Use and abuse of potential rates in soil microbiology

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Abstract

Potential rate assays are used in soil microbial ecology to determine the rates of a functional process in environmental samples under a defined set of conditions. While they can be used appropriately to provide mechanistic insights, potential rates are also often used to estimate the abundance of specific taxonomic groups and their *in situ* activity. These estimates incorrectly assume that all contributing organisms in a community are active at a maximum rate under one set of 'optimal' incubation conditions and that potential rates reflect activity in the soil. While investigators now recognise that populations within communities are physiologically diverse, they often ignore the consequent suboptimal activity, or even inactivity, of the majority of community members performing that function. In this short perspective article, we discuss when potential assays can be informative and highlight the underlying conceptual problems under circumstances where potential assays are misused, using potential nitrification rate (PNR) as an example. PNR was originally developed to estimate the size of active ammonia oxidising communities in environmental samples. It is routinely determined in short-term shaken slurry incubations by measuring assumed maximum rates of nitrate or nitrite production under optimal, non-substrate-limiting conditions. As with other functional processes, it is now recognised that a broad diversity of organisms contribute to aerobic ammonia oxidation in terrestrial and other habitats, and this diversity represents a substantial range of physiologies, including variation in substrate affinity, ammonia tolerance, cell specific activity and substrate preference. Despite this, PNR, and other potential rate assays, are often inappropriately used in an attempt to determine an ecologically relevant measurement of activity in soil. As with any potential assay, PNR has inherent biases towards particular functional groups and its use in investigating the ecology of ammonia oxidisers in natural systems should be carefully considered.

Main text

Measurement of the potential rate of microbially mediated soil processes has a long tradition. Commonly used examples are measurement of potential nitrification and denitrification rates and substrate-induced respiration. All determine a process rate, usually after addition of a relevant substrate, under what are sometimes considered to be optimal conditions that enable most organisms capable of performing the process to operate with maximum activity and/or specific growth rate. Potential rate obviously differs from 'actual' process rate, which will typically operate under non-optimal environmental conditions. Potential process rates have

value, and have traditionally been used, in the absence of alternatives, to estimate the biomass (or, less justifiably, abundance) of a functional microbial group. However, the assumptions underlying measurement of potential rate are often not considered, obeyed or tested and have been increasingly challenged as our understanding of the composition and diversity of microbial communities in natural environments has increased. The value, limitations and relevance of potential rate measurements in microbial ecology are illustrated below for soil nitrification. However, the factors defining the usefulness and limitations of potential assays will also apply to other processes and functional groups, some of which encompass far greater taxonomic or functional diversity. For example, potential denitrification assays are typically performed using one set of incubation condition only and attempt to target organisms that represent a far greater level of taxonomic and physiological diversity than nitrifiers. In such circumstances, predictive power will likely be even further reduced.

Potential nitrification rate

Nitrification is the sequential oxidation of ammonia (NH_3) to nitrite (NO_2^-) and nitrate (NO_3^-) and is an essential component of reactive nitrogen transformation in soil. While nitrite can accumulate in soil (Giguere et al., 2017; Ma et al., 2015; Taylor et al., 2019; Venterea and Rolston, 2000), particularly after N deposition (Venterea et al., 2020), ammonia oxidation is often assumed to be the rate-limiting step. As a consequence, the activity, diversity and community structure of ammonia oxidisers (AO) receives greatest attention with respect to understanding environmental factors that influence microbially-mediated nitrification. Potential nitrification rate (PNR) assays (also referred to as potential nitrification activity (PNA)) have been used for many decades as a rapid method for estimating net ammonia oxidation (≈nitrification) rates in soils.

While a range of PNR assays have been used, the most common approach is the shaken soil-slurry method (Belser and Mays, 1980; Hart et al., 1994). This typically involves incubation of a suspension of sieved soil in a solution containing NH_4^+, at non-limiting concentration, in a shaken flask, ensuring aeration and mixing, at an incubation temperature that is considered to be optimal. PNR is calculated as the gradient of the linear increase in nitrate concentration in samples taken regularly during a relatively short incubation period (often ≤24 h) (Hart et al., 1994). A potassium phosphate buffer, typically between pH 7.0 and 7.5, can be included in the assay solution, with the intention of optimizing pH for AO. Chlorate, an inhibitor of nitrite oxidation, is also commonly included and NO_2^- rather than NO_3^- is measured in each slurry sample (Belser and Mays, 1980). Growth is assumed to be negligible; hence the

assumption of a linear, rather an exponential increase in nitrite or nitrate concentration. PNR therefore measures the activity of the community and not specific growth rate. Linear kinetics are often not tested and it is not unusual for calculations to be based on nitrate concentrations measured only at the beginning and end of incubation or even using just the final value. Any growth, or other divergence from linear kinetics, invalidates calculation of a 'constant' PNR.

