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Substrate pulses as a selection factor for clades of marine Thaumarchaeota

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Abstract

Oxidation rates of N supplied as ammonium (AO) or urea (UO) by Thaumarchaeota-dominated nitrifying communities in samples of aerobic waters from continental shelf and slope waters of the Southern Ocean west of the Antarctic Peninsula were inhibited by substrate amendments in the low nM range. We found that the response varied consistently by water mass. Rates increased moderately (up to 2-fold) with 44 or 440 vs 6 nM NH4+ amendments to samples from the Winter Water (sampled at 70-80 m), but decreased (down to 7%) in samples from the Circumpolar Deep Water (400-600 m). AO rates decreased more than UO rates. Cell-specific AO rates were lower in CDW samples than in WW samples and chemoautotrophic carbon fixation was also inhibited by NH4+ amendments. We identified similar responses to substrate amendments in data collected elsewhere by others, indicating that inhibition of AO, and to a lesser extent UO, by substrate pulses may be a general phenomenon. Current estimates of nitrification in the epipelagic zone may be ~2-fold greater than in situ, while estimates for the mesopelagic may be ~25% of in situ. Our data suggest that differential adaptation to fluctuating resources may be the basis for the divergence of epipelagic and mesopelagic Thaumarchaeota ecotypes.

SUMMARY STATEMENT

Nitrification is a globally important biogeochemical process, helping to remove excess nitrogen from the biosphere. Thaumarchaeota are important contributors to ammonium oxidation, the first step in nitrification, especially in the ocean. A phylogenetic distinction between clades of marine Thaumarchaeota from shallow versus mesopelagic habitats emerged from the earliest analyses of sequence databases, yet the environmental factors driving these distributions, and their biogeochemical significance, are still debated. Steady-state ammonium concentrations are important determinants; however, environmental concentrations may fluctuate on short time scales, depending on localized coupling between production and consumption. Substrate pulses have been shown to inhibit the activity of Thaumarchaeota cultures via the accumulation of toxic intermediates. Here we provide field evidence that ammonia oxidation can be inhibited by ammonium amendments. We found greater inhibition with mesopelagic samples than with those from shallower water, potentially explaining the evolutionary divergence of marine Thaumarchaeota into deep- and shallow-water clades. Further, measurements of ammonium oxidation rates needed for biogeochemical models are typically made with substrate amendments that may yield artificially low rates, to 25% of the uninhibited rate, in mesopelagic samples. The inhibition also affects carbon fixation, which may thus be greater in the dark ocean than currently believed.

MAIN TEXT

Nitrification is a globally important biogeochemical process, helping to remove excess biologically available nitrogen from the ocean via coupled nitrification-denitrification [1,2]. The contribution of Thaumarchaeota to nitrification has been recognized for nearly 2 decades [3,4]. They have been shown to

oxidize reduced nitrogen in ammonia [4–6], urea [7–10] or cyanate [9] to nitrite, the first and rate-controlling step of nitrification.

A phylogenetic distinction between shallow- and deep-water clades of marine Thaumarchaeota emerged from the earliest analyses of sequence databases [11], yet the environmental factors driving these distributions and their biogeochemical consequences are still debated [12]. Steady-state ammonia concentration is clearly an important factor in the general distribution of these clades [12]; however, environmental concentrations of NH_4^+ and urea may fluctuate depending on localized coupling between regeneration and uptake or oxidation (e.g. [7,13,14]), subjecting nitrifiers, including Thaumarchaeota, to short-term temporal variation in substrate concentrations. It is not known whether these fluctuations might play a role in selecting for Thaumarchaeota ecotypes; however, experiments with cultures have shown that substrate pulses may inhibit the growth of Thaumarchaeota [15]. Further, as a purely technical matter, detection of N oxidation rates may require amendments of ^{15}N-labeled substrates that significantly increase the concentration of total (labeled plus unlabeled) substrate in samples [16], potentially affecting rate estimates.

