

# Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea

Jeremy A. Dodsworth,<sup>1</sup> Bruce A. Hungate<sup>2</sup> and Brian P. Hedlund<sup>1\*</sup>

<sup>1</sup>School of Life Sciences, University of Nevada, Las Vegas, Las Vegas, NV 89154-4004, USA.

<sup>2</sup>Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, AZ 86011, USA.

## Summary

Many thermophiles catalyse free energy-yielding redox reactions involving nitrogenous compounds; however, little is known about these processes in natural thermal environments. Rates of ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) were measured in source water and sediments of two ~80°C springs in the US Great Basin. Ammonia oxidation and denitrification occurred mainly in sediments. Ammonia oxidation rates measured using <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> pool dilution ranged from 5.5 ± 0.8 to 8.6 ± 0.9 nmol N g<sup>-1</sup> h<sup>-1</sup> and were unaffected or only mildly stimulated by amendment with NH<sub>4</sub>Cl. Denitrification rates measured using acetylene block ranged from 15.8 ± 0.7 to 51 ± 12 nmol N g<sup>-1</sup> h<sup>-1</sup> and were stimulated by amendment with NO<sub>3</sub><sup>-</sup> and complex organic compounds. The DNRA rate in one spring sediment measured using an <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> tracer was 315 ± 48 nmol N g<sup>-1</sup> h<sup>-1</sup>. Both springs harboured distinct planktonic and sediment microbial communities. Close relatives of the autotrophic, ammonia-oxidizing archaeon '*Candidatus Nitrosocaldus yellowstonii*' represented the most abundant OTU in both spring sediments by 16S rRNA gene pyrotag analysis. Quantitative PCR (qPCR) indicated that '*Ca. N. yellowstonii*' *amoA* and 16S rRNA genes were present at 3.5–3.9 × 10<sup>8</sup> and 6.4–9.0 × 10<sup>8</sup> copies g<sup>-1</sup> sediment. Potential denitrifiers included members of the *Aquificales* and *Thermales*. *Thermus* spp. comprised < 1% of 16S rRNA gene pyrotags in both sediments and qPCR for *T.*

*thermophilus narG* revealed sediment populations of 1.3–1.7 × 10<sup>6</sup> copies g<sup>-1</sup> sediment. These data indicate a highly active nitrogen cycle (N-cycle) in these springs and suggest that ammonia oxidation may be a major source of energy fuelling primary production.

## Introduction

Recently, research on the nitrogen biogeochemical cycle (N-cycle) in terrestrial geothermal habitats has been invigorated by the cultivation of thermophilic archaea capable of chemolithotrophic ammonia oxidation up to 74°C (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). Putative biomarkers for ammonia-oxidizing archaea (AOA) are globally distributed in physicochemically diverse geothermal habitats, including > 40 hot springs in Yellowstone National Park (de la Torre *et al.*, 2008), the US Great Basin (Zhang *et al.*, 2008), Tengchong, China (Zhang *et al.*, 2008), Kamchatka (Reigstad *et al.*, 2008; Zhang *et al.*, 2008) and Iceland (Reigstad *et al.*, 2008). Putative archaeal ammonia monooxygenase genes (*amoA*) have been amplified from DNA and cDNA from samples up to 94°C and with a pH range of 2.4–9.0, suggesting that AOA may be active at temperatures near boiling and in a wide variety of geochemical settings (Zhang *et al.*, 2008; Jiang *et al.*, 2010). Similarly, the biphytanyl lipid crenarchaeol, another possible biomarker for AOA, has been recovered from hot springs in the US Great Basin (Pearson *et al.*, 2004; 2008), Yellowstone (Pearson *et al.*, 2008) and Iceland (Reigstad *et al.*, 2008) up to 87°C. Crenarchaeol was shown to be most abundant at ~40°C (Zhang *et al.*, 2006) and much more abundant among archaeal lipids in Great Basin hot springs, as compared with Yellowstone hot springs (Pearson *et al.*, 2008). The role of biological versus abiotic oxidation of nitrite (NO<sub>2</sub><sup>-</sup>), either produced by AOA or from other sources, in geothermal systems is much less clear. A *Nitrospira* isolate catalyses NO<sub>2</sub><sup>-</sup> oxidation to NO<sub>3</sub><sup>-</sup> at temperatures reaching 58°C (Lebedeva *et al.*, 2011) and nitrifying enrichments supporting *Nitrospira* have been reported at temperatures up to 60°C (Lebedeva *et al.*, 2005). 16S rRNA genes for this group of nitrite oxidizing bacteria have been detected in geothermal systems up to 69°C (Kanokratana *et al.*, 2004; Hirayama *et al.*, 2005).

Received 26 November, 2010; accepted 5 April, 2011. \*For correspondence. E-mail brian.hedlund@unlv.edu; Tel. (+1) 702 895 0809; Fax (+1) 702 895 3950.