Employment of 'ideal' or 'optimal' conditions attempts to ensure that all AO within the sample are active, which is further encouraged by addition of substrate (NH_4^+). The assumption of universal activity was implicit in the traditional use of PNR to estimate AO abundance in soil. This assumption was always seen as a major limitation of this approach (Woldendrop and Laanbroek, 1989; Hart et al., 1994), particularly given the low growth rates of ammonia oxidisers. (The only alternative was use of the most probable number method, which also relies on incubation of dilutions of soil suspensions under 'ideal' conditions and similar assumptions.)

Physiological diversity of ammonia oxidisers challenges underlying assumptions of PNR

For more than a century, ammonia oxidation was thought to be performed mainly by ammonia oxidising bacteria (AOB) (Prosser and Nicol, 2012). The past 25 years have, however, demonstrated considerable phylogenetic diversity within AOB and also the capacity for ammonia oxidation by ammonia oxidising archaea (AOA) (Könneke et al., 2005) and complete ammonia oxidizers (comammox) of the genus *Nitrospira* (Daims et al., 2015; van Kessel et al., 2015). Evidence from both cultivation-based and ecological studies also provides evidence for substantial physiological diversity in AO. Physiological diversity of cultivated AOB is well established and was used for traditional classification of these organisms (Prosser 1989; Koops et al., 2003). Since the first isolation of an ammonia oxidising archaeon (AOA), *Nitrosopumilus maritimus* (Könneke et al., 2015), several enrichments and isolates of soil AOA have also been obtained. These include, for example, the neutrophile *Nitrososphaera viennensis* EN76 (Tourna *et al.* 2011) and the obligate acidophile *Candidatus* Nitrosotalea devanaterra ND1 (Lehtovirta-Morley *et al*., 2011). In addition, comammox *Nitrospira* are abundant and contribute to ammonia oxidation in soil (Pjevac et al., 2017; Wang et al., 2019a), and a gammaproteobacterial AOB (*Candidatus* Nitrosoglobus terrae) was isolated recently (Hayatsu *et al.*, 2017). This taxonomic diversity is reflected in ecophysiological diversity and the maximum specific growth rate, cell specific activity, inhibitory ammonia and nitrite concentrations and affinity for ammonia of AO all vary by over an order of magnitude among different soil isolates, both within and between the major ammonia oxidiser groups (summarised in Lehtovirta-Morley et al., 2016). In addition, many AO are inhibited by NO_2^-, which accumulates when chlorate is

136 used to inhibit nitrite oxidisers, thereby reducing PNR depending on community composition.
137 Thus, even if the incubation conditions (temperature, pH, oxygen concentration) are appropriate
138 for all AO, the PNR will represent only an aggregate of a wide range of cell activities and will
139 vary with community composition and not just abundance.

140 Physiological diversity does, however, also mean that incubation conditions required
141 for activity of all AO will not be identical. Cultivation-based studies have demonstrated
142 significant differences in effects of temperature (Jiang and Bakken, 1999; Tourna et al., 2011;
143 Lehtovirta-Morley et al., 2014), pH (Allison and Prosser, 1991; Lehtovirta-Morley et al., 2011)
144 and salinity (Mosier et al., 2012; Qin et al., 2014; Bello et al., 2019). Similar evidence of
145 physiological diversity is provided by ecological studies (Nicol et al., 2008; Ollivier et al., 2012;
146 Gubry-Rangin et al., 2017; Nacke et al., 2017; Taylor et al., 2017; Lu et al. 2018) and the
147 ubiquity of AO in the full range of soils, in itself, is evidence that a single set of incubation
148 conditions will be insufficient. For example, ammonia supply is an important factor influencing
149 niche differentiation between AOA and AOB populations in soil. AOB often dominate growth
150 at higher concentrations of added inorganic ammonium (e.g. Jia and Conrad, 2009; Xia et al.,
151 2011; Hink et al., 2017), while AOA appear to prefer ammonium that is derived from a
152 mineralised organic N source, i.e. that is part of the native organic component of the soil or
153 added organic N (Levičnik-Höfferle et al., 2012; Hink et al., 2018). In low pH soils, AOA make
154 a greater relative contribution to ammonia oxidation under specific incubation conditions
155 (Gubry-Rangin et al., 2010; Zhang et al., 2012) and, as such, a decreasing proportion of the
156 ammonia oxidising community may preferentially oxidise added inorganic N with decreasing
157 soil pH.