We evaluated the effect of substrate amendments on nitrite production from N supplied as ammonium (AO) and urea (UO) to samples from Antarctic coastal waters where Thaumarchaeota are abundant [17-19] and are the primary agents of ammonia oxidation [20]. We found marked differences in the responses to ^{15}N amendments of nitrifiers from Winter Water (WW, a remnant of the winter mixed layer found at depth following summer stratification, sampled at the water column temperature minimum) versus Circumpolar Deep Water (CDW, a mesopelagic water mass circling Antarctica at depths of 400-1500 m).

AO rates in WW samples increased with increasing amendments of $^{15}NH_4^+$, while AO rates were reduced by increasing $^{15}NH_4^+$ amendments to CDW samples (Fig. 1, Supplemental Fig. 1). This difference in response was significant at $p < 0.05$ (Fig. 1, Supplemental Table 1). A similar pattern emerged if AO rates with 44 nM amendments were compared to rates with 440 nM amendments (Supplemental Table 1); however, the difference was less pronounced. UO rates in WW samples also increased with increasing ^{15}N-urea amendments, while UO rates in CDW samples decreased (Fig. 1, Supplemental Table 1). CDW populations were inhibited more strongly by NH_4^+ than by urea amendments.

These amendments increased total NH_4^+ concentrations (([in situ] + [amendment])/[in situ]), on average, to 101, 106 and 160% in WW samples, and 108, 159 and 701% in CDW samples (Supplemental Table 2). Urea amendments to WW samples increased total urea concentration to 110, 176, and 854% of in situ, while the same amendments to CDW samples increased total urea to 122, 272, and 1,813% of in situ (Supplemental Table 2).

A related experiment (Fig. 2) tested the effect of NH_4^+ amendments on non-phototrophic incorporation of dissolved inorganic carbon (DIC) into biomass by microorganisms. As with ammonia oxidation, higher

NH_4^+ amendments (here 44 vs 440 nM) inhibited the incorporation of DIC, and the inhibition was stronger for the CDW sample (rate with 440 nM NH_4^+ amendments = 33% of rate with 44 nM amendments, 1-way ANOVA $p = 0.047$, F = 19.68) than the WW population (76%, $p = 0.522$, F = 1.54). A second experiment showed a slight increase in DIC incorporation with 444 vs 6 nM NH_4^+ amendments to WW (120%) and CDW (107%) samples. However, these differences were not statistically significant (1-way ANOVA $p = 0.67$, F = 0.45 and $p = 0.31$, F = 1.77 for WW and CDW samples, respectively) and the DIC incorporation rates in the CDW sample were below our estimate of the limit of detection. Although not conclusive, these data support the conclusion that NH_4^+ pulses can inhibit the overall metabolism of ammonia oxidizers.

Inhibition of AO and UO rates in response to substrate amendments has been observed previously, but the broader physiological and ecological significance of the phenomenon has not been addressed. AO and UO rates measured in samples from the 1% light level (51 m) during a period of active upwelling (March 2015) at the SPOT station off southern California decreased in response to elevated (250 vs 15 nM) amendments to samples with ambient NH_4^+ and urea-N concentrations of 10 and 190 nM (Figure 5 in Laperriere et al. [8]). Although not discussed in their paper, Shiozaki et al. [10] found that urea amendments of 1,560 nM inhibited UO rates to 50 - 77% of the rates measured with 31 nM amendments (ambient [urea] 84-110 nM) in 3 samples from the 0.1% light level in the Beaufort Sea (epipelagic, 72-101 m, calculated from their Supplemental Dataset 1). They did not test the effect of NH_4^+ amendments on AO rates on this cruise; however, they performed similar experiments with $^{15}NH_4^+$ amendments ranging from 31 to 1,560 nM using samples from the 0.1% light level (epipelagic, 30-170 m) at stations on a meridional transect of the North Pacific [14]. These experiments (reported in their Figure 4a and Supplemental Table 1) showed no clear response of AO to amendments: AO rates increased in 6 and decreased in 7 samples where rates were greater than the limit of detection. The mean change of AO rates with amendments of 1,560 nM versus 31 nM was 105% (range of 44-273%). The 31 nM $^{15}NH_4^+$ amendments used in this study represent larger enrichments (194% to infinity, since ambient [NH_4^+] was undetectable in some samples), than the 6 nM amendments used in our experiments (range 100-140% for both substrates).

