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1 **Limited accessibility of nitrogen supplied as amino acids, amides, and amines as energy**
2 **sources for marine *Thaumarchaeota***
3
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24 **SUMMARY**

25 Genomic and physiological evidence from some strains of ammonia-oxidizing *Thaumarchaeota*
26 demonstrate their additional ability to oxidize nitrogen (N) supplied as urea or cyanate, fueling
27 conjecture about their ability to conserve energy by directly oxidizing reduced N from other
28 dissolved organic nitrogen (DON) compounds. Similarly, field studies have shown rapid
29 oxidation of polyamine-N in the ocean, but it is unclear whether *Thaumarchaeota* oxidize
30 polyamine-N directly or whether heterotrophic DON remineralization is required. We tested
31 growth of two marine *Nitrosopumilus* isolates on DON compounds including polyamines, amino
32 acids, primary amines, and amides as their sole energy source. Though axenic cultures only
33 consumed N supplied as ammonium or urea, there was rapid but inconsistent oxidation of N
34 from the polyamine putrescine when cultures included a heterotrophic bacterium. Surprisingly,
35 axenic cultures oxidized ^{15}N-putrescine during growth on ammonia, suggesting co-metabolism
36 or accelerated breakdown of putrescine by reactive metabolic byproducts. Nitric oxide, hydrogen
37 peroxide, or peroxynitrite did not oxidize putrescine in sterile seawater. These data suggest that
38 the N in common DON molecules is not directly accessible to marine *Thaumarchaeota*, with
39 thaumarchaeal oxidation (and presumably assimilation) of DON-N requiring initial heterotrophic
40 remineralization. However, reactive byproducts or enzymatic co-metabolism may facilitate
41 limited thaumarchaeal DON-N oxidation.

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42 **INTRODUCTION**

43 While chemoautotrophy supported by ammonia (NH_3) oxidation is the primary
44 metabolism of most *Thaumarchaeota* (Francis *et al.*, 2007; Stahl and de la Torre, 2012; Santoro
45 *et al.*, 2019), there has also been speculation on their additional capacity to oxidize nitrogen (N)
46 from a variety of dissolved organic nitrogen (DON) compounds. Numerous experiments have
47 demonstrated that some *Thaumarchaeota* can grow via oxidation of N supplied as urea:
48 following the discovery of urease and urea transporter genes in the sponge symbiont
49 *Cenarchaeum symbiosum* (Hallam *et al.*, 2006), thaumarchaeal urease genes were documented
50 throughout the ocean (Yakimov *et al.*, 2011; Alonso-Saez *et al.*, 2012; Smith *et al.*, 2016), rate
51 measurements using ^{15}N tracers showed moderate urea-N oxidation in coastal waters (Tolar *et*
52 *al.*, 2017; Damashek *et al.*, 2019a; Kitzinger *et al.*, 2019; Laperriere *et al.*, 2021), and marine
53 *Thaumarchaeota* capable of growth via stoichiometric oxidation of urea-N to nitrite (NO_2^-) were
54 isolated (Qin *et al.*, 2014; Bayer *et al.*, 2016; Carini *et al.*, 2018). In addition to urea, a
55 thaumarchaeote isolated from a hot spring contains a cyanate hydratase gene (*cynS*) and can
56 grow via oxidation of cyanate-N (Palatinszky *et al.*, 2015). Oxidation of both urea- and cyanate-
57 N by *Thaumarchaeota* appears to drive NO_2^- production in the northern Gulf of Mexico
58 (Kitzinger *et al.*, 2019). These studies provide ample evidence of widespread thaumarchaeal
59 oxidation of urea-N, and likely cyanate-N, throughout the ocean.

60 In addition to urea and cyanate, there is interest in whether *Thaumarchaeota* can directly
61 oxidize N from other common DON compounds. Early studies of marine microbial assemblages
62 demonstrated thaumarchaeal amino acid assimilation (Ouverney and Fuhrman, 2000; Teira *et al.*,
63 2006; Kirchman *et al.*, 2007), and recent experiments in the coastal Pacific Ocean demonstrated
64 assimilation of N but not carbon (C) from amino acids, prompting speculation about the ability

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65 of *Thaumarchaeota* to oxidize amino acid-N directly (Dekas *et al.*, 2019). However, data on
66 thaumarchaeal DON-N oxidation rates are scarce. ^{15}N-tracer experiments in the coastal South
67 Atlantic Bight, where *Thaumarchaeota* are the dominant ammonia oxidizers (Hollibaugh *et al.*,
68 2011; Hollibaugh *et al.* 2014; Tolar *et al.*, 2017; Liu *et al.*, 2018; Damashek *et al.*, 2019a), not
69 only measured detectable oxidation of N supplied as urea and L-glutamic acid (L-GLU), but
70 found oxidation rates of N from the polyamine putrescine (1,4-diaminobutane; PUT) to be far
71 higher than oxidation rates of urea- or amino acid-N (Damashek *et al.*, 2019a). Polyamines
72 consist of a reduced C backbone with at least two amine substitutions (Tabor and Tabor, 1984).
73 Ubiquitously high intracellular concentrations (~mM) of polyamines such as PUT, spermine, and
74 spermidine (Tabor and Tabor, 1985; Liu *et al.*, 2016) and rapid turnover rates in seawater (Lee
75 and Jørgensen, 1995; Liu *et al.*, 2015) suggest fast microbial cycling of polyamines in the marine
76 environment, including oxidation of polyamine-N (Damashek *et al.*, 2019a). However, it is
77 unknown whether *Thaumarchaeota* can conserve energy by oxidizing polyamine- or amino acid-
78 N directly, or if prior heterotrophic remineralization to ammonium (NH_4^+) is required.
79 We tested the ability of two thaumarchaeal strains originally isolated from the northern
80 Adriatic Sea (*Nitrosopumilus piranensis* D3C and *N. adriaticus* NF5; Bayer *et al.*, 2016; Bayer
81 *et al.*, 2019a) to grow using a variety of DON compounds as sole energy sources. Given prior
82 evidence of rapid PUT-N oxidation in the field, our primary focus was polyamines. Axenic
83 thaumarchaeal cultures were unable to grow when supplied with single DON compounds, other
84 than the expected growth on urea by *N. piranensis* D3C. However, experiments with ^{15}N-labeled
85 compounds demonstrated that axenic cultures of both strains oxidized a significant amount of
86 PUT-N when growing on NH_3, suggesting PUT may be co-metabolized or broken down by
87 reactive byproducts produced during thaumarchaeal growth. Furthermore, enrichment cultures

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88 containing a heterotrophic bacterium occasionally showed rapid PUT-N oxidation, indicating an
89 important role for heterotrophic DON remineralization in this process. Our data indicate that
90 oxidation of reduced N by marine *Nitrosopumilaceae* is restricted to well-known substrates, but
91 suggest breakdown of DON and oxidation of its liberated N may occur as an indirect effect of
92 thaumarchaeal growth or due to tight coupling between DON remineralization by heterotrophs
93 and subsequent thaumarchaeal oxidation of the resulting NH_4^+.
94