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

162

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

164 The consequences of these limitations can be seen when correlations are determined between
165 published PNR for soils and sediments and AOA and AOB abundances (using *amoA* gene
166 abundance as a proxy for cell abundance). Results are highly variable. Of 107 studies published
167 from 2007 to 2020 (Supplementary Table 1), only 36% and 75% reported a significant positive
168 correlation between PNR and AOA or AOB abundances, respectively (Figure 1). Ten percent
169 and 46% report a positive correlation with only AOA or only AOB, respectively, 11%

170 correlated with neither and 4% and 5% reported a negative correlation with AOA and AOB,
171 respectively. No correlation has yet been observed between PNR and comammox bacteria
172 (Wang *et al*. 2019b; Luchibia *et al*. 2020; Zhang *et al.*, 2020). While these studies suggest that
173 some populations of AOA are using added ammonium, the majority of studies indicate that
174 only ammonia oxidising bacteria (AOB) contribute to PNR, often leading to the conclusion that
175 AOA are not contributing to ammonia oxidation in a soil *in situ*. If we consider the growth
176 characteristics of AO in both culture and soil, it is evident that correlating results from a single
177 defined assay with the abundance of communities containing substantial physiological diversity
178 is unlikely to be meaningful and likely to be misleading.

179 The observed dominance of AOB over AOA in contributing to PNR activity in many
180 soils may reflect differences in their ability to utilise added inorganic nitrogen. To investigate
181 this, soil was sampled across a pH 4.5 to 7.5 gradient that has been fully characterised with
182 respect to AOA and AOB community structure and abundance (Aigle et al., 2019; Gubry-
183 Rangin et al. 2011), where *amoA* transcript and gene abundance of AOA and AOB decrease
184 and increase directly with soil pH, respectively (Prosser and Nicol, 2012). PNR assays were
185 established with both standard 1 mM phosphate buffered and unbuffered 1.5 mM NH_4^+
186 solution, and parallel incubations were performed in the presence of alkynes to differentiate
187 contributions of all AO, AOA and AOB. Acetylene (C_2H_2) has been extensively used as an
188 inhibitor of all ammonia oxidisers that possess ammonia monooxygenase (AMO) and 1-octyne
189 (C_8H_2) inhibits growth of AOB, but not AOA, in both culture and soil (Taylor et al., 2013).
190 Nitrate accumulation during incubation for 24 h was used to calculate PNR, which increased
191 directly with soil pH across the gradient (Figure 2). However, as this activity was completely
192 inhibited in the presence of both C_8H_2, and C_2H_2, it was attributed solely to AOB. This provides
193 strong evidence that AOA do not contribute to PNR in some soils, PNR severely underestimates
194 their abundance and this prevents any meaningful interpretation of their abundance or actual
195 activity in soil at the time of sampling.

196 As indicated above, the source of ammonium is also an important factor in defining the
197 ecological niches of AO populations within a community. Using concentrations typical of
198 fertilisation application rates, we compared nitrification activity in both pH 4.5 and 7.5 soil
199 microcosms after the addition of ammonium nitrate or urea (which is fully mineralised to
200 ammonium within 0.5 days). After near stoichiometric conversion of ammonium to nitrate over
201 12 days, inorganic and urea-derived ammonium were oxidised at comparable rates for both 100
202 and 200 μg N g^{-1} dry soil applications in the pH 7.5 soil (Figure 3). The greatest change in pH
203 in soil microcosms was associated with those with the highest nitrification rates, indicating that

