

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9 520 exchangeable NH_4^+ - in both, nil plot measurements significantly lower at each sampling time point

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11 521 than for any treatments; (d) - NO_3^- , nil plots significantly lower and the urea and urea + UI plots

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13 522 significantly higher than plots with urea + NI or urea + NI + UI. ANOVA results are reported in Table 2.

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15 523 **Fig. 2.** Wheat grain and straw yields at 85% dry matter expressed as t ha^{-1} (n = 4). Different letters

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17 524 above bars denote significantly different means ($\alpha = 0.05$) according to Tukey's post-hoc test on

18
19 525 ANOVA for each set of yields. ANOVA results: grain yield $F_{4,12} = 95.0$, $P < .001$; straw yield $F_{4,12} = 29.7$,

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21 526 $P < .001$.

22
23 527 **Fig. 3.** Abundance of bacterial *amoA* gene copies estimated using qPCR (n = 4) at 2, 8 and 15 days

24
25 528 after application of urea fertilizer alone or in combination with nitrification inhibitor DCD (NI) and / or

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27 529 urease inhibitor NBPT (UI). Nil – no urea control. All points were subjected to Tukey's pot-hoc test on

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29 530 ANOVA, significant results reported where $\alpha = 0.05$. The abundance of *amoA* in soils treated with urea

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31 531 alone or in combination with UI fell significantly between 2 and 8 days and was significantly greater

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33 532 than the other treatments at these days. ANOVA results are reported in Table 3.

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35 533 **Fig. 4.** Abundance of bacterial *amoA* transcripts estimated using qPCR (n = 4) at 2, 8 and 15 days after

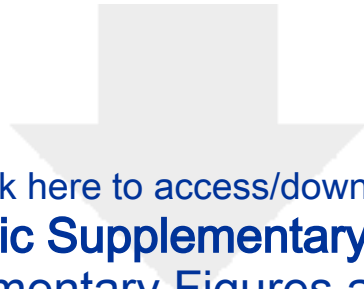
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37 534 application of urea fertilizer alone or in combination with nitrification inhibitor DCD (NI) and / or

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39 535 urease inhibitor NBPT (UI). Nil – no urea control. The soils treated with urea + NI +UI did not yield

40
41 536 sufficient mRNA to be included in this figure and mRNA recovery was too low to infer statistical

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43 537 significance. ANOVA results are reported in Table 3.