95 RESULTS

96 GROWTH EXPERIMENTS

97 Growth experiments were conducted with axenic cultures of *Nitrosopumilus* strains D3C
98 and NF5 (Bayer *et al.*, 2019a) grown in NH_4^+-free Synthetic Crenarchaeota Medium (SCM;
99 Könneke *et al.*, 2005) amended with a variety of single DON compounds (1 or 2 mM added N;
100 Table 1) as their sole energy and N source. Small amounts of NO_2^- (10-50 μM) were linearly
101 produced over 70 days in all treatments (including negative controls with no added NH_4^+ or
102 DON), presumably due to NH_4^+ contamination or breakdown and oxidation of media
103 components such an antibiotics. Both strains converted NH_3 into NO_2^- stoichiometrically within
104 7 days, and D3C converted urea-N into NO_2^- within ~14 days (Table 1; Fig. S1), consistent with
105 previous data from these isolates (Bayer *et al.*, 2016; Bayer *et al.*, 2019a). A small fraction (20-
106 30%) of the glutamine-N amendment in both strains was converted to NO_2^- linearly with time.
107 None of the other DON amendments led to NO_2^- production (Table 1; Fig. S1).

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Table 1 NO_2^- produced (mean ± standard deviation of triplicates) by axenic thaumarchaeal strains in media containing single organic N compounds as the sole energy source. Bolded text shows treatments with at least one isolate accumulating NO_2^- to a level greater than the negative control treatments.

			D3C		NF5	
Substrate	Amendment (µM)	N Addition (µM)	NO_2^- Produced (µM)	Available N Oxidized (%)	NO_2^- Produced (µM)	Available N Oxidized (%)
Ammonium	1000	1000	**951.8 ± 10.4**	**95.2 ± 1.0**	**982.1 ± 19.3**	**98.2 ± 1.9**
Urea	500	1000	**1034.5 ± 16.9**	**103.5 ± 1.7**	8.2 ± 7.0	0.8 ± 0.7
1,2-diaminoethane	500	1000	16.7 ± 4.1	1.7 ± 0.4	17.2 ± 1.4	1.7 ± 0.1
1,3-diaminopropane	500	1000	23.4 ± 2.3	2.3 ± 0.2	23.4 ± 3.0	2.3 ± 0.3
Putrescine	500	1000	43.9 ± 1.9	4.4 ± 0.2	16.0 ± 3.1	1.6 ± 0.3
Cadaverine	500	1000	21.7 ± 1.2	2.2 ± 0.1	23.4 ± 2.2	2.3 ± 0.2
1,7-diaminoheptane	500	1000	23.4 ± 0.6	2.3 ± 0.1	22.1 ± 0.8	2.2 ± 0.1
Spermine	500	2000	48.3 ± 3	2.4 ± 0.2	23.9 ± 7.6	1.2 ± 0.4
Spermidine	500	1000	45.9 ± 1.8	4.6 ± 0.2	13.7 ± 3.6	1.4 ± 0.4
Methylamine	1000	1000	39.9 ± 1.8	4.0 ± 0.2	13.5 ± 3.8	1.4 ± 0.4
L-Glutamate	1000	1000	43.9 ± 3	4.4 ± 0.3	13.0 ± 5.7	1.3 ± 0.6
L-Glutamine	500	1000	**299.1 ± 6.1**	**29.9 ± 0.6**	**212.5 ± 4.4**	**21.3 ± 0.4**
L-Arginine	500	2000	48.7 ± 3.1	2.4 ± 0.2	26.8 ± 0.7	1.3 ± 0.0
L-Asparagine	500	1000	32.3 ± 2.5	3.2 ± 0.3	45.2 ± 2.1	4.5 ± 0.2
L-Glycine	1000	1000	32.5 ± 2.8	3.3 ± 0.3	42.8 ± 1.4	4.3 ± 0.1
L-Ornithine	500	1000	27.1 ± 2	2.7 ± 0.2	38.0 ± 1.3	3.8 ± 0.1
Acetamide	1000	1000	28.2 ± 2.4	2.8 ± 0.2	41.2 ± 1.4	4.1 ± 0.1
Formamide	1000	1000	45.1 ± 0.7	4.5 ± 0.1	52.8 ± 0.8	5.3 ± 0.1
Nicotinamide	1000	2000	33.1 ± 2.3	1.7 ± 0.1	41.2 ± 1.8	2.1 ± 0.1
Propionamide	1000	1000	34.7 ± 3.4	3.5 ± 0.3	37.9 ± 1.8	3.8 ± 0.2
Butyramide	1000	1000	39.4 ± 5.4	3.9 ± 0.5	37.3 ± 4.9	3.7 ± 0.5
Water	0	0	42.3 ± 3.4		41.9 ± 2.4	

108 Further growth experiments 109 were conducted with enrichment 110 cultures of *Nitrosopumilus* strains 111 D3C and NF5 containing ~5-15% 112 bacterial 16S rRNA genes (Table 113 2) belonging to the heterotrophic 114 bacterium *Oceanicaulis alexandrii* 115 (Bayer *et al.*, 2016). Growth on 116 NH_3 or urea proceeded rapidly and 117 matched published rates (Bayer *et* 118 *al.*, 2016). NO_2^- accumulation 119 during growth of enrichments on

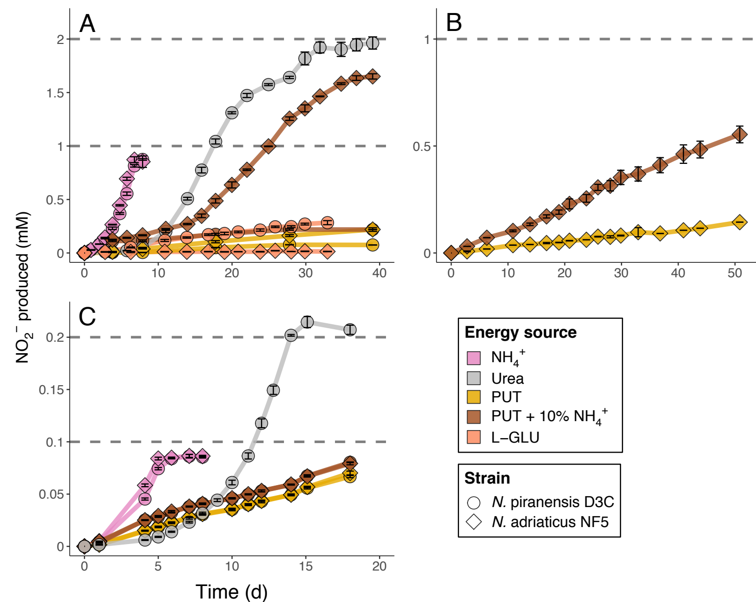

Fig. 1 NO_2^- accumulation by enrichment cultures of *N. piranensis* D3C (circles) and *N. adriaticus* NF5 (diamonds) over time. Color denotes the compound included as sole energy source in media. Points show the average of triplicate incubation bottles while error bars show standard deviation. Dashed lines show maximal NO_2^- production given added N (two lines are present to show total added N when NH_4^+ and urea were included). **A)** Growth with either 1 mM NH_4^+, 1 mM urea, 1 mM PUT, 1 mM PUT plus 100 µM NH_4^+, or 1 mM L-GLU. **B)** Growth with either 1 mM PUT or 1 mM PUT plus 100 µM NH_4^+ (*N. adriaticus* NF5 only). **C)** Growth with either 100 µM NH_4^+, 100 µM urea, 100 µM PUT, or 100 µM PUT plus 10 µM NH_4^+. Bacterial and thaumarchaeal 16S rRNA gene quantities for selected experiments (panels A, C) are shown in Table 2.

