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1 Use and abuse of potential rates in soil microbiology

2

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33

34 **Abstract**

35 Potential rate assays are used in soil microbial ecology to determine the maximum rate of a  
36 functional process in environmental samples. They assume that all contributing organisms in a  
37 community are active at a maximum rate under one set of ‘optimal’ incubation conditions.  
38 Potential rates are then often compared to the abundance of specific taxonomic groups to infer  
39 their relative contribution *in situ*. However, while investigators now recognise that populations  
40 within communities are physiologically diverse, they often ignore the consequent suboptimal  
41 activity, or even inactivity, of the majority of community members performing that function.  
42 In this short perspective article, we highlight the underlying conceptual problems associated  
43 with potential assays and use potential nitrification rate (PNR) as an example. PNR was  
44 originally developed to estimate the size of active ammonia oxidising communities in  
45 environmental samples. They are routinely performed in short-term shaken slurry incubations  
46 by measuring assumed maximum rates of nitrate or nitrite production under optimal, non-  
47 substrate-limiting conditions. As with other functional processes, it is now recognised that a  
48 broad diversity of organisms contribute to aerobic ammonia oxidation in terrestrial and other  
49 habitats, and this diversity represents a substantial range of physiologies, including variation in  
50 substrate affinity, ammonia tolerance, cell specific activity and substrate preference. Despite  
51 this, PNR is routinely used in an attempt to determine an ecologically relevant measurement of  
52 nitrification activity in soil. As with other potential assays, PNR has inherent biases towards  
53 particular functional groups and its use in investigating the ecology of ammonia oxidisers in  
54 natural systems is severely restricted.

55

56 **Main text**

57 Measurement of the potential rate of microbially mediated soil processes has a long tradition.  
58 Commonly used examples are measurement of potential nitrification and denitrification rates  
59 and substrate-induced respiration. All aim to determine process rate, usually after addition of a  
60 relevant substrate, under what are considered to be optimal conditions that enable all organisms  
61 capable of performing the process to operate with maximum activity and/or specific growth  
62 rate. Potential rate obviously differs from ‘actual’ process rate, operating under non-optimal  
63 environmental conditions, although actual rates are required for most ecological questions.  
64 Potential process rates may have some value, and have traditionally been used, in the absence  
65 of alternatives, to estimate the biomass (or, less justifiably, abundance) of a functional microbial  
66 group. However, the assumptions underlying measurement of potential rate are rarely  
67 considered, rarely obeyed and even more rarely tested and have been increasingly challenged

68 as our understanding of the composition and diversity of microbial communities in natural  
69 environments has increased. The limitations and relevance of potential rate measurements in  
70 microbial ecology are illustrated below for soil nitrification, but apply equally to other  
71 processes, functional groups and ecosystems.

72

### 73 *Potential nitrification rate*

74 Nitrification is the sequential oxidation of ammonia ( $\text{NH}_3$ ) to nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ )  
75 and is an essential component of reactive nitrogen transformation in soil. While nitrite can  
76 accumulate in soil (Giguere et al., 2017; Ma et al., 2015; Taylor et al., 2019; Venterea and  
77 Rolston, 2000), particularly after N deposition (Venterea et al., 2020), ammonia oxidation is  
78 often assumed to be the rate-limiting step. As a consequence, the activity, diversity and  
79 community structure of ammonia oxidisers (AO) receives greatest attention with respect to  
80 understanding environmental factors that influence microbially-mediated nitrification.  
81 Potential nitrification rate (PNR) assays (also referred to as potential nitrification activity  
82 (PNA)) have been used for many decades as a rapid method for estimating net ammonia  
83 oxidation ( $\approx$ nitrification) rates in soils.