A mechanism that might explain the response of mesopelagic AOA to substrate amendments is sensitivity to reactive oxygen (ROS) and nitrogen (RNS) species. AOA are known to be inhibited by ROS and RNS species produced as a consequence of their metabolism [15,21] and previous work in our study area [22] verifies that these AOA populations are sensitive to the ROS species HOOH at nM levels. We hypothesize that ROS/RNS accumulated during incubations with elevated substrate concentrations, including the 31 nM additions used as the lowest amendment by Shiozaki et al. [14], can reach toxic levels, inhibiting further oxidation of N supplied as NH_4^+ or urea. This response is similar to the response of Thaumarchaeota cultures to elevated [NH_4^+] reported in Fig. 3B of Kim et al. [15]. Substrate concentrations, especially NH_4^+, were generally lower in CDW samples than in WW samples, thus the same $^{15}NH_4^+$ or ^{15}N-urea amendment represents a greater increase in substrate concentration in CDW than in WW samples (Supplemental Table 2). The greater inhibition of CDW populations by NH_4^+ vs urea may be due to the

slower rate at which N from urea versus NH_4^+ is oxidized, and thus ROS/RNS is produced, (21.2 vs 1.6 nmol L^{-1} d^{-1} for AO vs UO, respectively, in WW samples; 7.9 vs 2.5 in CDW samples, Fig. 1, Supplemental Table 2).

It is likely that sensitivity to, or production of, ROS/RNS varies among Thaumarchaeota clades [15]. Gene ratios from samples collected on LMG1801, as well as more rigorous analyses performed previously [20,23,24], demonstrate that WW and CDW Thaumarchaeota populations are phylogenetically distinct. This difference may influence the cell-specific rates at which they oxidize NH_4^+ or urea-N and produce or detoxify ROS/RNS. Detoxification of ROS and RNS, regardless of its source, is also likely a community-level process [25,26]. Thus, differences in the composition of bacterioplankton communities in these two water masses may also play a role in the response of Thaumarchaeota to elevated substrate concentrations. Bacterioplankton and Thaumarchaeota populations in the winter mixed layer that becomes the Winter Water following water column stratification during spring [27,28] may have been exposed to elevated concentrations of ROS generated by photochemistry, including photosynthesis. The concentration of one ROS compound, HOOH, has been shown to be higher in the surface mixed layer of the study area than at greater depths [22,29] and Thaumarchaeota populations are greatly attenuated in the surface waters at our study site following summer stratification [23,27]. In contrast, the CDW water mass is always below the photic zone, thus CDW bacterioplankton and Thaumarchaeota would not have been exposed to photochemically produced ROS. These differences in exposure histories may exert selective pressure for ROS/RNS-tolerant bacterioplankton and Thaumarchaeota ecotypes in the WW (epipelagic) relative to the CDW (mesopelagic).

Our data strongly suggest that even small increases (6 vs 44 nM) in substrate concentration can inhibit ammonia oxidation in CDW populations. We tested two large data sets of ammonia oxidation rate measurements we made in the same area on LMG1101[20] and LMG1801 for additional evidence of differential inhibition of CDW vs WW populations by substrate amendments. Rates measured on LMG1101 used 50 nM $^{15}NH_4^+$ amendments. We normalized the AO and UO rates we measured to the abundance of Thaumarchaeota 16S rRNA genes, measured in the same sample by quantitative PCR as described in Tolar et al.[20]. We found that cell-specific AO rates were significantly higher in WW samples than in CDW samples on both cruises ($p < 0.01$, Table 1). In contrast, cell-specific rates of UO did not differ between samples taken from these two water masses on LMG1801.