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120 NH_3-free SCM amended with either 500 µM PUT or 500 µm PUT + 50 µM NH_4^+ was often
121 linear and relatively slow (Fig. 1), but occasionally resembled a typical microbial growth curve
122 (e.g., *N. adriaticus* NF5 grown with PUT and NH_4^+ amendment; Fig. 1 A). During growth on
123 NH_3, thaumarchaeal and bacterial 16S rRNA genes in selected treatments (those shown in Fig. 1
124 A, C) generally retained the same relative abundance as the respective inoculum (~90-95%
125 thaumarchaeal genes) and attained abundances comparable to the initial inoculum by the end of
126 the experiment (Table 2). Thaumarchaeal and bacterial genes gradually increased in treatments
127 containing PUT, with bacterial genes often reaching higher abundances than the initial inoculum
128 (Table 2). Nitrite concentration increased rapidly when *N. adriaticus* NF5 was amended with
129 PUT + NH_4^+ (Fig. 1 A). Both populations of genes increased in this treatment; notably, bacterial
130 genes increased from
131 6.7×10^6 genes mL^{-1} in
132 the inoculum (8.2% of
133 16S genes) to 1.4–
134 4.5×10^8 genes mL^{-1}
135 (79.3–81.0% of 16S
136 genes) during the
137 incubation (Table 2).
138
139

Table 2 Quantification of bacterial and thaumarchaeal 16S rRNA genes via qPCR for selected experiments with enrichment cultures. NO_2^- accumulation in these experiments is shown in Fig. 1 A, C.

Strain	Substrate	Nutrients	Time (d)	16S rRNA genes mL^{-1} Bacterial	16S rRNA genes mL^{-1} Thaumarchaeal	% *Thaumarchaeota*
D3C	All D3C treatments	Fig. 1 A	Inoculum	1.5×10^7	9.4×10^7	86.3
NF5	All NF5 treatments	Fig. 1 A	Inoculum	6.7×10^6	7.5×10^7	91.8
NF5	NH_4^+	Fig. 1 A	8	7.8×10^6	5.8×10^7	88.1
NF5	NH_4^+	Fig. 1 A	8	6.6×10^6	6.5×10^7	90.9
NF5	Putrescine + NH_4^+	Fig. 1 A	37	4.5×10^8	1.2×10^8	20.7
NF5	Putrescine + NH_4^+	Fig. 1 A	37	1.4×10^8	3.4×10^7	19.0
D3C	All D3C treatments	Fig. 1 C	Inoculum	1.5×10^6	1.3×10^7	94.2
D3C	NH_4^+	Fig. 1 C	8	8.7×10^5	1.4×10^7	96.6
D3C	NH_4^+	Fig. 1 C	8	1.3×10^6	1.5×10^7	95.5
D3C	Urea	Fig. 1 C	8	1.3×10^6	7.3×10^6	91.9
D3C	Urea	Fig. 1 C	8	1.7×10^6	7.9×10^6	90.1
D3C	Urea	Fig. 1 C	14	1.1×10^6	3.4×10^7	98.4
D3C	Urea	Fig. 1 C	14	1.2×10^6	3.7×10^7	98.4
D3C	Urea	Fig. 1 C	18	1.1×10^6	3.1×10^7	98.3
D3C	Urea	Fig. 1 C	18	1.3×10^6	4.0×10^7	98.3
D3C	Putrescine	Fig. 1 C	8	1.0×10^6	6.1×10^6	91.4
D3C	Putrescine	Fig. 1 C	8	1.8×10^6	8.9×10^6	90.9
D3C	Putrescine	Fig. 1 C	14	1.2×10^6	8.6×10^6	93.6
D3C	Putrescine	Fig. 1 C	14	1.1×10^6	8.3×10^6	93.6
D3C	Putrescine	Fig. 1 C	18	3.6×10^6	1.2×10^7	87.0
D3C	Putrescine	Fig. 1 C	18	1.4×10^6	1.4×10^7	95.0
NF5	All NF5 treatments	Fig. 1 C	Inoculum	1.5×10^6	1.6×10^7	95.1
NF5	NH_4^+	Fig. 1 C	8	1.0×10^6	1.4×10^7	96.1
NF5	NH_4^+	Fig. 1 C	8	1.5×10^6	1.5×10^7	94.8
NF5	Putrescine	Fig. 1 C	8	1.6×10^6	6.4×10^6	89.1
NF5	Putrescine	Fig. 1 C	8	1.6×10^6	5.9×10^6	88.3
NF5	Putrescine	Fig. 1 C	14	1.0×10^6	7.2×10^6	93.6
NF5	Putrescine	Fig. 1 C	14	1.1×10^6	7.7×10^6	93.2
NF5	Putrescine	Fig. 1 C	18	3.0×10^6	9.7×10^6	86.7
NF5	Putrescine	Fig. 1 C	18	2.5×10^6	1.1×10^7	89.8

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140 ISOTOPE EXPERIMENTS

141 Both axenic *Nitrosopumilus* strains oxidized 100 µM NH_3 to NO_2^- within 6 days. When
142 1.25 µM ^{15}N-NH_4^+ was added to the growth media containing 100 µM unlabeled NH_4^+, all of the
143 ^{15}N was converted to ^{15}N-NO_2^- by stationary phase (NF5: 1.20 ^{15}N-NO_2^- µM produced; D3C:
144 1.09 µM; Fig. 2). Oxidation of polyamine-N was tested by adding ^{15}N-labeled 1,2-diaminoethane
145 (DAE), 1,3-diaminopropane (DAP), or PUT to the growth media. L-GLU was used as a control
146 for remineralization, given its rapid catabolism and NH_4^+ remineralization by marine
147 heterotrophs (Hollibaugh, 1978; Goldman *et al.*, 1987; Goldman and Dennett, 1991). Growth
148 with ^{15}N-labeled DAE, DAP, or GLU did not produce ^{15}N-NO_2^-, with values similar to the
149 negative control (^{14}N-NH_4^+ amendment, no added ^{15}N). During growth with ^{15}N-PUT, ~35% of
150 the added ^{15}N was oxidized to ^{15}N-NO_2^- as NH_3 was consumed (NF5: 0.45 ^{15}N-NO_2^- µM
151 produced; D3C: 0.43 µM; Fig. 2).