84 While a range of PNR assays have been used, the most common approach is the shaken  
85 soil-slurry method (Belser and Mays, 1980; Hart et al., 1994). This typically involves  
86 incubation of a suspension of sieved soil in a solution containing  $\text{NH}_4^+$ , at non-limiting  
87 concentration, in a shaken flask, ensuring aeration and mixing, at an incubation temperature  
88 that is considered to be optimal. PNR is calculated as the gradient of the linear increase in nitrate  
89 concentration in samples taken regularly during a relatively short incubation period (often  $\leq 24$   
90 h) (Hart et al., 1994). A potassium phosphate buffer, typically between pH 7.0 and 7.5, can be  
91 included in the assay solution, with the intention of optimizing pH for AO. Chlorate, an  
92 inhibitor of nitrite oxidation, is also commonly included and  $\text{NO}_2^-$  rather than  $\text{NO}_3^-$  is measured  
93 in each slurry sample (Belser and Mays, 1980). Growth is assumed to be negligible; hence the  
94 assumption of a linear, rather than exponential increase in nitrite or nitrate concentration. PNR  
95 therefore measures the activity of the community and not specific growth rate. Linear kinetics  
96 are often not tested and it is not unusual for calculations to be based on nitrate concentrations  
97 measured only at the beginning and end of incubation or even using just the final value. Any  
98 growth, or other divergence from linear kinetics, invalidates calculation of a 'constant' PNR.

99 Employment of 'ideal' or 'optimal' conditions attempts to ensure that all AO within the  
100 sample are active, which is further encouraged by addition of substrate ( $\text{NH}_4^+$ ). The assumption  
101 of universal activity was implicit in the traditional use of PNR to estimate AO abundance in

102 soil. This assumption was always seen as a major limitation of this approach (Woldendorp and  
103 Laanbroek, 1989; Hart et al., 1994), particularly given the low growth rates of ammonia  
104 oxidisers. (The only alternative was use of the most probable number method, which also relies  
105 on incubation of dilutions of soil suspensions under ‘ideal’ conditions and similar assumptions.)  
106

### 107 *Physiological diversity of ammonia oxidisers challenges underlying assumptions of PNR*

108 For more than a century, ammonia oxidation was thought to be performed mainly by ammonia  
109 oxidising bacteria (AOB) (Prosser and Nicol, 2012). The past 25 years have, however,  
110 demonstrated considerable phylogenetic diversity within AOB and also the capacity for  
111 ammonia oxidation by ammonia oxidising archaea (AOA) (Könneke et al., 2005) and complete  
112 ammonia oxidizers (comammox) of the genus *Nitrospira* (Daims et al., 2015; van Kessel et al.,  
113 2015). Evidence from both cultivation-based and ecological studies also provides evidence for  
114 substantial physiological diversity in AO. Physiological diversity of cultivated AOB is well  
115 established and was used for traditional classification of these organisms (Prosser 1989; Koops  
116 et al., 2003). Since the first isolation of an ammonia oxidising archaeon (AOA), *Nitrosopumilus*  
117 *maritimus* (Könneke et al., 2015), several enrichments and isolates of soil AOA have also been  
118 obtained. These include, for example, the neutrophile *Nitrososphaera viennensis* EN76 (Tourna  
119 et al. 2011) and the obligate acidophile *Candidatus Nitrosotalea devanaterrea* ND1 (Lehtovirta-  
120 Morley et al., 2011). In addition, comammox *Nitrospira* are abundant and contribute to  
121 ammonia oxidation in soil (Pjevac et al., 2017; Wang et al., 2019a), and a gammaproteobacterial  
122 AOB (*Candidatus Nitrosoglobus terrae*) was isolated recently (Hayatsu et al., 2017). This  
123 taxonomic diversity is reflected in ecophysiological diversity and the maximum specific growth  
124 rate, cell specific activity, inhibitory ammonia and nitrite concentrations and affinity for  
125 ammonia of AO all vary by over an order of magnitude among different soil isolates, both  
126 within and between the major ammonia oxidiser groups (summarised in Lehtovirta-Morley et  
127 al., 2016). In addition, many AO are inhibited by  $\text{NO}_2^-$ , which accumulates when chlorate is  
128 used to inhibit nitrite oxidisers, thereby reducing PNR depending on community composition.  
129 Thus, even if the incubation conditions (temperature, pH, oxygen concentration) are appropriate  
130 for all AO, the PNR will represent only an aggregate of a wide range of cell activities and will  
131 vary with community composition and not just abundance.