The presence of AOA in geothermal environments suggests the importance of a biogeochemical nitrogen cycle in these systems, where nitrite and nitrate produced by nitrification of ammonia supplied by the source water serve as terminal electron acceptors for anaerobic respiration. The products of respiration of nitrate and nitrite include  $N_2O$  and  $N_2$  in the case of denitrification or  $NH_4^+$  in the case of dissimilatory nitrate reduction to ammonium (DNRA). The presence of 16S rRNA gene and lipid biomarkers for bacteria involved in anaerobic ammonia oxidation (anammox) in Great Basin hot springs suggests that this process may also play a role in  $NH_4^+$  and  $NO_2^-$  consumption (Jaeschke *et al.*, 2009). A wide variety of thermophiles have the ability to respire nitrate, including both archaea and bacteria, from both marine and terrestrial ecosystems. In terrestrial geothermal habitats, the archaea *Pyrobaculum aerophilum* and *Ferroglobus placidus* respire nitrate to  $N_2$  and  $N_2O$ , respectively, although each appears to accumulate high concentrations of intermediates. *Pyrobaculum aerophilum* accumulates  $N_2O$  (Völkl *et al.*, 1993; Afshar *et al.*, 1998) and *F. placidus* accumulates  $NO_2^-$  with lower concentrations of  $NO$  (Hafenbradl *et al.*, 1996; Vorholt *et al.*, 1997). Among terrestrial thermophilic bacteria, the *Thermales*, *Aquificales* and *Bacillales* include members capable of nitrate reduction to nitrite (Ramirez-Arcos *et al.*, 1998), nitrate reduction to nitrous oxide (Gokce *et al.*, 1989; Nakagawa *et al.*, 2004) or complete denitrification to  $N_2$  (Suzuki *et al.*, 2001; Takai *et al.*, 2003; Cava *et al.*, 2008; Mishima *et al.*, 2009). The known upper temperature limit of cultivated nitrate-reducing microorganisms is 113°C by the marine archaeon *Pyrobolus fumarii*, which catalyses DNRA during chemolithotrophic growth with  $H_2$  as electron donor (Blöchl *et al.*, 1997). Several thermophilic bacteria are also capable of DNRA, including the marine isolates *Thermovibrio ruber* (Huber *et al.*, 2002), *T. ammonificans* (Vetriani *et al.*, 2004) and *Caldithrix abyssii* (Miroshnichenko *et al.*, 2003), as well as *Ammonifex degensii*, isolated from a terrestrial hot spring (Huber *et al.*, 1996).

Despite mounting evidence that ammonia oxidation and possibly other N transformations may be widespread and important in terrestrial geothermal ecosystems, we are aware of only two studies directly measuring rates of these processes in terrestrial thermal environments. Reigstad and colleagues (2008) measured nitrification rates in two acidic, clay-rich springs in Iceland using  $^{15}N$ -nitrate pool dilution. Burr and colleagues (2005) measured rates of denitrification by acetylene block and ammonification by a pool flux technique in an acidic geothermal soil in Yellowstone. The extent to which ammonia oxidation and denitrification are coupled in these systems, and the importance of these processes to overall productivity, is therefore largely unknown.

Here, we quantified ammonia oxidation and nitrate reduction in two springs in the Great Boiling Spring system in the US Great Basin, Great Boiling Spring (GBS) and Sandy's Spring West (SSW). These rate measurements were combined with censuses of microbial populations in the spring water and sediment/water interface and quantitative PCR (qPCR) specific for microorganisms implicated in these processes. Previously, a cultivation-independent census of the sediment/water interface in the source pools of these springs estimated moderately diverse microbial populations with 20 species-level groups in GBS and 53 species-level groups in SSW (Costa *et al.*, 2009). Although uncultivated phyla were abundant among 16S rRNA genes recovered from both springs, a high proportion (~55%) of archaeal genes from GBS were closely related to the AOA '*Candidatus Nitrosocaldus yellowstonii*'. This, along with a supply of ammonia/ammonium in spring water (25–60  $\mu M$ ), relatively high concentrations of nitrite (up to 35  $\mu M$ ), and the general abundance of crenarchaeol in Great Basin springs (Pearson *et al.*, 2008), suggested that the N-cycle might be particularly active in this spring system (Costa *et al.*, 2009). These two springs also provide an excellent system to study the effects of flow rate, or more precisely water residence time, on microbial activities and community composition since they share a common subterranean reservoir and nearly identical redox-inactive chemical composition, but have dramatically different water residence time and redox-active chemical composition (Anderson, 1978; Costa *et al.*, 2009).

In this study we addressed the following questions: (i) do nitrification, denitrification and DNRA occur in these spring systems and, if so, at what rates? (ii) how are these activities and the microorganisms catalysing them distributed in water and sediment? (iii) are these processes limited by availability of electron donors or acceptors? (iv) is denitrification driven by chemoorganotrophy or chemolithotrophy? And (v) how do rates of these processes compare with similar non-thermal environments? Together, these experiments provide the first integrated study of ammonia oxidation, nitrate reduction and microbial community composition in any geothermal environment and provide a foundation for investigating the N-cycle in other geothermal systems.

## Results and discussion

We found that ammonia oxidation, denitrification and DNRA were active in two US Great Basin hot springs, GBS and SSW, at rates comparable to those in non-thermal environments. A summary of the rates measured, the methods used and the spring temperatures at sampling and incubation sites is shown in Table 1.