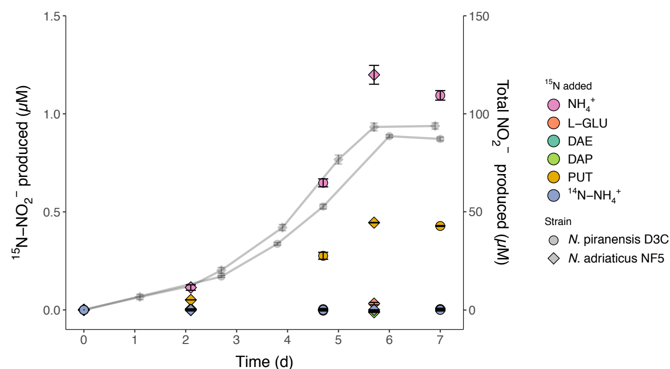

Fig. 2 ^{15}N-NO_2^- production from ^{15}N-labeled organic N compounds during growth of axenic cultures of *N. piranensis* D3C (circles) and *N. adriaticus* NF5 (diamonds) on NH_3. Gray points show total NO_2^- (^{14}N and ^{15}N; right y-axis) while points with color show concomitant production of ^{15}N-NO_2^- (left y-axis). All points represent the midpoint of duplicate incubation bottles, with error bars representing the range. The ^{14}N-NH_4^+ treatment was a negative control containing no added ^{15}N.

152 To test whether this ^{15}N-PUT oxidation was due to abiotic reactions with short-lived
153 reactive metabolic intermediates, ^{15}N-PUT was incubated in filtered-sterilized, oligotrophic
154 seawater or filtered-sterilized SCM dosed with nitric oxide (NO), peroxynitrite ($ONOO^-$), or
155 hydrogen peroxide (H_2O_2). Amendment with only NO_2^- or PUT provided controls with no
156 reactive compounds (Fig. 3 F). There was no significant difference in $\delta^{15}N_{NOx}$ values between

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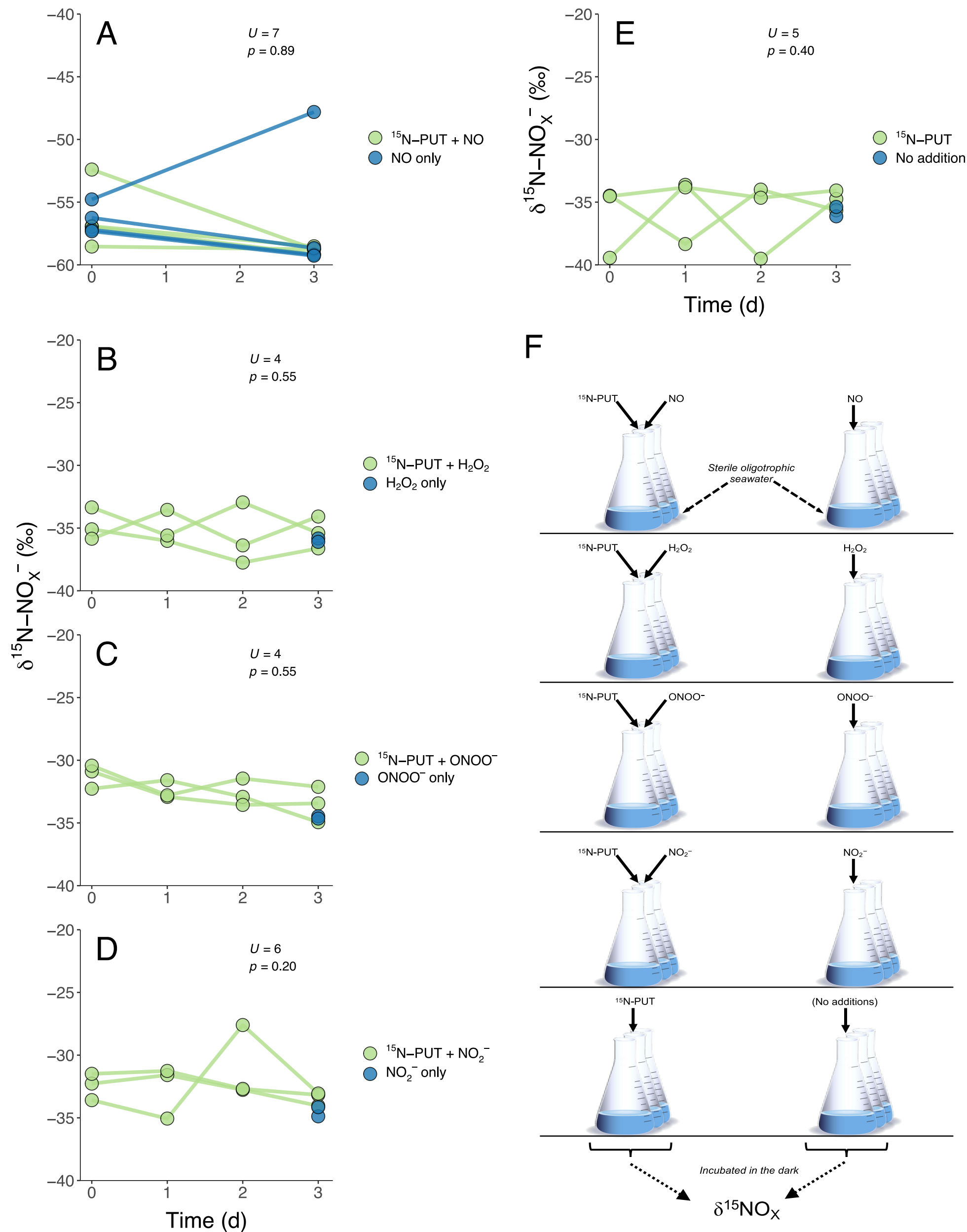

Fig. 3 A-E) $\delta^{15}NO_x$ (‰) over time in abiotic incubations containing ^{15}N-labeled PUT and a single reactive compound (blue) or a reactive compound only (green). Results of a Mann-Whitney *U*-test are shown in each panel. **F)** Schematic of abiotic incubation experiments. The left column shows treatments including ^{15}N-labeled PUT and a reactive compound while the right column shows control treatments containing a reactive compound and no ^{15}N-labled PUT.

157 incubations of culture medium or filtered seawater receiving NO additions (U=26, p=0.574);

158 therefore, NO addition experiments using either matrix were combined in data analyses.