132 Physiological diversity does, however, also mean that incubation conditions required  
133 for activity of all AO will not be identical. Cultivation-based studies have demonstrated  
134 significant differences in effects of temperature (Jiang and Bakken, 1999; Tourna et al., 2011;  
135 Lehtovirta-Morley et al., 2014), pH (Allison and Prosser, 1991; Lehtovirta-Morley et al., 2011)

136 and salinity (Mosier et al., 2012; Qin et al., 2014; Bello et al., 2019). Similar evidence of  
137 physiological diversity is provided by ecological studies (Nicol et al., 2008; Ollivier et al., 2012;  
138 Gubry-Rangin et al., 2017; Nacke et al., 2017; Taylor et al., 2017; Lu et al. 2018) and the  
139 ubiquity of AO in the full range of soils, in itself, is evidence that a single set of incubation  
140 conditions will be insufficient. For example, ammonia supply is an important factor influencing  
141 niche differentiation between AOA and AOB populations in soil. AOB often dominate growth  
142 at higher concentrations of added inorganic ammonium (e.g. Jia and Conrad, 2009; Xia et al.,  
143 2011; Hink et al., 2017), while AOA appear to prefer ammonium that is derived from a  
144 mineralised organic N source, i.e. that is part of the native organic component of the soil or  
145 added organic N (Levičnik-Höfferle et al., 2012; Hink et al., 2018). In low pH soils, AOA make  
146 a greater relative contribution to ammonia oxidation under specific incubation conditions  
147 (Gubry-Rangin et al., 2010; Zhang et al., 2012) and, as such, a decreasing proportion of the  
148 ammonia oxidising community may preferentially oxidise added inorganic N with decreasing  
149 soil pH.

150 It is therefore impossible to identify incubation conditions that will be ‘ideal’ for all  
151 organisms and the majority of AO will have sub-optimal or no activity and will not contribute  
152 to measured PNR. To this must be added the assumption that ammonia available and oxidised  
153 in PNR assays will also be available for the same populations in soil.

154

155 *Potential nitrification rate does not reflect ammonia oxidiser activity in situ*

156 The consequences of these limitations can be seen when correlations are determined between  
157 published PNR for soils and sediments and AOA and AOB abundances (using *amoA* gene  
158 abundance as a proxy for cell abundance). Results are highly variable. Of 107 studies published  
159 from 2007 to 2020 (Supplementary Table 1), only 36% and 75% reported a significant positive  
160 correlation between PNR and AOA or AOB abundances, respectively (Figure 1). Ten percent  
161 and 46% report a positive correlation with only AOA or only AOB, respectively, 11%  
162 correlated with neither and 4% and 5% reported a negative correlation with AOA and AOB,  
163 respectively. No correlation has yet been observed between PNR and comammox bacteria  
164 (Wang *et al.* 2019b; Luchibia *et al.* 2020; Zhang *et al.*, 2020). While these studies suggest that  
165 some populations of AOA are using added ammonium, the majority of studies indicate that  
166 only ammonia oxidising bacteria (AOB) contribute to PNR, often leading to the conclusion that  
167 AOA are not contributing to ammonia oxidation in a soil *in situ*. If we consider the growth  
168 characteristics of AO in both culture and soil, it is evident that correlating results from a single

169 defined assay with the abundance of communities containing substantial physiological diversity  
170 is unlikely to be meaningful and likely to be misleading.