The response of Thaumarchaeota to amendments is likely a complex interaction between the kinetic effect of higher substrate concentrations on rates and inhibition via the release of toxic ROS/RNS. The data suggest that the effect is very nonlinear (Fig. 1, Table 1; Fig. 2 in Laperriere et al. [8]; Fig. 5 in Shiozaki et al. [10]; Kim et al. [15]). Comparisons between rates measured with 30–50 nM amendments and rates measured with much higher amendments may show little change because the threshold for inhibition is lower than 30–50 nM (Fig. 1, Supplemental Table 1; compare rates measured with 44 or 47 nM amendments with those measured with 440 or 470 amendments; Fig. 4a in Shiozaki et al. [10]).

Our experiments suggest that inhibition of CDW populations by amendments appears to be stronger than stimulation of WW populations (Fig. 1, supplemental Table 1). Inhibition of cell-specific AO rates in CDW samples is consistent with these experimental results; however, other factors may also be at play: substrate concentrations were lower in CDW than WW samples and the Thaumarchaeota populations in these water masses are phylogenetically distinct [20,24,30], which may affect cell-specific rates. In contrast, the medians of UO rates and of cell-specific UO rates from CDW samples were both 83% of rates measured from WW samples (not significantly different, $p > 0.01$). This is also consistent with the results of our experiments (Fig. 1, Supplemental Table 1) and may reflect the slower rate at which the nitrogen in urea is made available to nitrifiers, or to other differences in the way N supplied as urea is metabolized.

Table 1

Mann-Whitney tests of the significance of differences in the oxidation rates of N supplied as ammonium or urea to samples from WW versus CDW water masses. Thaums = Thaumarchaeota 16S rRNA genes, 103 copies L− 1; AO > LD = oxidation rate of N supplied as ammonium where rates were > limit of detection (LD, 4.4 nmol L− 1 d− 1); UO > LD = oxidation of N supplied as urea where rates were > limit of detection (0.6 nmol L− 1 d− 1); AO cell− 1 and UO cell− 1: cell-specific oxidation rates of N supplied as ammonium or urea for rates > LD, pmol N cell− 1 d− 1. Values of p < 0.01 are shown in BOLD

LMG18-01	**Thaums**	**AO > LD**	**UO > LD**	**AO cell^{-1}**	**UO cell^{-1}**
Count WW	38.00	59.00	53.00	52.00	45.00
Count CDW	36.00	46.00	64.00	40.00	54.00
Median WW	9484.00	15.11	2.14	1.24	2.07
Median CDW	9317.26	7.48	1.77	0.52	1.73
Ratio: CDW/WW	0.98	0.49	0.83	0.42	0.83
p1	0.4960	**0.0007**	0.2843	**0.0027**	0.3898
p2	0.9920	**0.0015**	0.5687	**0.0054**	0.1949
LMG11-01					
Count WW	28	23		21	
Count CDW	25	23		22	
Median WW	1495000	10.1		3.92	
Median CDW	13794118	16.5		1.49	
Ratio: CDW/WW	9.2	1.6		0.38	
p1	< 0.0001	0.0764		**0.0026**	
p2	< 0.0001	0.1527		**0.0053**	

These findings have global implications for analyses of oceanic nitrogen budgets and models of nitrogen biogeochemistry (e.g. [31,32]). Rate measurements made in open ocean samples where [NH_4^+] and [urea] are in the low nM range typically use substrate amendments that range from 30 to 50 nM, following recommendations from [16]. Reported rates are thus likely to have been affected by the increase in substrate concentration due to the tracer amendment. Over our entire data set from LMG1801 (107 samples, 214 rate measurements), amendments of 44 nM $^{15}NH_4^+$ increased substrate concentrations 110 ± 11% (mean ± SD) in WW samples and 150 ± 28% in CDW samples. ^{15}N-urea amendments (47 nM) increased WW concentrations by 310 ± 370% and CDW concentrations by 290 ± 190%. Assuming that the Thaumarchaeota in all of our samples responded similarly to amendments as those in our experiments, we predict that the AO and UO rates we measured in WW samples overestimate in situ rates by 180% and 130%, on average, while the AO and UO rates we measured in CDW samples are 25% and 77% of in situ rates, on average (Supplemental Table 2). There is no reason to believe that the response of ammonia oxidizing Thaumarchaeota to substrate amendments is restricted to ecotypes from Antarctic coastal waters or, for that matter, to marine Thaumarchaeota.