159 There was no significant difference in the ratio of $^{15}N/^{14}N$ in the nitrate plus nitrite (NO_X) pool

160 ($\delta^{15}N_{NOx}$) at the termination of any of the incubations containing ^{15}N-PUT versus those without

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161 ^{15}N-PUT (Mann-Whitney *U*-tests, all $p \geq 0.2$; Fig. 3 A-E). Furthermore, there was no increase in
162 $\delta^{15}N_{NOx}$ values when NO gas was directly mixed with a variety of ^{15}N-labeled polyamines, L-
163 GLU, or NH_4^+ in sterile oligotrophic seawater (Mann-Whitney *U*-test, p=0.64; Fig. 4). NO_2^- in
164 NO-amended incubation endpoints was likely
165 produced via spontaneous NO oxidation (Lewis and
166 Deen, 1994), as NO_2^- was not detectable in
167 incubations without added NO (regardless of ^{15}N
168 addition). Therefore, NO_2^- concentration in
169 incubation endpoints likely reflects the concentration
170 of the initial NO amendment, and was fairly well
171 correlated to $\delta^{15}N_{NOx}$ values (Spearman's $\rho = -0.70$,
172 p=0.02; Fig. 4).

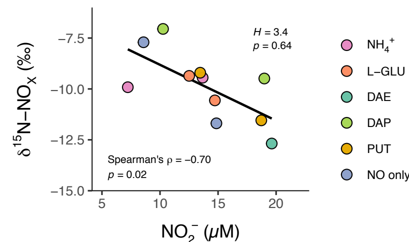

Fig. 4 $\delta^{15}NO_x$ (‰) as a function of NO_2^- concentration following 24 hours of abiotic incubation of sterile seawater amended with ^{15}N-labeled compounds and NO gas. NO (1% in N_2) was directly bubbled into seawater. Color denotes ^{15}N addition. Results of a Kruskal-Wallis test (comparing $\delta^{15}NO_x$ between treatment groups) and Spearman's correlation coefficient are shown. NO_2^- in these incubation endpoints was likely due to abiotic NO oxidation and therefore reflects the amount of added NO.

173

174 **DISCUSSION**

175 *Organic N oxidation by* Thaumarchaeota *relies on remineralization*

176 Polyamines are ubiquitous in cells (Tabor and Tabor, 1985; Nishibori and Nishijima,
177 2004; Liu *et al.*, 2016) and highly labile in aqueous environments (Lu *et al.*, 2015; Liu *et al.*,
178 2015; Krempaska *et al.*, 2017; Madhuri *et al.*, 2019). The amine groups at the ends of
179 polyamines are essentially NH_3 with an aliphatic C chain substituted for a hydrogen, and since
180 their pK_a values (~10.5, depending on chain length and temperature) are close to that of NH_3
181 (~9.25), they are similarly protonated in seawater. Thus, recent field evidence of rapid oxidation
182 of polyamine-N in *Thaumarchaeota*-rich ocean waters (Damashek *et al.*, 2019a) begs the
183 question of whether these archaea can use polyamine-N directly as an energy source.

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184 We tested this hypothesis using two *Nitrosopumilus* strains phylogenetically similar to
185 the *Thaumarchaeota* typical of South Atlantic Bight inshore waters (Hollibaugh *et al.*, 2011;
186 Hollibaugh *et al.*, 2014; Liu *et al.*, 2018; Damashek *et al.*, 2019b), where previously-measured
187 polyamine-N oxidation rates were high (Damashek *et al.*, 2019a). Efforts to grow *N. piranensis*
188 D3C and *N. adriaticus* NF5 with single polyamines as their sole energy source conclusively
189 demonstrated that they cannot grow by oxidizing N from PUT or other common polyamines
190 (Fig. S1). Previous experiments with the same strains found only minimal [^{3}H]leucine
191 incorporation, particularly when archaea were starved of NH_3, similarly suggesting no ability to
192 use leucine as a carbon or energy source (Bayer *et al.*, 2019a). There is long-standing
193 biogeochemical and genomic evidence that some marine *Thaumarchaeota* may use some organic
194 compounds as C sources (e.g., Ouverney and Fuhrman, 2000; Teira *et al.*, 2004; Swan *et al.*,
195 2014; Seyler *et al.*, 2018; Dekas *et al.*, 2019), and potentially even as an energy source (in
196 addition to NH_3; Dekas *et al.*, 2019). As with polyamines, we found no evidence of growth by
197 either *Nitrosopumilus* strain on a wide variety of common amino acids, amines, or amides (Table
198 1; Fig. S1). This suggests that NH_3, urea, and cyanate remain the only known growth substrates
199 of marine *Thaumarchaeota*.
200 The lack of evidence for direct oxidation of DON-N by pure cultures suggests that
201 observed rates of DON-N oxidation (and potentially assimilation) by *Thaumarchaeota* in mixed
202 communities are not a consequence of direct oxidation by *Thaumarchaeota* alone, but instead
203 couple heterotrophic DON-N remineralization to NH_4^+ with subsequent thaumarchaeal oxidation
204 of this newly-produced NH_4^+. Strong evidence for this conclusion comes from growth
205 experiments with enrichment cultures: the presence of a heterotrophic bacterium led to a
206 substantial accumulation of NO_2^- in the medium and an increase in the abundance of bacterial

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207 16S rRNA genes, especially when cultures were amended with both PUT and NH_4^+ (Fig. 1;
208 Table 2). In contrast, pure cultures of the same *Thaumarchaeota* were unable to oxidize N
209 supplied as PUT in the absence of NH_3 (Fig. S1). In addition to indicating a reliance of
210 thaumarchaeal DON-N oxidation on external remineralization, these findings may have similar
211 implications for experiments measuring DON assimilation by *Thaumarchaeota* in mixed
212 communities.

213 Polyamines are highly labile and energy-rich, as their backbone contains multiple fully-
214 reduced C atoms. It is not surprising that adding this rich C source led to rapid bacterial growth
215 in enrichment cultures, similar to the stimulation of heterotrophic communities documented in
216 field studies (Mou *et al.*, 2011; Mou *et al.*, 2015). Notably, the C:N ratio of PUT (2) is lower
217 than that of natural marine microbial cells (typically ~4-5; Goldman *et al.*, 1987; White *et al.*,
218 2019), suggesting PUT catabolism by marine bacteria will lead to production of excess NH_4^+
219 (Hollibaugh, 1978; Goldman and Dennett, 1991). Therefore, NO_2^- production in enrichment
220 cultures could result from bacterial catabolism of PUT, followed by oxidation of the resulting
221 NH_4^+ by *Thaumarchaeota*, typically leading to slow and linear NO_2^- accumulation (Fig. 1). The
222 occasional rapid accumulation of NO_2^- when NH_4^+ was added alongside PUT (Fig. 1 A) may
223 indicate the bacterium in this enrichment was unable to use PUT alone as an N source (i.e.,
224 needed free NH_4^+ to catabolize PUT), or may suggest bacterial dependence on *Thaumarchaeota*
225 (growing on the low concentration of NH_3) as a source of nutrients or organic substrates (e.g.,
226 Doxey *et al.*, 2015; Heal *et al.*, 2018; Bayer *et al.*, 2019b).