171 The observed dominance of AOB over AOA in contributing to PNR activity in many  
172 soils may reflect differences in their ability to utilise added inorganic nitrogen. To investigate  
173 this, soil was sampled across a pH 4.5 to 7.5 gradient that has been fully characterised with  
174 respect to AOA and AOB community structure and abundance (Aigle et al., 2019; Gubry-  
175 Rangin et al. 2011), where *amoA* transcript and gene abundance of AOA and AOB decrease  
176 and increase directly with soil pH, respectively (Prosser and Nicol, 2012). PNR assays were  
177 established with both standard 1 mM phosphate buffered and unbuffered 1.5 mM  $\text{NH}_4^+$   
178 solution, and parallel incubations were performed in the presence of alkynes to differentiate  
179 contributions of all AO, AOA and AOB. Acetylene ( $\text{C}_2\text{H}_2$ ) has been extensively used as an  
180 inhibitor of all ammonia oxidisers that possess ammonia monooxygenase (AMO) and 1-octyne  
181 ( $\text{C}_8\text{H}_2$ ) inhibits growth of AOB, but not AOA, in both culture and soil (Taylor et al., 2013).  
182 Nitrate accumulation during incubation for 24 h was used to calculate PNR, which increased  
183 directly with soil pH across the gradient (Figure 2). However, as this activity was completely  
184 inhibited in the presence of both  $\text{C}_8\text{H}_2$ , and  $\text{C}_2\text{H}_2$ , it was attributed solely to AOB. This provides  
185 strong evidence that AOA do not contribute to PNR in some soils, PNR severely underestimates  
186 their abundance and this prevents any meaningful interpretation of their abundance or actual  
187 activity in soil at the time of sampling.

188 As indicated above, the source of ammonium is also an important factor in defining the  
189 ecological niches of AO populations within a community. Using concentrations typical of  
190 fertilisation application rates, we compared nitrification activity in both pH 4.5 and 7.5 soil  
191 microcosms after the addition of ammonium nitrate or urea (which is fully mineralised to  
192 ammonium within 0.5 days). After near stoichiometric conversion of ammonium to nitrate over  
193 12 days, inorganic and urea-derived ammonium were oxidised at comparable rates for both 100  
194 and 200  $\mu\text{g N g}^{-1}$  dry soil applications in the pH 7.5 soil (Figure 3). In contrast, urea-derived  
195 ammonium was oxidised at a faster rate in the pH 4.5 soil, and ammonia oxidation was inhibited  
196 by application of 200  $\mu\text{g}$  inorganic N  $\text{g}^{-1}$  dry soil (Figure 3). While these data do not differentiate  
197 the contribution of different AO groups, they reveal that sources of N other than inorganic  
198  $\text{NH}_4^+$ , as used in PNR, are preferred by some AO populations, again preventing meaningful  
199 interpretation of PNR data.

200 Despite the major limitations discussed above, potential process rates are still frequently  
201 used in attempting to address ecological questions. Again, nitrification studies illustrate their  
202 lack of usefulness. Attempts to understand the relative contribution of AOA and AOB to

203 nitrification involve correlation of their abundances with PNR, often after a treatment or  
204 perturbation (e.g. fertiliser application or temperature change) (Supplementary Table 1). This  
205 approach is also used to study niche specialisation, which implicitly assumes that changes in  
206 relative abundance of different phylotypes will result from differences in activity under  
207 different conditions. Incubation conditions used to measure PNR will favour different members  
208 of the AOA and AOB communities and will differentially influence activities of AOA and  
209 AOB. It will therefore not be possible to determine whether relationships between abundance  
210 and PNR are due to differences in preferences of the different communities for the specific  
211 incubation conditions used or due to links between abundance and actual activity. A more  
212 fundamental issue is the assumption, implicit in niche specialisation, that changes in community  
213 composition result from differences in actual activity, determined by different *in situ*,  
214 environmental conditions and do not depend on potential activity under a single set of  
215 laboratory conditions.

216

217 *The limitations of potential assays should discourage their use*

218 PNR does not characterise the contribution of AO to nitrification in soil and knowledge of the  
219 diversity of AO physiology and factors controlling their growth clearly challenges a long-  
220 established assumption that a single incubation assay measures the activity of all AO. Critically,  
221 potential rates also have little relevance to actual rates *in situ* and therefore have limited  
222 meaning in most nitrification studies. These fundamental and significant limitations should  
223 discourage the use of PNR and apply equally to other potential assays. Our expanding  
224 knowledge of the vast diversity and composition of microbial communities in natural  
225 environments, emphasises that communities contributing to a functional process comprise a  
226 diverse array of ecophysologies with substantial niche differentiation between populations.  
227 This challenges the underlying assumptions in potential rate measurements and severely  
228 restricts their relevance and value in studying soil microbial ecology.