204 pH change was not an inhibiting factor (Supplementary Figure 1). Nevertheless, these results
205 highlight an additional concern, as changing conditions during incubation will influence
206 activity and kinetics will no longer be linear. In contrast, urea-derived ammonium was oxidised
207 at a faster rate in the pH 4.5 soil, and ammonia oxidation was inhibited by application of 200
208 μg inorganic N g^{-1} dry soil (Figure 3). While these data do not differentiate the contribution of
209 different AO groups, they reveal that sources of N other than inorganic NH_4^+, as used in PNR,
210 are preferred by some AO populations, again preventing meaningful interpretation of PNR data.
211 These two experiments, performed using only one experimental agricultural soil system,
212 are clearly not meant to be considered representative of all other managed or natural soil
213 systems. They do, however, demonstrate that PNR does not effectively characterise the
214 contribution of all AO populations to nitrification, but selects for those that are most
215 competitive under one set of incubation conditions. Despite the major limitations discussed
216 above, potential process rates are still frequently used inappropriately to address ecological
217 questions. Attempts to understand the relative contribution of AOA and AOB to nitrification
218 often involves correlation of their abundances with PNR, often after a treatment or perturbation
219 (e.g. fertiliser application or temperature change) (Supplementary Table 1). This approach is
220 also used to study niche specialisation, which implicitly assumes that changes in relative
221 abundance of different phylotypes will result from differences in activity under different
222 conditions. Incubation conditions used to measure PNR will favour different members of the
223 AOA and AOB communities and will differentially influence activities of AOA and AOB. It
224 will therefore not be possible to determine whether relationships between abundance and PNR
225 are due to differences in preferences of the different communities for the specific incubation
226 conditions used or due to links between abundance and actual activity. A more fundamental
227 issue is the assumption, implicit in niche specialisation, that changes in community composition
228 result from differences in actual activity, determined by different *in situ*, environmental
229 conditions and do not depend on potential activity under a single set of laboratory conditions.
230
231 *When can potential rate measurements be useful?*
232 Potential functional assays aim to employ incubation conditions that enable maximum or
233 optimal activity for all organisms performing a particular microbial function in an
234 environmental sample. They therefore provide a means of estimating the biomass of functional
235 groups and standard conditions have been developed for such assays. This is exemplified by
236 traditional use of potential nitrification rate, potential denitrification rate and substrate induced
237 respiration as measures of biomass of nitrifiers, denitrifiers and 'total' microbial biomass in

238 soil. This article has described the major disadvantages of this approach and such assays have
239 very limited value as measures of biomass. Alternative methods are available and have largely
240 been replaced for many functional groups by estimates of functional gene abundance. While
241 these have their own limitations, they do not rely on or describe activity.

242 These traditional potential assays must, however, be distinguished from the use of
243 laboratory incubations, in liquid culture, soil slurries or soil microcosms to study soil
244 microbiology. Many such studies measure activity following substrate amendment and under
245 controlled incubation conditions. Indeed, such studies are often used to investigate the effects
246 of substrate type and concentration and environmental conditions on functional groups, and
247 subgroups, to distinguish their activities and to investigate the influence of soil conditions.
248 While such studies effectively measure 'potential' activity, this term is rarely used and is never
249 used to imply that all organisms will be active or that the activity of each is optimal and
250 identical. Laboratory incubations are ecologically meaningful if the limitations of the approach
251 are critically evaluated with respect to their ability to assess activities in the environment
252 (Taylor et al., 2019). For example, with respect to nitrification, they have demonstrated
253 differences in the optimum temperature of AOA and AOB activity (Taylor *et al.*, 2017),
254 ammonia source preference (Levičnik-Höfferle *et al.*, 2012) and nitrification-associated N_2O
255 yields (Hink *et al.*, 2017). For other functional groups they have, for example, revealed the
256 presence of both high- and low-affinity methane oxidisers in soil (Bender and Conrad, 1992)
257 and greater influence on denitrification by edaphic factors than N-reducing community
258 composition (Attard *et al.*, 2011). In these instances, it is the relative changes in activity that
259 provide valuable insights into the ecophysiology of different functional groups and not
260 inference of a direct measurement of activity in the natural environment.

261 The use of *ex situ* potential activity assays for inferring *in situ* activity is not justified
262 and better measures are available. For example, soil nitrification activity can be measured as
263 gross nitrification rates using ^{15}N pool dilution methods (Hart et al., 1996). These techniques
264 may be more time-consuming and require specialist equipment, unlike PNR assays, but they
265 provide environmentally relevant results. Where PNR has been used to evaluate the effect of
266 an environmental factor, observations of relative differences in PNR will reflect effects on a
267 particular set of nitrifying populations, and the data must be recognised as only reflecting the
268 changing activities of these populations and not an overall community response. This is, of
269 course, the same situation for any functional group utilising a specific substrate, but curiously
270 this is often ignored. PNR does not characterise the contribution of all AO to nitrification in
271 soil and knowledge of the diversity of AO physiology and factors controlling their growth

clearly challenges a long-established assumption that a single incubation assay measures the activity of all AO. Critically, potential rates can have little relevance to actual rates *in situ* and therefore have limited meaning in many nitrification studies which should discourage the use of PNR in many circumstances.