227 Since many DON compounds are highly labile C sources, it is imperative to
228 systematically account for rapid heterotrophic remineralization of N from DON in incubations of
229 mixed communities before concluding that *Thaumarchaeota* are capable of direct oxidation or

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230 assimilation of DON, ideally by using pure cultures to test field hypotheses. Urea is a clear
231 exception, since pure cultures of some marine *Thaumarchaeota* can grow with urea-N as their
232 only energy and N source (Qin *et al.*, 2017a; Bayer *et al.*, 2019a). Direct cyanate-N
233 oxidation/assimilation may also occur in marine *Thaumarchaeota*, but it is unclear how marine
234 *Thaumarchaeota* could directly oxidize cyanate-N. While pure cultures of *Nitrosopumilus*
235 *maritimus* SCM1 produced ^{15}N-NO_2^- when ^{15}N-cyanate was added to their growth media, this
236 only happened when they were already growing on NH_3 (Kitzinger *et al.*, 2019; see discussion
237 below). The pathway of cyanate oxidation (or NH_4^+ production from cyanate) is unclear since
238 their genomes lack canonical cyanase genes (Kitzinger *et al.*, 2019).
239 Of the myriad polyamines, amino acids, amines, and amides we tested as thaumarchaeal
240 growth substrates, the only compound leading to appreciable NO_2^- accumulation was glutamine:
241 when both axenic strains were supplied with glutamine as their sole energy source, there was a
242 slow, linear accumulation of NO_2^- (Fig. S1). Since the increase in NO_2^- concentration over time
243 did not resemble a growth curve, it is unlikely that the archaea were directly using glutamine-N
244 as a growth substrate. Although the mechanism is not clear, glutamine was probably slowly
245 remineralized to NH_4^+, which was then oxidized. A simple explanation is abiotic glutamine
246 deamination, which occurs relatively rapidly in liquid media containing phosphate and
247 bicarbonate at neutral pH (Gilbert *et al.*, 1949), such as the SCM used here. However, our data
248 cannot rule out a biological explanation. Glutamine is a common biochemical amino/amido
249 donor, and *Thaumarchaeota* encode many genes involved in these reactions (e.g., glutamine
250 amidotransferases or transaminases in amino acid and cofactor biosynthesis pathways; Walker *et*
251 *al.*, 2010; Kerou *et al.*, 2016) as well as a variety of putative amino acid transporters (Offre *et al.*,
252 2014). If excess glutamine is transported into thaumarchaeal cells, some may be recycled to

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253 NH_4^+ for either assimilatory or dissimilatory use. This amino acid “recycling” is common in
254 energy-starved microbes (Lever *et al.*, 2015) and was recently hypothesized to play a role in
255 thaumarchaeal survival in deep sea sediments (Kerou *et al.*, 2021). It is possible that energy-
256 starved *Thaumarchaeota* in our experiment transported glutamine into their cells but were unable
257 to incorporate it into biomass, so they hydrolyzed the glutamine amide N and shunted the
258 resulting NH_4^+ into energy production, leading to slow growth. Direct tests of thaumarchaeal
259 glutamine uptake and transformation compared to abiotic breakdown in SCM would be needed
260 to uncover the mechanism behind the slow glutamine oxidation documented here.
261

262 *Potential co-metabolism or abiotic breakdown of polyamines*

263 Although axenic cultures of marine *Nitrosopumilus* were unable to grow using PUT as
264 their sole energy and N source, the significant fraction of ^{15}N-PUT oxidized during growth on
265 NH_3 (Fig. 2), combined with the correlation between PUT-N and NH_3 oxidation rates in the
266 ocean (Damashek *et al.* 2019a), suggests PUT may be co-metabolized by the archaeal ammonia
267 monooxygenase enzyme (AMO) or decomposed by reactive intermediates produced during NH_3
268 oxidation (e.g., Martens-Habbena *et al.*, 2015; Kim *et al.*, 2016). Therefore, *Thaumarchaeota* in
269 environments with high polyamine availability and enough NH_3 for rapid growth may indirectly
270 oxidize a significant amount of PUT-N to NO_2^- despite their inability to use PUT-N as a direct
271 energy source, increasing the flux of N from DON into the NO_X pool and potentially
272 contributing to measured PUT-N oxidation rates in the ocean (Damashek *et al*., 2019a).
273 Co-metabolism of a variety of compounds due to non-specific oxidation by ammonia
274 monooxygenase is well documented in ammonia-oxidizing bacteria (AOB). Some AOB can, for
275 example, co-metabolize methane (Hyman and Wood, 1983; Ward, 1987), and some AOB and

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276 *Thaumarchaeota* can co-metabolize a variety of organic compounds (e.g., Rasche *et al.*, 1991;
277 Wright *et al.*, 2020). Furthermore, co-metabolism of some DON compounds has been
278 documented: two AOB strains and the thaumarchaeote *Nitrososphaera gargensis* can co-
279 metabolize the tertiary amines mianserin and ranitidine during growth on NH_3 (Men *et al.*,
280 2016). Given the close structural and chemical similarity between NH_3 and the primary amine
281 groups on PUT-N, it seems conceivable that archaeal ammonia monooxygenase may similarly be
282 able to oxidize PUT-N.

283 There has been recent recognition of the role played by reactive metabolic intermediates
284 (primarily NO and H_2O_2) in thaumarchaeal physiology, and of the importance of chemical
285 transformations catalyzed by these reactive compounds in culture experiments. For example,
286 pure thaumarchaeal isolates produce H_2O_2 (Kim *et al.*, 2016; Bayer *et al.*, 2019c) but lack
287 catalase to facilitate its rapid detoxification. Exposure of isolates or field populations to high
288 concentrations of H_2O_2 therefore arrests their growth and activity (Kim *et al.*, 2016; Tolar *et al.*,
289 2016; Qin *et al.*, 2017b; Bayer *et al.*, 2019c). NO, an obligate intermediate in the archaeal
290 ammonia oxidation pathway, accumulates during growth of thaumarchaeal cultures (Martens-
291 Habbena *et al.*, 2015; Kozlowski *et al.*, 2016; Sauder *et al.*, 2016; Hink *et al.*, 2017) and is found
292 in high concentrations in marine regions with high nitrification rates or abundant
293 *Thaumarchaeota* (Ward and Zafiriou, 1988; Lutterbeck *et al.*, 2018). NO is a reactive radical that
294 rapidly forms reactive nitrogen oxide species (RNOS; e.g., NO_2 or N_2O_3) upon exposure to O_2 or
295 superoxide (Wink and Mitchell, 1998). RNOS are highly destructive of many biological
296 molecules; relevant to our data, RNOS react with primary amines to produce nitrosamines that
297 are subsequently deaminated to NH_4^+ (Ridnour *et al.*, 2004). These reactions may be a
298 mechanism for liberating NH_4^+ from polyamines due to NO production during thaumarchaeal

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299 growth on NH_3. Similarly, reactions of NO with various compounds have been posited to explain
300 chemical transformations during thaumarchaeal growth: the abiotic reaction between NO and
301 reduced iron in growth media is thought to form nitrous oxide in cultures (Kozlowski *et al.*,
302 2016), and NO may react with cobalamin within thaumarchaeal cells, leading to nitrocobalamin
303 production (Heal *et al.*, 2018).