**Table 1.** Summary of process rates measured in GBS and SSW sediments in this study.

Date and process	Method	Spring (temperature)	Rate (nmol N g <sup>-1</sup> h <sup>-1</sup> )	Figure(s)
October 2008 <sup>a</sup>				
NH <sub>3</sub> oxidation <sup>b</sup>	<sup>15</sup> N-NO <sub>3</sub> <sup>-</sup> pool dilution	GBS (81°C)	6.7 ± 3.1	Fig. 1
NH <sub>3</sub> oxidation <sup>b</sup>	<sup>15</sup> N-NO <sub>3</sub> <sup>-</sup> pool dilution	SSW (79°C)	8.6 ± 0.9	Fig. 1
Denitrification	Acetylene block	GBS (81°C)	51 ± 12 <sup>c</sup>	Fig. 2A
Denitrification	Acetylene block	SSW (79°C)	15.8 ± 0.7 <sup>c</sup>	Fig. 2A
April 2009				
NH <sub>3</sub> oxidation <sup>b</sup>	<sup>15</sup> N-NO <sub>3</sub> <sup>-</sup> pool dilution	GBS (82°C)	5.5 ± 1.1	Fig. 1
Denitrification	Acetylene block	GBS (82°C)	226 ± 25 <sup>d</sup>	Fig. 2B
DNRA	<sup>15</sup> N-NO <sub>3</sub> <sup>-</sup> tracer	GBS (82°C)	315 ± 48 <sup>d</sup>	Fig. 4

a. Microbial community analysis by 16S rRNA gene pyrotag sequencing (Fig. 5) and qPCR (Fig. 6) correspond to this date.

b. Gross production of NO<sub>x</sub> from <sup>15</sup>N-NH<sub>4</sub><sup>+</sup>; data are from unamended sediment slurries.

c. Rate from unamended, dispersed sediment cores.

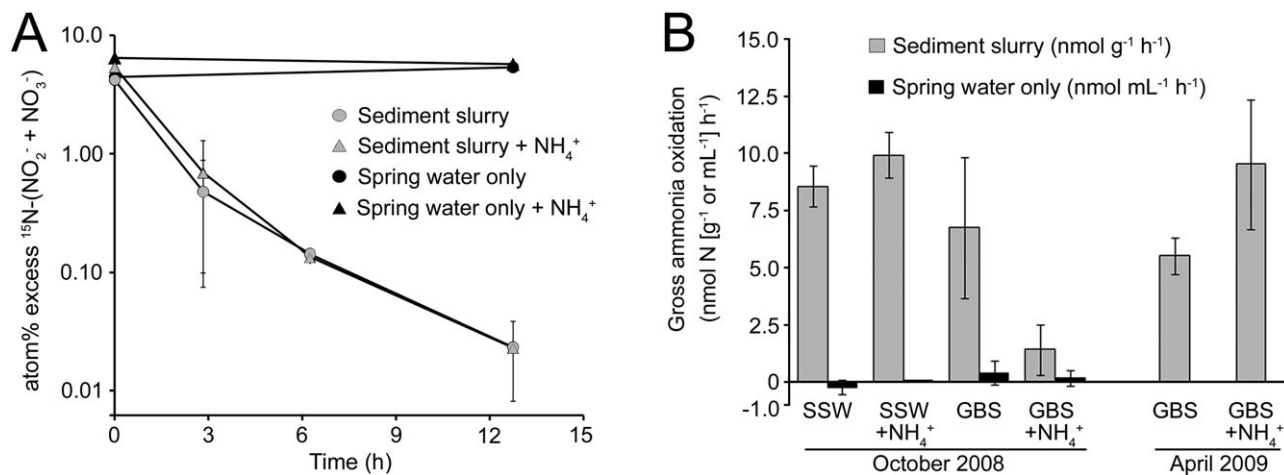
d. Rate from dispersed sediment cores amended with 30 μM NO<sub>3</sub><sup>-</sup>.

Chemistry data associated with these experiments are shown in Table S1.

#### Ammonia oxidation rates

Significant ammonia oxidation rates were observed in both SSW and GBS sediment slurries, but not in the spring water, as measured using <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> pool dilution (Barraclough, 1991). In experiments with SSW sediment slurries, either unamended or amended with NH<sub>4</sub><sup>+</sup>, an approximately log-linear decrease in atom% (at%) excess-<sup>15</sup>N in the NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup> (NO<sub>x</sub>) pool over time was observed (Fig. 1A), consistent with a constant flux of unlabelled N into the NO<sub>x</sub> pool. Experiments using spring water only showed little change in the at% excess-<sup>15</sup>N-NO<sub>x</sub><sup>-</sup> throughout the course of the experiment, indicating

the activity resulting in dilution of the <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> pool in the sediment slurries was contained primarily in the sediment fraction. Ammonia oxidation rates calculated from the pool dilution data in unamended SSW sediments were 8.6 ± 0.9 nmol g<sup>-1</sup> h<sup>-1</sup>, and were insensitive to the addition of 1 mM NH<sub>4</sub><sup>+</sup> (9.9 ± 1.0 nmol g<sup>-1</sup> h<sup>-1</sup>; Table 1 and Fig. 1B). In contrast, rates were either much lower or undetectable in spring water (0.073 ± 0.011 nmol ml<sup>-1</sup> h<sup>-1</sup> and -0.25 ± 0.31 nmol ml<sup>-1</sup> h<sup>-1</sup> when amended or unamended with NH<sub>4</sub><sup>+</sup> respectively). Although the observed dilution of the <sup>15</sup>N-NO<sub>x</sub> pool predicted a flux corresponding to an increase in [NO<sub>x</sub>] of ~10 μM, the NO<sub>x</sub> pool remained at or below the level of detection (2 μM) throughout the experiment. This suggests that production of NO<sub>x</sub> by ammonia oxidation was coupled with denitrification activity present in anaerobic microenvironments in



**Fig. 1.** Gross ammonia oxidation rates measured using the <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> pool dilution technique.