Our expanding knowledge of the vast diversity and composition of microbial communities in natural environments emphasises that communities contributing to a functional process comprise a diverse array of ecophysiologies with substantial niche differentiation between populations. This challenges the underlying assumptions in using traditional potential rate measurements to estimate microbial biomass or *in situ* activity. However, controlled and manipulated laboratory studies that consider the underlying assumptions implicit in their use have been, and will continue to be, an important tool for mechanistic studies of soil microbiology.

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Figures

Figure 1. Meta-analysis of soil and sediment studies finding significant positive correlations of AOA and/or AOB abundances with PNR. All studies used a shaken slurry method and measured NO_3^- or NO_2^- with the addition of chlorate to inhibit NO_2^- oxidation. A total of 107 studies were included (Supplementary Table S1).

Figure 2. Demonstration of only AOB contributing to PNR. Soils were sampled across a pH gradient and PNR determined using the shaken slurry method. Flasks were established in triplicate using 144 ml serum vial bottles with 6 g soil (dry weight equivalent) and 50 ml of 1.5 mM NH_4Cl, with or without 1 mM potassium phosphate (pH 7.2) buffer. (a) Samples were taken after incubation for 0, 3, 6, 9, 12 and 24 h to determine nitrate concentrations (nitrite was not detected). Means (±SE) with different letters within a buffer treatment are significantly different based on Tukey's *post hoc* test following one-way ANOVA ($p<1.1 \times 10^{-7}$). Means annotated with a line are significantly different between buffer treatments based on two sample *t*-tests ($p>0.01<0.05$). (b) Example of complete inhibition of nitrification activity in flasks incubated in the presence of 0.01% C_2H_2 or 0.03% C_8H_2 with pH 7.5 soil (unbuffered). Nitrification activity was not detected in any soil in the presence of either alkyne.

Figure 3. Nitrate (NO_3^-) production in soil microcosms amended with varying concentrations of ammonium sulphate or urea (0 – 200 ug N g^{-1} dry soil) in pH 7.5 and 4.5 soil ((a) and (b), respectively). Soil microcosms were prepared (in triplicate for each treatment and timepoint) with sterile 144 ml serum vial glass bottles using 10 g soil (dry weight equivalent) which was sampled at 0-10 cm depth and sieved (2 mm mesh size). Microcosms were incubated at 30°C for 12 days with 30% soil water content (w/w). Microcosms were aerated every 3 days and destructively sampled before extracting NO_3^- in a 1 M KCl solution. Nitrate concentration was determined colorimetrically following Hink et al. (2018) immediately after sampling (NO_2^- was not detected). Error bars represent standard error of the mean. The greatest change in pH in soil microcosms was associated with those with the highest nitrification rates, indicating that pH change was not an inhibiting factor (Supplementary Figure 1). Nevertheless, these results highlight an additional concern, as changing conditions during incubation will influence activity and kinetics will no longer be linear.

510 **Supplementary Figure 1.** Soil pH in soil microcosms amended with varying concentrations of
511 ammonium sulphate or urea (0 – 200 ug N g-1 dry soil) in pH 7.5 and 4.5 soil ((a) and b),
512 respectively). The standard errors of the mean pH of triplicate microcosms were ≤0.06 and are
513 not visible behind markers.
514
515 **Supplementary Table 1.** Analysis of 107 studies correlating PNR with the abundance of AOA,
516 AOB or comammox bacteria as (inferred from qPCR analysis of *amoA* gene abundance).
517 Published studies were found with a SCOPUS search (TITLE-ABS-KEY ammonia AND
518 oxidi* AND archaea AND bacteria AND potential AND nitrification) combined with manual
519 refinement for only soil and sediment studies that used a shaken soil-slurry method.
520 Publications are grouped by soil or sediment and NO_2^- or NO_3^- measured as the end product.
521 The majority of correlations were positive (X) although nine negative correlations (-X) were
522 found. There were no correlations with comammox bacteria.
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