304 Given the capability of NO-derived RNOS to react with primary amines, speculation
305 about the reactivity of NO in thaumarchaeal cultures, and the documented ability of polyamines
306 to scavenge oxygen radicals (Khan *et al.*, 1992; Ha *et al.*, 1998; Chattopadhyay *et al.*, 2003), we
307 hypothesized that NO produced during NH_3 oxidation could react abiotically with ^{15}N-PUT to
308 produce the $^{15}N\text{-}NO_2^-$ we observed (Fig. 2). However, experiments with cell-free artificial media
309 and sterilized seawater showed no evidence of reaction between NO and ^{15}N-polyamines (Fig. 3
310 A, Fig. 4). NO_2^- was produced rapidly in treatments with added NO since NO auto-oxidizes to
311 NO_2^- and H^+ in oxic water (Lewis and Deen, 1994), but this NO_2^- did not contain the ^{15}N
312 initially added as ^{15}N-PUT. The correlation between NO_2^- and $\delta^{15}N_{NOx}$ values shown in Fig. 4
313 thus reflects the $\delta^{15}N$ value of spontaneously oxidized NO, with no apparent ^{15}N enrichment due
314 to oxidation of any ^{15}N-labeled substrates. We then tested whether ^{15}N-polyamines react with
315 H_2O_2 or $ONOO^-$ (formed by reacting NO_2^- and H_2O_2; Robinson and Beckman, 2005) to yield
316 $^{15}N\text{-}NO_2^-$, as these reactive compounds are also produced during thaumarchaeal growth (Kim *et*
317 *al.*, 2016; Heal *et al.* 2018; Bayer *et al.*, 2019b), but found no detectable $^{15}N\text{-}NO_2^-$ in these
318 treatments either (Fig. 3). This suggests that some PUT oxidation seen in field experiments
319 (Damashek *et al.* 2019a), with mixed cultures (Fig. 1), or during growth on NH_3 (Fig. 2) may be
320 explained by enzymatic co-metabolism of PUT, or due to reactions with reactive species that we
321 did not test (e.g., superoxide or other RNOS compounds).

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322 Our isotope experiments were restricted to PUT, DAP, and DAE due to limited
323 commercial availability of ^{15}N-labeled polyamines. Of these, only PUT is commonly found in
324 high concentrations in phytoplankton and bacterial cells or marine waters (Nishibori *et al.*, 2001;
325 Lu *et al.*, 2014; Liu *et al.*, 2016; Lin and Lin, 2019). It remains unknown whether other common
326 polyamines (e.g., spermine, spermidine, norspermine, or norspermidine) are comparably
327 oxidized during thaumarchaeal NH_3 oxidation. However, the oxidation of cyanate-N by marine
328 *Thaumarchaeota* may be analogous, given that axenic *N. maritimus* SCM1 cultures only
329 oxidized ^{15}N-cyanate while growing on NH_3 (Kitzinger *et al.*, 2019), comparable to our results
330 with ^{15}N-PUT. Kitzinger *et al*. (2019) hypothesized that *N. maritimus* could break down cyanate
331 extracellularly, since ^{15}N-NH_4^+ was produced in cultures amended with ^{15}N-cyanate despite no
332 known cyanate hydratase genes existing in the *N. maritimus* SCM1 genome. Whether due to
333 abiotic reactions with metabolic intermediates (intra- or extracellularly), some as yet
334 undiscovered mechanism of extracellular remineralization, or co-metabolism by ammonia
335 monooxygenase, the dual evidence of oxidation of PUT-N (Fig. 2) and cyanate (Kitzinger *et al.*,
336 2019) during thaumarchaeal growth on NH_3 suggests some DON compounds can be oxidized
337 indirectly by growing *Thaumarchaeota*, but definitive demonstrations of mechanisms remain
338 elusive.
339

340 **CONCLUSIONS**

341 Given the ability of *Thaumarchaeota* to grow using reduced N supplied as urea and
342 cyanate, there has been interest in their potential to access other forms of organic N, spurred by
343 numerous field studies reporting putative thaumarchaeal DON assimilation or oxidation. Our
344 experiments with two axenic isolates suggest marine *Thaumarchaeota* cannot directly access N

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345 supplied as polyamines, amino acids, amides, or primary amines as an energy source. Inclusion
346 of a heterotrophic bacterium in enrichment cultures of *Thaumarchaeota* resulted in the oxidation
347 of PUT-N, demonstrating the importance of DON remineralization to NH_4^+ by heterotrophs for
348 *Thaumarchaeota* to oxidize N supplied as DON. Therefore, claims of DON use by
349 *Thaumarchaeota* in mixed communities must strictly account for heterotrophic remineralization.
350 Despite lacking the ability to grow on PUT alone, the surprising finding that both pure strains
351 oxidized ^{15}N supplied as PUT while growing on NH_3 suggests some DON may be co-
352 metabolized or broken down abiotically, possibly mediated by reactive species produced during
353 NH_3 oxidation. Abiotic experiments ruled out some of the known reactive oxygen and nitrogen
354 intermediates of thaumarchaeal metabolism as oxidants, but did not identify the mechanism
355 leading to ^{15}N-PUT oxidation in cultures. This study suggests that oxidation of most DON-N for
356 energy conservation by marine *Thaumarchaeota* requires initial DON remineralization to NH_4^+
357 by heterotrophs, but also indicates a potential role for co-metabolism or reactive metabolic
358 byproducts in thaumarchaeal DON-N oxidation.
359

360 EXPERIMENTAL PROCEDURES

361 GROWTH EXPERIMENTS

362 Axenic cultures of *Nitrosopumilus piranensis* D3C and *N. adriaticus* NF5 (Bayer *et al.*,
363 2019a) were grown in SCM amended with an array of single DON compounds as sole energy
364 and N sources, including NH_4^+, urea, amino acids, primary amines (including polyamines), or
365 amides added to 1 or 0.5 mM final concentration (1 or 2 mM N; Table 1). All treatments were
366 run in triplicate for each strain and included pyruvate (200 µM) to scavenge reactive oxygen
367 species (Kim *et al.*, 2016) and 50 µg/mL each of streptomycin and kanamycin. NH_4Cl was added

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368 to positive controls but was not included in other treatments. Experiments were initiated using a
369 10% (v/v) transfer of stationary phase cultures (with NH_4^+ completely consumed) to ensure
370 against NH_4^+ carryover. Growth experiments were conducted in the dark at 29°C for ~70 days
371 and subsampled for NO_2^- determination as described above. Culture purity was tested throughout
372 the duration of the experiment by adding Marine Broth 2216 to aliquots of the culture (10% v/v)
373 and monitoring for bacterial growth by measuring OD_{600}.