A. Dilution of <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> in the NO<sub>x</sub> pool in SSW water and sediment slurries, with or without addition of NH<sub>4</sub>Cl to 1 mM (+ NH<sub>4</sub><sup>+</sup>), performed during the October 2008 sampling trip. Data are expressed as the change in at% excess (above the natural <sup>15</sup>N abundance of 0.3663%) of <sup>15</sup>N in the NO<sub>x</sub> pool as a function of time. Error bars show the standard deviation of the mean (*n* = 3).

B. Gross ammonia oxidation rates calculated from pool dilution experiments on amended (+ NH<sub>4</sub><sup>+</sup>) or unamended GBS and SSW water and sediment slurries. Amendments were to 1 mM NH<sub>4</sub>Cl in October 2008 and 3 mM NH<sub>4</sub>Cl in April 2009. Bars represent the mean and standard error calculated using data from four time points with three replicates each (October 2008) or two time points with two replicates each (April 2009).

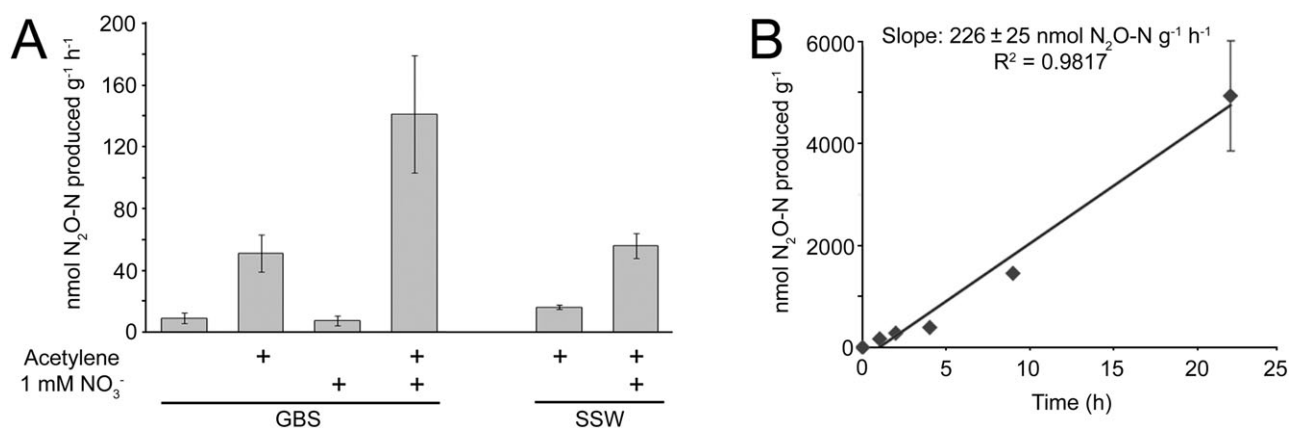
the sediment slurries. Rates were similar or somewhat lower in GBS sediments unamended or amended with  $\text{NH}_4^+$  respectively. For these samples, an unexpected ~1.6-fold increase in the at%  $^{15}\text{N}$ - $\text{NO}_x$  in the initial stages of incubation was observed. This increase was concomitant with a decrease in  $[\text{NO}_x]$  from  $25 \pm 3 \mu\text{M}$  to  $12 \pm 3 \mu\text{M}$ , and could be explained by preferential use of the unlabelled  $\text{NO}_2^-$  in the  $\text{NO}_x$  pool by denitrifiers, increasing the concentration of the  $^{15}\text{N}$ - $\text{NO}_x$ . Because of this, rates for GBS sediments were calculated where the rate of change in at%  $^{15}\text{N}$ - $\text{NO}_x$  was approximately linear, excluding data from the initial time point. Similar to SSW, rates of ammonia oxidation in GBS were much lower in spring water than in sediments, and were not stimulated by addition of  $\text{NH}_4^+$  (Fig. 1B).

Ammonia oxidation rates measured in GBS sediments in April 2009 were comparable to those observed in October 2008 even though a different incubation and sampling procedure was used (Table 1 and Fig. 1B). In contrast to the October 2008 experiments, ammonia oxidation appeared to be stimulated ( $9.5 \pm 2.9 \text{ nmol g}^{-1} \text{ h}^{-1}$ ) by amendment with  $\text{NH}_4^+$  to 3 mM (Fig. 1B). This stimulation in the April 2009 experiments may be due to the higher amounts of  $\text{NH}_4^+$  used for amendment or to the lower  $[\text{NH}_4^+]$  in GBS water on that date. The  $[\text{NH}_4^+]$  measured in GBS spring water in April 2009 was  $27 \mu\text{M}$ , lower than that in GBS and SSW water in October 2008 (~40 and ~80  $\mu\text{M}$  respectively). Also, the  $\text{NH}_4^+$  pool decreased approximately 10-fold in unamended sediments during the pool dilution experiment in April 2009 while  $[\text{NH}_4^+]$  remained relatively constant in the October 2008 experiments. These results are consistent with ammonia oxidation being limited by substrate, at least under certain conditions.