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Figure 1

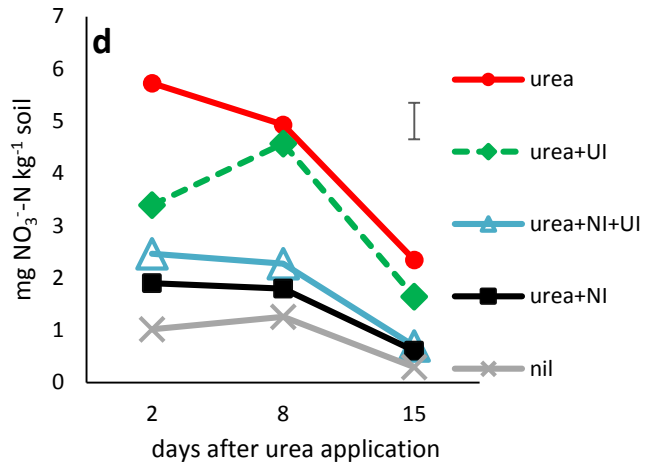
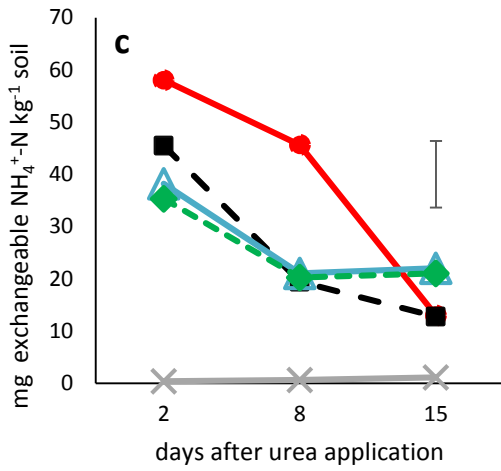
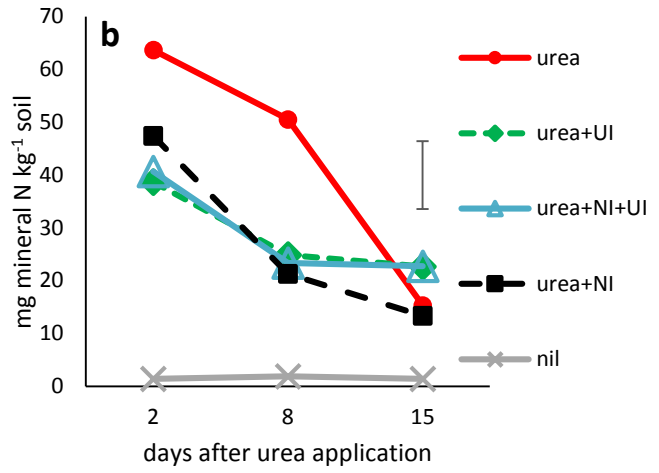
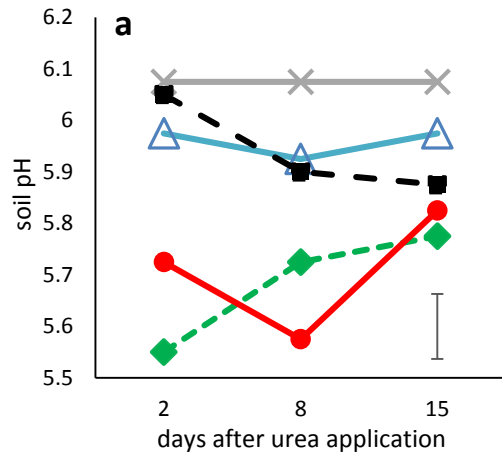
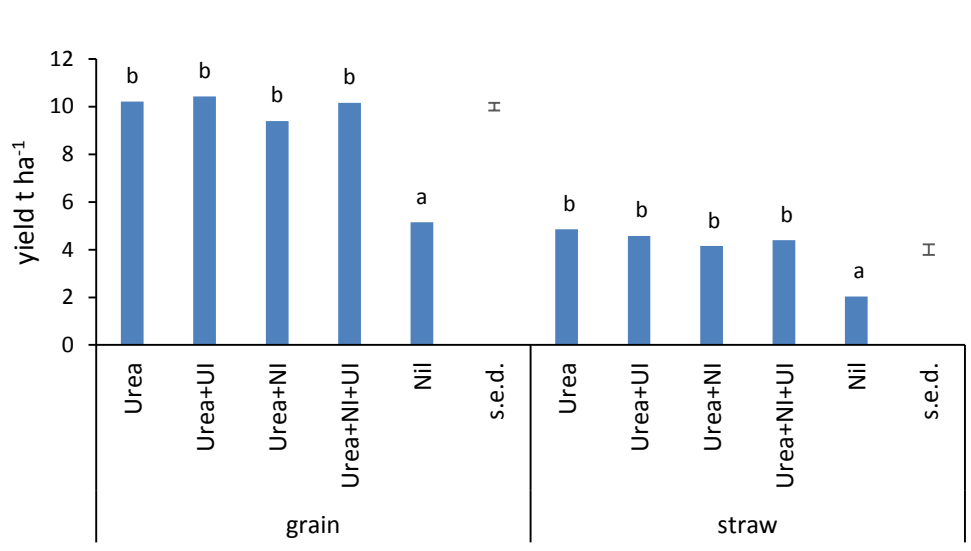
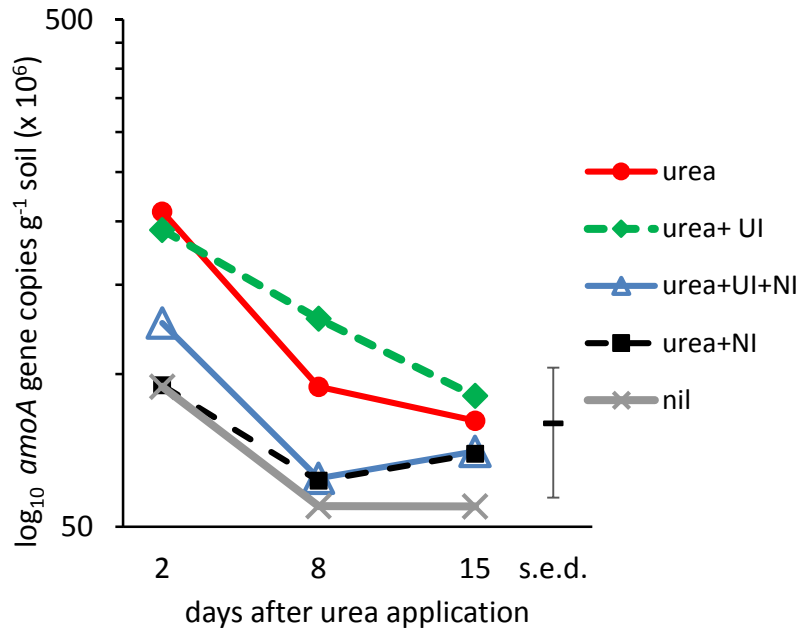