229

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232

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413 **Figures**

414

415 **Figure 1.** Meta-analysis of soil and sediment studies finding significant positive correlations of  
416 AOA and/or AOB abundances with PNR. All studies used a shaken slurry method and  
417 measured  $\text{NO}_3^-$  or  $\text{NO}_2^-$  with the addition of chlorate to inhibit  $\text{NO}_2^-$  oxidation. A total of 107  
418 studies were included (Supplementary Table S1).

419

420 **Figure 2.** Demonstration of only AOB contributing to PNR. Soils were sampled across a pH  
421 gradient and PNR determined using the shaken slurry method. Flasks were established in  
422 triplicate using 144 ml serum vial bottles with 6 g soil (dry weight equivalent) and 50 ml of 1.5  
423 mM  $\text{NH}_4\text{Cl}$ , with or without 1 mM potassium phosphate (pH 7.2) buffer. (a) Samples were  
424 taken after incubation for 0, 3, 6, 9, 12 and 24 h to determine nitrate concentrations (nitrite was  
425 not detected). Means ( $\pm\text{SE}$ ) with different letters within a buffer treatment are significantly  
426 different based on Tukey's *post hoc* test following one-way ANOVA ( $p < 1.1 \times 10^{-7}$ ). Means  
427 annotated with a line are significantly different between buffer treatments based on two sample  
428 *t*-tests ( $p > 0.01 < 0.05$ ). (b) Example of complete inhibition of nitrification activity in flasks  
429 incubated in the presence of 0.01%  $\text{C}_2\text{H}_2$  or 0.03%  $\text{C}_8\text{H}_2$  with pH 7.5 soil (unbuffered).  
430 Nitrification activity was not detected in any soil in the presence of either alkyne.

431

432 **Figure 3.** Nitrate ( $\text{NO}_3^-$ ) production in soil microcosms amended with varying concentrations  
433 of ammonium sulphate or urea (0 – 200  $\mu\text{g N g}^{-1}$  dry soil) in pH 7.5 and 4.5 soil ((a) and (b),  
434 respectively). Soil microcosms were prepared (in triplicate for each treatment and timepoint)  
435 with sterile 144 ml serum vial glass bottles using 10 g soil (dry weight equivalent) which was  
436 sampled at 0-10 cm depth and sieved (2 mm mesh size). Microcosms were incubated at 30°C  
437 for 12 days with 30% soil water content (w/w). Microcosms were aerated every 3 days and  
438 destructively sampled before extracting  $\text{NO}_3^-$  in a 1 M KCl solution. Nitrate concentration was  
439 determined colorimetrically following Hink et al. (2018) immediately after sampling ( $\text{NO}_2^-$  was  
440 not detected). Error bars represent standard error of the mean. The greatest change in pH in soil  
441 microcosms was associated with those with the highest nitrification rates, indicating that pH  
442 change was not an inhibiting factor (Supplementary Figure 1). Nevertheless, these results  
443 highlight an additional concern, as changing conditions during incubation will influence  
444 activity and kinetics will no longer be linear.

445

446 **Supplementary Figure 1.** Soil pH in soil microcosms amended with varying concentrations of  
447 ammonium sulphate or urea (0 – 200 ug N g<sup>-1</sup> dry soil) in pH 7.5 and 4.5 soil ((a) and b),  
448 respectively). The standard errors of the mean pH of triplicate microcosms were  $\leq 0.06$  and are  
449 not visible behind markers.

450

451 **Supplementary Table 1.** Analysis of 107 studies correlating PNR with the abundance of AOA,  
452 AOB or comammox bacteria as (inferred from qPCR analysis of *amoA* gene abundance).  
453 Published studies were found with a SCOPUS search (TITLE-ABS-KEY ammonia AND  
454 oxid\* AND archaea AND bacteria AND potential AND nitrification) combined with manual  
455 refinement for only soil and sediment studies that used a shaken soil-slurry method.  
456 Publications are grouped by soil or sediment and NO<sub>2</sub><sup>-</sup> or NO<sub>3</sub><sup>-</sup> measured as the end product.  
457 The majority of correlations were positive (X) although nine negative correlations (-X) were  
458 found. There were no correlations with comammox bacteria.