The phylogenetic distinction between shallow- and deep-water clades of marine Thaumarchaeota emerged from the earliest analyses of sequence databases [11], and has biogeochemical implications [33], yet the environmental factors driving these distributions are still debated [12]. Seasonal blooms of estuarine and coastal Thaumarchaeota appear to be driven by water temperature and irradiance [34,35], with the effect of irradiance likely indirect via generation of ROS [22]. Metagenomic analyses suggest that the depth distributions of open ocean clades are not controlled by pressure (depth) or temperature [36], but rather point to ambient, steady-state ammonium concentrations as a primary factor in niche partitioning [36,37]. Our data suggest that, compared to shallow water ecotypes, mesopelagic Thaumarchaeota are poorly adapted to short-term temporal variability (hours or less) in ammonium concentration of 10's of nM or less. Localized fluctuations in ammonium or urea concentrations of this magnitude might arise from uncoupling (e.g. [7,13,14]) between consumption and production, as plumes from sinking particles[38,39] or by zooplankton excretion. Inherent differences in the densities and distribution of sinking particles and zooplankton, and the presence of additional sinks for NH_4^+, in epipelagic versus mesopelagic environments may be key factors in the variability of NH_4^+ and urea concentrations and instrumental in selecting for deep-water versus shallow water Thaumarchaeota ecotypes[33].

MATERIALS AND METHODS

Sample Collection. We sampled the continental shelf and slope west of the Antarctic Peninsula during the austral summer of 2018 (ARV Laurence M Gould cruise LMG1801, PAL-LTER cruise 26, DOI: 10.7284/907858). Seawater was sampled using 20 L Niskin bottles (General Oceanics Inc., Miami, FL,

USA). Water for rate measurements was drained into aged, acid-washed, sample-rinsed 250 mL polycarbonate bottles (Nalge).

Nitrogen oxidation rates. AO and UO were measured using ^{15}N-labeled substrates. Substrates were added to duplicate bottles within ~ 1 hr of collection to yield 6, 44 or 440 nM of $^{15}NH_4^+$ or 6, 47 or 470 nM of urea (12, 94 or 940 nM of urea ^{15}N). Bottles were incubated in the dark at (mean ± SD) 0.23 ± 0.71 ^oC for ~ 48 hr. Incubations were terminated by decanting ~ 40 mL subsamples into plastic tubes that were immediately frozen at -80°C. We ran time course experiments with samples from 2 depths at 3 stations to verify that ^{15}N oxidation rates did not change significantly during incubations. The characteristics of the samples used in these experiments compared favorably (*t*-test, $p > 0.01$) with mean conditions over all samples from the same water mass.

^{15}N in nitrite plus nitrate. The ^{15}N content of NO_2^- plus NO_3^- ($^{15}NO_x$) of our samples was measured using the 'denitrifier method' [40] with *Pseudomonas aureofaciens* as described previously [41]. The N_2O produced was analyzed using a Gas Bench II coupled to a Finnegan MAT 252 mass spectrometer [42,43] following the recommendations of [44].

Rate calculations. Our rate measurements are based on the production of $^{15}NO_x$ from ^{15}N labeled substrates. We calculated oxidation rates by comparing $\delta^{15}N$ values of the NO_x pool at the ends of the incubations with values in unamended samples ("natural abundance"), as described previously [41]. We assumed that the $\delta^{15}N$ value of naturally occurring ammonium and urea is the same as that of AIR. Ammonium concentrations were measured on the cruise using the *o*-phthaldialdehyde method [45]. Urea was determined manually from frozen samples by the diacetyl monoxime method [46,47]. The concentrations of NO_2^- + NO_3^- (NO_x) in our samples were determined from frozen samples by PAL-LTER personnel using an autoanalyzer. Some of the chemical data needed for rate calculations were missing for some samples so we substituted water mass averages determined from other samples taken on the cruise. The rates we report are for N oxidized (NO_x produced), regardless of whether it was supplied as NH_4^+ or urea.