Figure 2



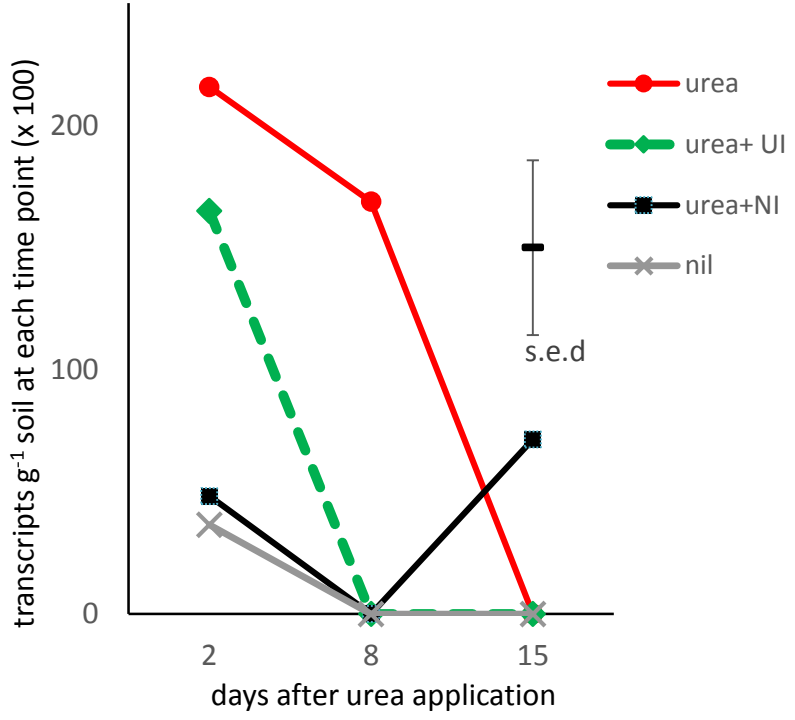
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Figure 3