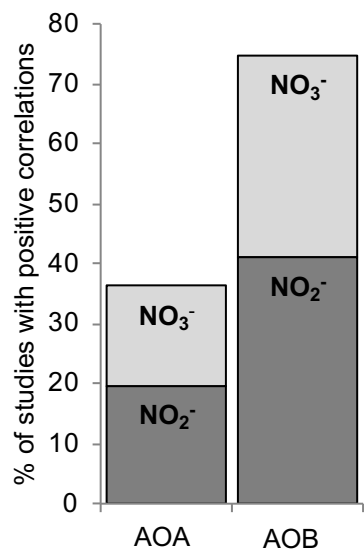
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**Figure 1.**



**Figure 2.**

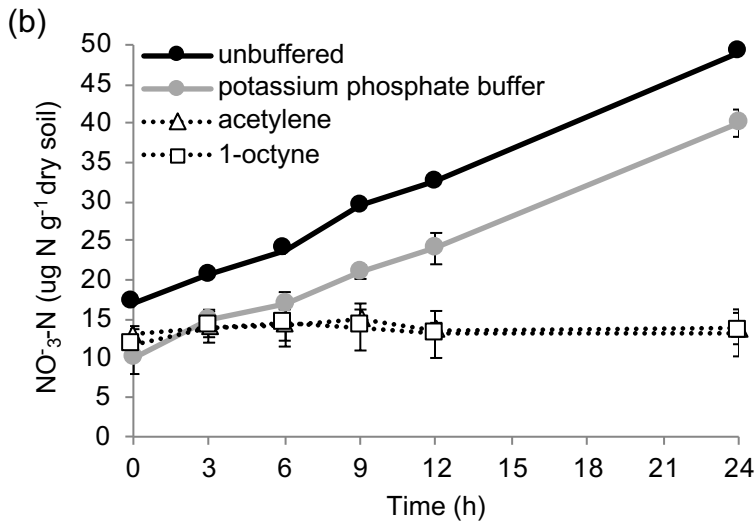
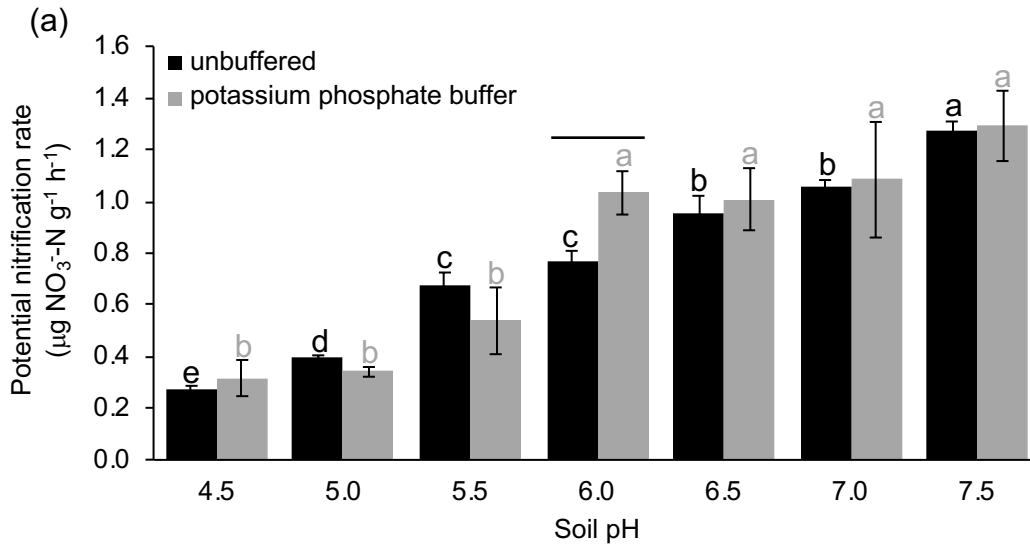
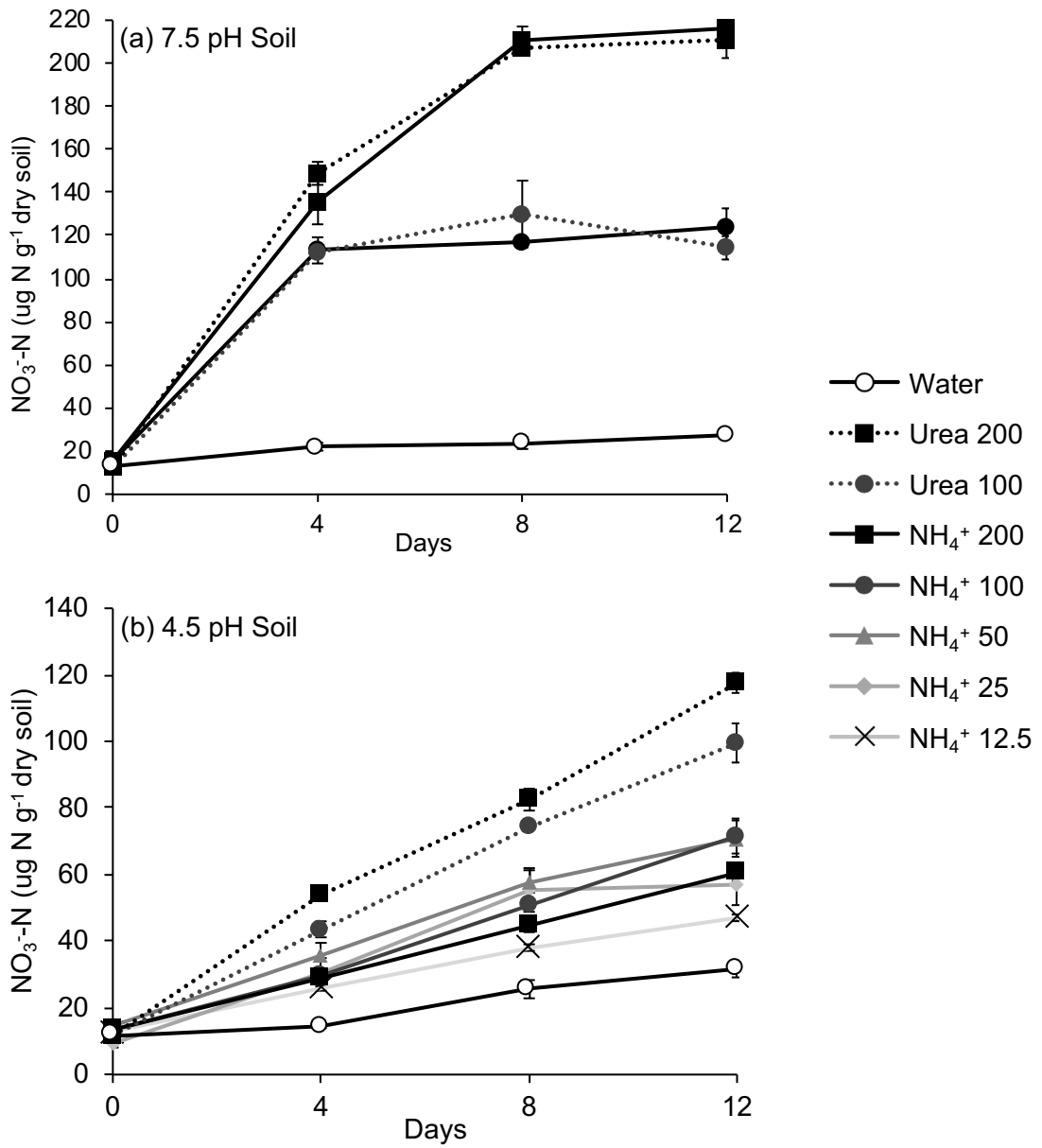


Figure 3.



Supplementary figure 1