Chemoautotrophy

Water was collected directly from the Niskin bottle into 250 mL amber polyethylene bottles filled to the top (volume ~ 270 mL). Each experiment used two replicate treatments and a control bottle. Controls consisted of samples that were incubated along with the ^{14}C-amended treatments, except that no ^{14}C was added until immediately before filtering the set. Each bottle received ~ $7.4x10^5$ Bq of $NaH^{14}CO_3$ (Perkin-Elmer) in 100 uL. Label was added in a darkened lab van illuminated with a dim, red-filtered light. Lights were off except when working. Samples were mixed by inverting gently then placed in an ice bath contained in an insulated cooler wrapped in aluminum foil, then covered with black polyethylene.

At the end of the incubation (~ 48 hours) the bottles were removed, triplicate samples of 100 uL were taken from one of the treatments and radioassayed to verify the amount of tracer added. Samples were filtered through 25 mm diameter, 0.22 um pore size Millipore filters under dim red light. Filters were rinsed 2 X with filtered SW, removed and placed into vials, then 100 uL of 10% HCl was added to each vial, soaking the filter in the process. Each vial received 4 mL of Ultima Gold (Perkin-Elmer) scintillation cocktail, then were counted in a Perkin-Elmer LSC.

Precision and accuracy. Analytical uncertainty of $\delta^{15}N$ measurements ranged from 0.36‰ to 0.56‰. Accuracy was 0.42‰ (at-% ^{15}N = 0.00019, n = 56). The precision of nitrite + nitrate analyses run by LTER personnel was reported to be 100 nM. We determined the precision of ammonium and urea analyses as the mean standard deviation of replicate (2 or 3) analyses of a given sample. They are: ammonium, 65 nM; urea, 10 nM. We ran Monte Carlo simulations (10,000) to estimate the precision of rate measurements, which are 2.2 nmol L^{-1} d^{-1} for AO and 0.31 nmol L^{-1} d^{-1} for UO, for limits of detection (95% confidence interval) of 4.4 and 0.6 nmol L^{-1} d^{-1}, respectively. The limit of detection for chemoautotrophy measurements was determined as the 95% CI of the intercept of a model 2 regression of replicate 1 vs replicate 2 of each sample (n = 34) and was 0.2 nmol C fixed L^{-1} d^{-1}.

Data archives. The data presented here are archived by the Biological and Chemical Oceanography Data Management Office (BCO-DMO) under project acronym “Oxidation of Urea N,” doi:10.26008/1912/bco-dmo.840629.2, https://www.bco-dmo.org/dataset/840629/data.

Declarations

COMPETING INTERESTS

The authors declare no competing interests.

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AUTHOR CONTRIBUTIONS

JTH designed the research; JTH, BNP and HD conducted the sampling program; JTH, JD, NJW and BNP contributed to sample analysis; JTH and BNP analyzed the data, JTH wrote the paper with input from the coauthors.

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Figures

Figure 1. Oxidation rates of N supplied as NH_4^+ or urea as functions of substrate amendments. Amendment reported as nmol L^{-1} of the substrate, not of N in the case of urea. Winter Water samples (WW, epipelagic) are from 70-80 m depth, Circumpolar Deep-Water samples (CDW, mesopelagic) are from 400-600 m depth. Box and whisker plots show median, 2nd and 3rd quartiles and range. Results of Kruskal-Wallis ranks tests of the significances of differences between distributions by amendment within a water mass are shown where $p<0.05$. Additional tests are presented in Supplemental Table 1. Key: ● Station 149.-050; ●, ♦ Station 200.-040; ●, ♦ - Station 600.040B ●, ♦ Station 600.180.