The ammonia oxidation rates measured in GBS and SSW were comparable to nitrification rates reported in two acidic (pH 3), ~85°C Iceland hot springs ( $2.8$ – $7.0 \text{ nmol NO}_3^- \text{ produced g}^{-1} \text{ h}^{-1}$ ), after conversion from volume using the reported mass of solids in their slurries (Reigstad *et al.*, 2008). Similar to rates in GBS sediments in April 2009, nitrification rates were increased approximately twofold in one of these Iceland springs upon amendment with  $0.5 \text{ mM NH}_4^+$ . The rates reported here are also within the range of gross nitrification rates observed in a variety of non-thermal environments, including woodland, grassland and agricultural soils [ $0.3$ – $180 \text{ nmol g}^{-1} \text{ h}^{-1}$  (Booth *et al.*, 2005)] and wetland sediments [ $6.3$ – $85 \text{ nmol g}^{-1} \text{ h}^{-1}$  (White and Reddy, 2003)]. It should be noted that the measurements reported here indicate ammonia oxidation rates specifically, rather than complete nitrification (ammonia oxidation plus nitrite oxidation), and do not address whether  $\text{NO}_2^-$  oxidation is also occurring.

#### Denitrification rates

In coordination with the ammonia oxidation rate measurements described above, high denitrification rates were measured in GBS and SSW sediments using the acetylene block technique, where the rate of accumulation of  $\text{N}_2\text{O}$  over time in the presence of acetylene, which inhibits  $\text{N}_2\text{O}$  reductase, is used as a proxy for denitrification (Balderson *et al.*, 1976; Yoshinari and Knowles, 1976; Dodsworth *et al.*, 2011). In experiments performed in October 2008, the  $\text{N}_2\text{O}$  pool size in sediments with 10% acetylene ( $51 \pm 12 \text{ nmol N g}^{-1} \text{ h}^{-1}$ ) was approximately sixfold higher than in unamended sediments ( $9.0 \pm 3.3 \text{ nmol N g}^{-1} \text{ h}^{-1}$ ) after a 20 h incubation (Fig. 2A). This indicated that the acetylene block was



**Fig. 2.** A.  $\text{N}_2\text{O}$  production during acetylene block experiments under anaerobic conditions in GBS and SSW sediments in October 2008. Data are expressed as  $\text{nmol N}$  in  $\text{N}_2\text{O}$  ( $\text{N}_2\text{O-N}$ ) per gram of sediment (dry weight) per hour, and the stimulatory effects of 10% acetylene and  $1 \text{ mM NO}_3^-$  (added as  $\text{NaNO}_3$ ) are shown. Bars represent the mean and standard error of the mean ( $n=3$ ) for a 20 h incubation. B. Linear increase in the  $\text{N}_2\text{O}$  produced over time using the acetylene block method on GBS sediment in April 2009, amended with  $30 \mu\text{M NO}_3^-$  and 10% (v/v) acetylene.

successful and that most of the  $\text{N}_2\text{O}$  produced in GBS sediments without acetylene was further reduced to  $\text{N}_2$  as a final product of denitrification. Because acetylene also blocks ammonia oxidation by inhibition of ammonia monooxygenase (Hynes and Knowles, 1978; Walter *et al.*, 1979), the acetylene block technique may underestimate denitrification rates in systems where this process is coupled to  $\text{NO}_3^-$  production by nitrification. To test whether this might be the case in GBS and SSW, acetylene block experiments were also performed on sediments in the presence of 1 mM  $\text{NO}_3^-$ . In GBS sediments,  $\text{NO}_3^-$  did not result in an increase in the  $\text{N}_2\text{O}$  pool in the absence of acetylene; however, under blocked conditions,  $\text{N}_2\text{O}$  accumulation was stimulated approximately 2.7-fold. A similar effect was observed in SSW sediments (~3.5-fold increase). When taken together with the fact that denitrification rates in GBS and SSW were quantitatively much higher than corresponding ammonia oxidation rates (Fig. 1), these data suggest that denitrification is coupled to and limited by nitrification in these two springs.

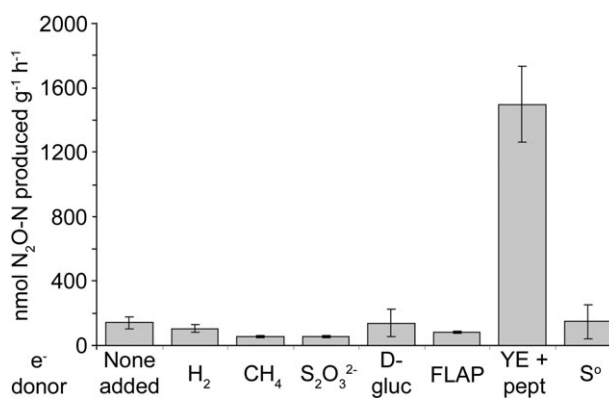
Preliminary experiments performed in June 2008 (data not shown), and subsequent experiments in April 2009 (Fig. 2B) and June 2009 (data not shown) demonstrated that the accumulation of  $\text{N}_2\text{O}$  was approximately linear over time in acetylene-blocked sediments amended with  $\text{NO}_3^-$  (1 mM for June 2008; 30  $\mu\text{M}$  for April and June 2009). This justifies the calculation of denitrification rates using  $\text{N}_2\text{O}$  accumulated at the end-point of the 20 h incubations as done with the October 2008 experiments. The denitrification rate measured in April 2009 of 226  $\text{nmol N g}^{-1} \text{h}^{-1}$  (Fig. 2B) in GBS sediment amended with 30  $\mu\text{M}$   $\text{NO}_3^-$  was somewhat higher, but generally similar to those observed in October 2008.