374 Growth experiments were also conducted with early enrichment cultures of *N. piranensis*
375 strain D3C and *N. adriaticus* strain NF5, in which approximately 5-15% of the cells were the
376 heterotrophic bacterium *Oceanicaulis alexandrii* (Bayer *et al.*, 2016; Bayer *et al.*, 2019c).
377 Triplicate cultures were grown in SCM containing either 1 mM NH_4^+, 1 mM urea, 1 mM PUT,
378 and 1 mM PUT + 100 µM NH_4^+ as N or energy sources. Experiments were conducted at 29°C in
379 the dark and subsampled over time for immediate NO_2^- determination using standard methods
380 (Griess reagent; Strickland and Parsons, 1972). In selected rounds of these experiments,
381 subsamples were taken for qPCR analysis by mixing 0.8 mL of a culture with 0.8 mL 2X lysis
382 buffer (1.5 M sucrose, 80 mM EDTA, 100 mM Tris; pH 8.3) and freezing immediately at -80°C.
383 DNA was extracted using standard phenol/chloroform techniques (Tolar *et al.*, 2013; Damashek
384 *et al.*, 2019a). Bacterial and thaumarchaeal 16S rRNA genes were quantified using primers
385 BACT1369F/PROK1492R/TM1389F and GI_334F/GI_554R/TM519AR (Suzuki *et al.*, 2000),
386 respectively (see Table S1 for amplification conditions). Reactions were run in triplicate on a
387 C1000 Touch Thermal Cycler/CFX96 Real-Time System (Bio-Rad) using the Platinum qPCR
388 SuperMix-UDG (Thermo Fisher). Standard curves consisted of a dilution series of a linearized
389 plasmid containing a previously-sequenced amplicon.
390

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391 ISOTOPE EXPERIMENTS

392 The ability of *Thaumarchaeota* to oxidize DON during growth on NH_3 was assessed
393 using ^{15}N isotope tracers (98-99% ^{15}N, Cambridge Isotope Laboratories). Axenic cultures were
394 grown on SCM as described above except using 100 µM ammonium chloride and 20 µM
395 pyruvate. Culture purity was tested as described above. Triplicate bottles per substrate were
396 amended to 1.25 µM of ^{15}N-labeled substrate: DAE, DAP, PUT, ammonium chloride, L-GLU, or
397 ^{14}N ammonium chloride (a negative control with no added ^{15}N). Both amine groups of DAE,
398 DAP, and PUT were ^{15}N-labeled, leading to total ^{15}N additions of 2.5 µM in these treatments.
399 Growth was estimated by measuring NO_2^- production over time. At multiple timepoints,
400 subsamples were frozen at -80°C in polypropylene tubes for isotopic analysis.

401 $\delta^{15}NO_x$ values were measured using the bacterial denitrifier method (Sigman *et al.*,
402 2001). Briefly, NO_X was converted to nitrous oxide by *Pseudomonas aureofaciens* and its N
403 isotopic ratio was measured using a Finnigan MAT-252 isotope ratio mass spectrometer coupled
404 with a modified GasBench II interface (Casciotti *et al.*, 2002; Beman *et al.*, 2011; McIlvin and
405 Casciotti, 2011). The concentration of ^{15}N-NO_2^- (µM) was calculated by multiplying the NO_X
406 atom fraction ^{15}N by the NO_2^- concentration (nitrate was not detectable).

407 Abiotic oxidation of ^{15}N-PUT by multiple reactive compounds was tested by amending
408 triplicate flasks containing 75 mL of 0.22-µm filtered aged surface water from the Gulf Stream
409 or Station ALOHA (NH_4^+ and NO_X below the limit of detection), or sterile SCM, with ^{15}N-PUT
410 and a single reactive compound (NO, H_2O_2, or $ONOO^-$; Fig. 3 F). Flasks were then incubated at
411 23°C in the dark. Two control treatments were run that did not contain reactive compounds: one
412 amended with NO_2^-, and one with no addition. For each treatment (including the two negative
413 controls), parallel replicated incubations were conducted with and without added ^{15}N-PUT (Fig.

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414 3 F). NO was generated from the NO donor (Z)-1-[N-(3-aminopropyl)-N-(n-
415 propyl)amino]diazen-1-ium-1,2-diolate (PAPA NONOate; Cayman Chemical, Ann Arbor). 100
416 μM of PAPA NONOate was added to each flask to produce 200 μM NO (Hrabie *et al.*, 1993).
417 The PAPA NONOate stock was prepared by slowly injecting 5.7 mL 0.01 M NaOH into an
418 airtight vial containing 50 mg PAPA NONOate. This stock was wrapped in foil and stored at 4°C
419 prior to use within hours. NO addition experiments were also conducted using blank SCM media
420 (no added N, as described above). In H_2O_2 (Millipore Sigma, Burlington, MA), $ONOO^-$, NO_2^-,
421 and no addition control treatments, 1 μM of the respective compound was added to flasks
422 containing 100 nM ^{15}N-PUT, while NO treatment flasks contained 1.25 μM ^{15}N-PUT. $ONOO^-$
423 was generated by mixing NO_2^- and H_2O_2 (Robinson and Beckman, 2005). At the beginning and
424 end of each incubation, subsamples were frozen at -80°C for $\delta^{15}NO_x$ determination (as described
425 above). Twenty μM sodium nitrate with a known $\delta^{15}N_{NOx}$ value was added to samples prior to
426 conversion of NO_X to nitrous oxide to enable isotopic measurements in experiments with no
427 NO_2^- accumulation (H_2O_2, $ONOO^-$, NO_2^-, and no addition control).
428 In tests of the oxidative ability of NO on multiple ^{15}N-labeled compounds, NO gas (1%
429 v/v in N_2; Airgas, Radnor Township, PA) was directly bubbled through a syringe into
430 Erlenmeyer flasks containing filtered aged surface water from the Gulf Stream (NH_4^+ and NO_X
431 below the limit of detection). ^{15}N-labeled NH_4^+, L-GLU, DAE, DAP, and PUT were added to
432 duplicate flasks (as well as a negative control with no added ^{15}N) and incubated at 23°C in the
433 dark. After 24 hours, subsamples were frozen at -80°C for $\delta^{15}NO_x$ determination (as described
434 above).
435
436 DATA ANALYSIS

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437 Mann-Whitney *U*-tests were conducted using R (R Core Team, 2019) to determine
438 whether $\delta^{15}NO_x$ values differed between abiotic incubation endpoints of treatments with and
439 without ^{15}N-PUT (shown in Fig. 3) and between abiotic incubation endpoints of treatments
440 containing different ^{15}N-labeled substrates (shown in Fig. 4). Correlations were determined by
441 calculating Spearman's rank correlation coefficient (ρ) in R. Plots were made using the ggplot2
442 R package (Wickham, 2016).
443

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449

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655 SUPPLEMENTAL TABLES AND FIGURES

656 Table S1

657 Primers, cycling information, and assay data for qPCR reactions

658

659 **Fig. S1** NO_2^- accumulation in axenic cultures of *N. piranensis* D3C (circles) and *N. adriaticus*
660 NF5 (diamonds) over time. Colors denote the compounds added to the media as sole energy and
661 N sources. Points show the average of triplicate incubation bottles while error bars show
662 standard deviation.

663