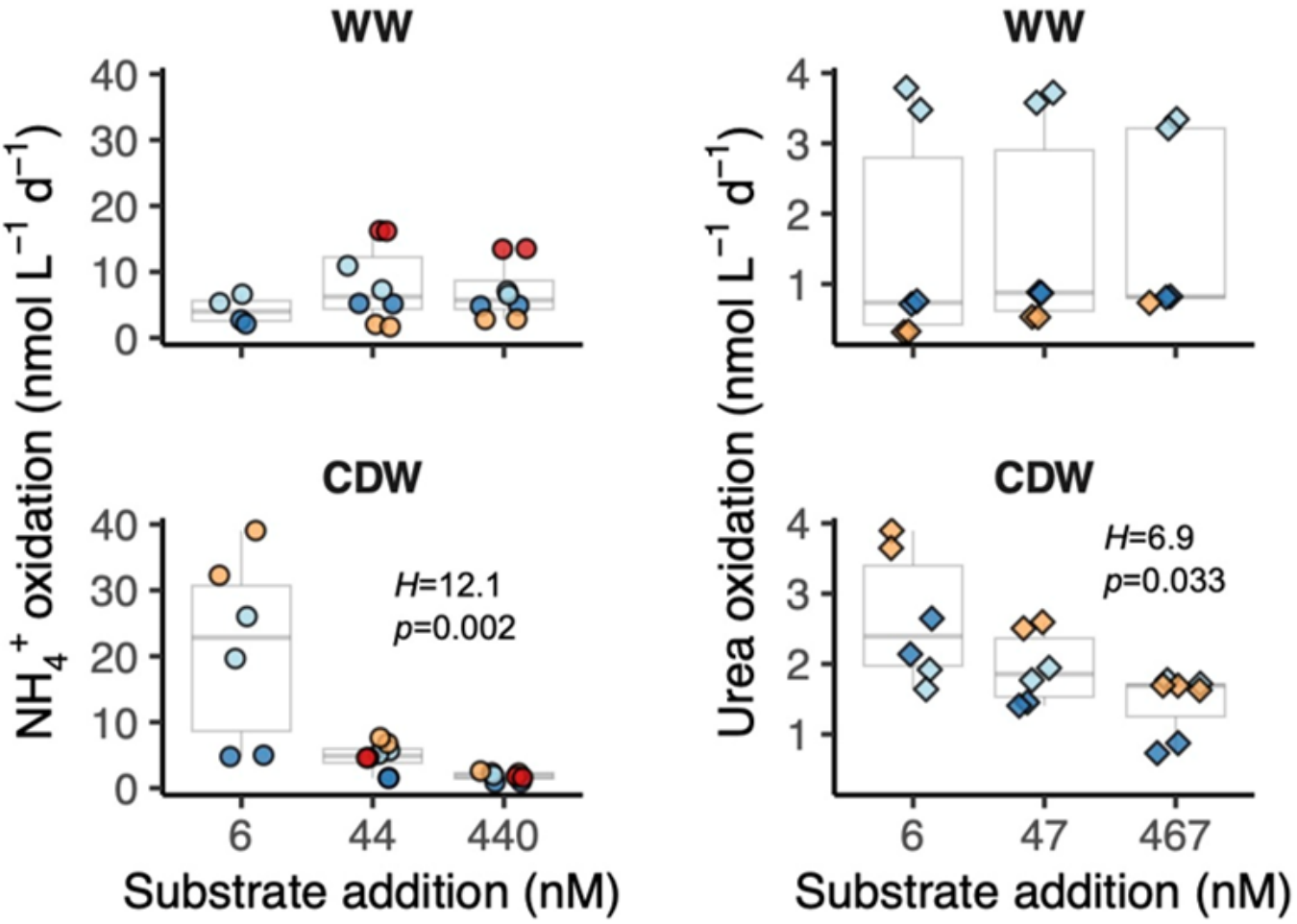

Figure 1

See image above for figure legend.