Acetylene block experiments with spring water only amended with 1 mM  $\text{NO}_3^-$  yielded only very low accumulation of  $\text{N}_2\text{O}$  over time, corresponding to rates of  $0.021 \pm 0.003$  and  $0.007 \pm 0.003$   $\text{nmol N}_2\text{O-N ml}^{-1} \text{h}^{-1}$  for GBS and SSW respectively. Thus, similar to ammonia oxidation rates discussed above, denitrification occurs predominately in the spring sediment. This is consistent with the presence of dissolved oxygen in the water, which, although not measured during this study, is commonly detectable in both springs, e.g. 53 and 9.4  $\mu\text{M}$  for GBS and SSW, respectively, measured on May 2006 (Costa *et al.*, 2009). Although aerobic denitrification has been shown to occur (Robertson *et al.*, 1995), the values measured in GBS and SSW are above the threshold  $\text{O}_2$  concentration of 6.25  $\mu\text{M}$  (0.2 ppm), below which denitrification is generally considered active (Seitzinger *et al.*, 2006).

Microorganisms isolated from terrestrial thermal environments have been shown to use both inorganic compounds, such as  $\text{H}_2$  and thiosulfate (Takai *et al.*, 2003;

Nakagawa *et al.*, 2004), and organic compounds (e.g. Cava *et al.*, 2008) as electron donors coupled to denitrification. To determine whether nitrous oxide production might be limited by electron donor, acetylene block experiments were performed on GBS sediments amended with various reduced inorganic and organic compounds. A mixture of yeast extract and peptone stimulated  $\text{N}_2\text{O}$  production approximately 11-fold, while dextrose, simple organic acids and several inorganic compounds as potential electron donors did not increase  $\text{N}_2\text{O}$  production (Fig. 3). Thus, in these springs, chemoorganotrophic denitrification is limited by the presence of complex organics as an electron donor. These data do not exclude the possibility that chemolithotrophic denitrification is also occurring. However, they do suggest that this process is not substrate-limited under the experimental conditions, that the nitrous oxide reductase of these organisms is not inhibited by acetylene, or that nitrate reduction coupled to oxidation of inorganic compounds does not result in  $\text{N}_2\text{O}$  or  $\text{N}_2$  as final products.

Denitrification rates in GBS and SSW were considerably higher than those determined in a YNP geothermal soil sample, ~0.8–1.1  $\text{nmol N}_2\text{O-N g}^{-1} \text{h}^{-1}$  (Burr *et al.*, 2005). When normalized to units of area, denitrification rates in October 2008 without added  $\text{NO}_3^-$  were  $98 \pm 10$   $\mu\text{mol N m}^{-2} \text{h}^{-1}$  in GBS and  $71 \pm 1$   $\mu\text{mol N m}^{-2} \text{h}^{-1}$  in SSW. These rates are on the high end of those observed in a variety of non-thermal environments including soils [0–195  $\mu\text{mol m}^{-2} \text{h}^{-1}$ , with an average of 1.5 and 10.6  $\mu\text{mol m}^{-2} \text{h}^{-1}$  for forest and agricultural soils respectively (Barton *et al.*, 1999)], estuaries [18–620  $\mu\text{mol m}^{-2} \text{h}^{-1}$  (Nixon *et al.*, 1996)] and lakes

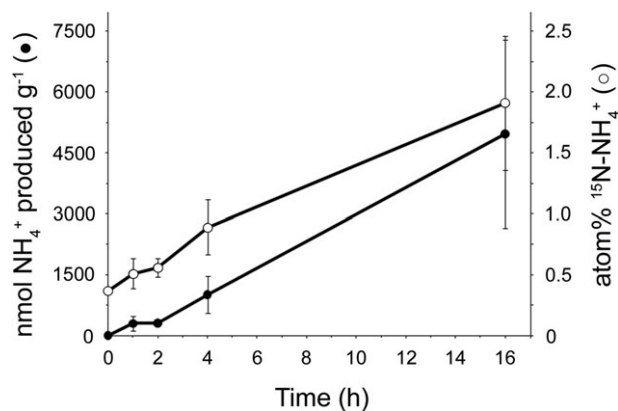


**Fig. 3.** Stimulatory effects of possible electron donors on  $\text{N}_2\text{O}$  production in nitrate-amended (1 mM) sediments under anaerobic, acetylene-blocked conditions. Sediment core slurries were amended with 1 mM of each potential electron donor except hydrogen (17% headspace v/v; added as an 80:20 mix of  $\text{H}_2$  :  $\text{CO}_2$ ), methane (21% headspace v/v), yeast extract (YE; 0.1%), peptone (pept; 0.05%) and sulfur (10% w/v). D-gluc: dextrose. FLAP: 1 mM each of formate, lactate, acetate and propionate. Results represent the mean and standard error of the mean ( $n = 3$ ) for a 20 h incubation.