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Figure 4



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gene	primer	sequence	reference
16S rRNA Bacteria	341F	CCT AYG GGR BGC ASC AG	Glaring et al. (2015)
	806R	GGA CTA CNN GGG TAT CTA AT	
16S rRNA Archaea	arch349F	GYG CAS CAG KCG MGA AW	Takai & Horikoshi. (2000)
	arch806R	GGA CTA CVS GGG TAT CTA AT	
16S rRNA Archaea	Parch519F	CAG CMG CCG CGG TAA	Ovreås et al. (1997)
	Arch1060R	GGC CAT GCA CCW CCT CTC	Reysenbach and Pace (1995)
ITS Fungi	ITS1f	TCC GTA GGT GAA CCT GCG G	Gardes and Bruns (1993)
	5.8s	CGC TGC GTT CTT CAT CG	Vilgalys and Hester (1990)
amoA Bacteria	amoA-1F	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. (1997)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	
amoA Archaea	arch-amoAF	STA ATG GTC TGG CTT AGA CG	Francis et al. (2005)
	arch-amoAR	GCG GCC ATC CAT CTG TAT GT	
ureC Bacteria	ureC_Collier_F	AAG STS CAC GAG GAC TGG GGA	Collier et al. (1999)
	ureC_Collier_R	AGG TGG TGG CAS ACC ATS AGC AT	
nxr- Nitrospira-	nxr-spira-for5	CAR TCS AAC TTC CGG TAY GG	Fu et al. (2018)
	nxr-spira-rev6	AGC CAC TTG ATC ATG AAY TC	
nxr- Nitrobacter	nxr-bacter-for1	GAC SCG YAC CCC SGA CGT GCA CYT CAT	
	nxr-bacter-rev3	ATG ACG TGR TTG RCC GCC ATC CA	
amoA- comammox	comaB-244F	TAY TTC TGG ACR TTY TA	Pjevac et al. (2017)
	comaB-659R	ARA TCC ARA CDG TGT G	

Table 1. Primers used for qPCR to assess gene abundance and activity.

Source of variation	d.f.	pH	mineral N	NO ₃ ⁻	exchangeable NH ₄ ⁺
Time	F _{2,45}	NS	8.17, <i>P</i> < .001	45.42, <i>P</i> < .001	3.61, <i>P</i> = 0.035
Treatment	F _{4,45}	10.79, <i>P</i> < .001	57.09, <i>P</i> < .001	31.9, <i>P</i> < .001	73.04, <i>P</i> < .001
Time x Treatment	F _{8,45}	NS	NS	NS	2.35, <i>P</i> = 0.033

Table 2. ANOVA for soil edaphic factors at 2, 8 and 15 days after urea application from all samples (see Fig. 1). Mineral N = NO₃⁻ + NO₂⁻ + exchangeable NH₄⁺.

NS – not statistically significant.

gene copies g ⁻¹ soil	d.f.	16S Bacteria	16S Archaea	ITS	AOA <i>amoA</i>	AOB <i>amoA</i>	<i>ureC</i>	<i>nxr-bacter</i>	<i>nxr-spira</i>	comammox
time	F _{2,51}	5.13, <i>P</i> =0.010	NS	4.96, <i>P</i> =0.011	NS	19.96, <i>P</i> <.001	4.10, <i>P</i> =0.023	18.49, <i>P</i> <.001	NS	NS
treatment	F _{4,51}	NS	NS	NS	NS	8.71, <i>P</i> <.001	NS	NS	NS	NS
time x treat	F _{8,51}	NS	NS	NS	NS	NS	NS	NS	NS	NS

transcripts g ⁻¹ soil	d.f.	16S bacteria	16S archaea	ITS	AOA <i>amoA</i>	AOB <i>amoA</i>
time	F _{2,51}	21.58, <i>P</i> <.001	27.83 <i>P</i> <.001	13.29, <i>P</i> <.001	28.50, <i>P</i> <.001	23.68, <i>P</i> =0.006
treatment	F _{4,51}	NS	NS	NS	NS	33.97, <i>P</i> =0.003
time x treat	F _{8,51}	NS	NS	NS	NS	NS

Table 3. ANOVA for gene and transcript copies in all treatments and times (see Supplementary Fig. 4, 5). Time had a significant effect on most genes, fertilizer treatment did not, affecting only the AOB which were significantly more abundant and active where urea was applied without NI. NS – not statistically significant