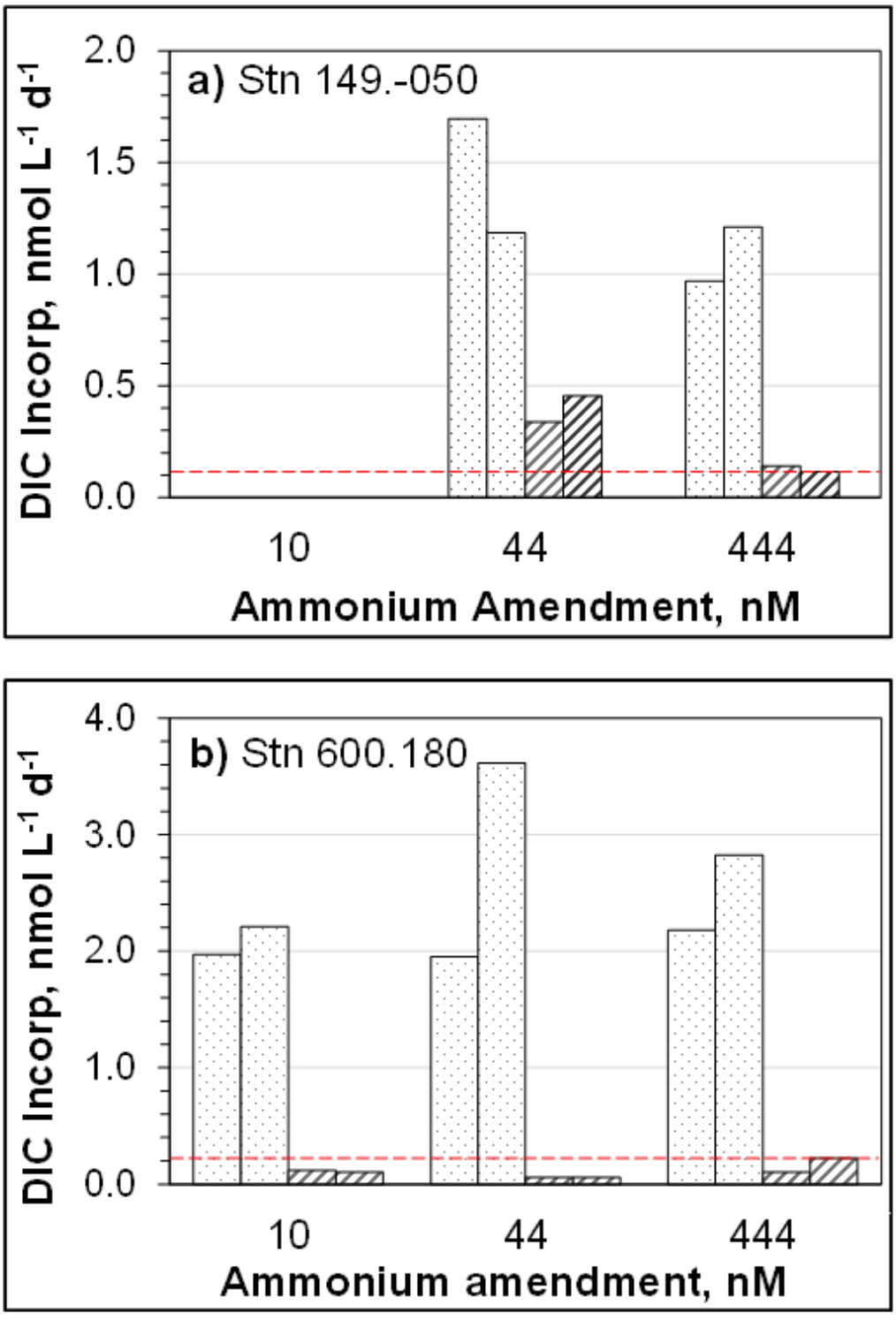

Figure 2

Effect of substrate amendments on chemoautotrophy. These samples were also used in tests of the effect of ammonium amendments on ammonia oxidation rates shown Figure 1. Stippled bars are WW samples, cross-hatched bars are CDW samples. Red lines at the bottom of each panel indicate the limit of detection for DI^{14}C incorporation rates.

Supplementary Files

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