[6–86  $\mu\text{mol m}^{-2} \text{h}^{-1}$  (Seitzinger *et al.*, 2006)]. Use of the acetylene block technique for measuring denitrification rates has been criticized, although mainly because it may underestimate true rates where denitrification is coupled to nitrification (Groffman *et al.*, 2006). During prolonged incubations (> 4 days) metabolism of acetylene has been shown to stimulate  $\text{N}_2\text{O}$  production in certain circumstances, and therefore result in potential overestimation of denitrification rates (Culbertson *et al.*, 1981; Topp and Germon, 1986). It is unlikely that this is occurring in the present work, where incubations were typically 20 h in length and  $\text{N}_2\text{O}$  accumulation was immediate and linear. As mentioned above,  $[\text{NO}_3^-]$  decreased in the initial stage of the  $^{15}\text{N}\text{-NO}_3^-$  pool dilution experiment. If this decrease is assumed to be due to denitrification, it corresponds to a rate of  $49 \pm 19 \text{ nmol N g}^{-1} \text{h}^{-1}$ . This rate calculated by the change in pool size is quantitatively similar to the rate obtained from the acetylene block without  $\text{NO}_3^-$  amendment, suggesting that the acetylene block technique is not grossly over- or underestimating denitrification. Because the acetylene block experiments reported here were performed on dispersed sediment cores under anaerobic conditions, they may not reflect *in situ* denitrification rates, which may be limited by diffusion. Nonetheless, these data demonstrate the potential for highly active denitrification in GBS and SSW and suggest that nitrate and/or nitrite are important electron acceptors for anaerobic respiration in sediments of these springs. The relative importance of nitrite and nitrate in this process may be driven by the extent to which biological or abiotic nitrite oxidation is coupled to ammonia oxidation in these environments; future studies will be designed to address this.

#### Dissimilatory nitrate reduction to ammonium (DNRA)

DNRA rates were also measured in dispersed GBS sediment cores on April 2009 using an  $^{15}\text{N}\text{-NO}_3^-$  tracer technique (Groffman *et al.*, 2006). An approximately linear increase over time in  $\text{at}\%^{15}\text{N}$  of the  $\text{NH}_4^+$  pool was observed, corresponding to an approximately constant calculated flux of N into this pool from the  $^{15}\text{N}$ -labelled  $\text{NO}_3^-$  pool (Fig. 4). These data yield a DNRA rate of  $315 \pm 48 \text{ nmol N g}^{-1} \text{h}^{-1}$ . Because of the relatively short incubation time (16 h), it is likely that this is due to direct, dissimilatory reduction of  $\text{NO}_x$  to  $\text{NH}_4^+$ , rather than assimilatory reduction followed by mineralization (ammonification). During the first 4 h of incubation,  $[\text{NH}_4^+]$  increased from  $31.8 \pm 1.0 \mu\text{M}$  to  $39.2 \pm 2.1 \mu\text{M}$ , while  $[\text{NO}_x]$  decreased from  $20.7 \pm 0.1 \mu\text{M}$  to  $13.8 \pm 0.3 \mu\text{M}$ , consistent with stoichiometric conversion of  $\text{NO}_x$  to  $\text{NH}_4^+$ . In sediment slurries harvested at the end time point,  $[\text{NO}_x]$  further decreased to  $7.0 \pm 1.8 \mu\text{M}$ , while  $[\text{NH}_4^+]$  changed



**Fig. 4.**  $^{15}\text{N}\text{-NO}_3^-$  tracer experiment in GBS, April 2009. Sediment samples were incubated up to 16 h under an  $\text{N}_2$  headspace in 160 ml serum vials with 60 ml of anaerobic, filtered spring water amended with  $33 \mu\text{M NO}_3^-$  at 10  $\text{at}\%^{15}\text{N}$ . Vials were sampled destructively over time and the pool sizes and  $\text{at}\%^{15}\text{N}$  were determined for  $\text{NH}_4^+$  and  $\text{NO}_x$ .  $\text{at}\%^{15}\text{N}$  of the  $\text{NH}_4^+$  pool determined by GC-MS and the  $\text{nmol NH}_4^+$  produced from the labelled  $\text{NO}_x$ , calculated from the  $\text{at}\%^{15}\text{N}$  of the  $\text{NO}_x$  pool and the  $\text{at}\%$  and size of the  $\text{NH}_4^+$  pool. Error bars represent the standard error of the mean ( $n=3$ ).

little from the 4 h time point ( $37.2 \pm 3.5 \mu\text{M}$ ). Because the  $\text{at}\%^{15}\text{N}$  of the  $\text{NH}_4^+$  pool increased linearly over the course of the experiment, the absence of further accumulation of  $\text{NH}_4^+$  after 4 h may reflect assimilation of  $\text{NH}_4^+$ . Rates of DNRA in thermal environments have not been as thoroughly investigated as denitrification, although DNRA may be the dominant pathway of dissimilatory nitrate removal in some environments (Burgin and Hamilton, 2007). Several recent studies report DNRA rates of 0–33 and 2.2–320  $\mu\text{mol N m}^{-2} \text{h}^{-1}$  in freshwater and marine environments respectively (Scott *et al.*, 2008; Dong *et al.*, 2009; Stief *et al.*, 2010). When normalized to activity per unit area, the DNRA rate in GBS,  $318 \pm 51 \mu\text{mol N m}^{-2} \text{h}^{-1}$ , lies at the high end of this range.

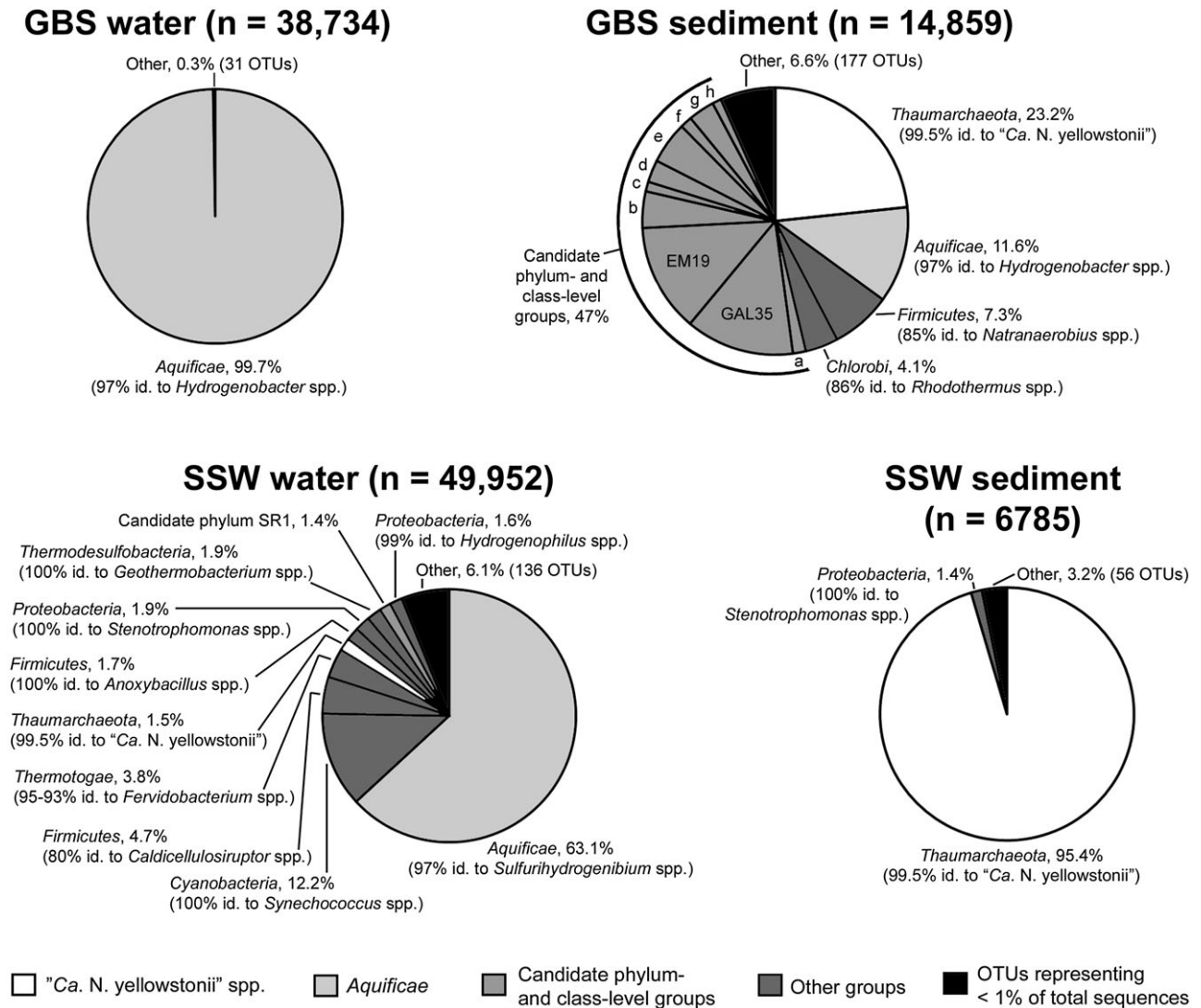
Because direct comparisons of DNRA and denitrification rates were not made on the same samples, these data do not directly address the relative importance of DNRA and denitrification. They nonetheless indicate a high potential in GBS sediments for dissimilatory reduction of  $\text{NO}_x$ , with DNRA rates likely comparable to those of denitrification. DNRA represents a functional 'link' in the N-cycle, as it produces  $\text{NH}_4^+$  from  $\text{NO}_x$ , which can be used as a substrate for ammonia oxidation and assimilated into biomass, whereas the gaseous products of denitrification,  $\text{NO}$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$ , are not directly available for assimilation and are more easily lost from the environment. In non-thermal environments, DNRA appears to be favoured under conditions of high sulfide and organic content (Tiedje *et al.*, 1982; Brunet and Garcia-Gil, 1996; Christensen *et al.*, 2000; Gardner *et al.*, 2006; Porubsky *et al.*, 2009). The relative importance of DNRA or denitrification

in GBS sediments may therefore be determined by location within the spring (e.g. depth in the sediment), conditions in sediment particle microenvironments or variation in spring conditions over time (e.g. temperature and flow rate).

*Microbial community composition*

To determine which members of the microbial community might be involved in ammonia oxidation and denitrification, the relative abundance of *Bacteria* and *Archaea*

in GBS and SSW was assessed by pyrosequencing of PCR-amplified 16S rRNA gene fragments. The overwhelming majority of sequences either grouped within the *Aquificae* and *Thaumarchaeota*, or were not closely related to cultured microbes (Fig. 5). In both springs, *Aquificae* dominated the planktonic community, whereas *Thaumarchaeota* or novel lineages dominated sediment communities. Close relatives of the thermophilic, autotrophic, ammonia-oxidizing archaeon 'Ca. N. yellowstonii' (de la Torre *et al.*, 2008) represented the single most abundant OTU in GBS and SSW sediments, as



**Fig. 5.** 16S rRNA gene pyrotag analysis of microbial communities in GBS and SSW, October 2008. DNA was extracted from sediment or material collected on a 0.2 µm filter after passage of spring water. 16S rRNA genes were amplified using primers 515F and 1391R modified for pyrosequencing, and products were sequenced from the forward primer using a 454 GS FLX pyrosequencer. The phylum-level classifications of individual OTUs representing > 1% of the total number of amplicons (n) analysed by PyroTagger (Kunin and Hugenholtz, 2010) using a 97% identity cut-off are shown. For phyla with cultured representatives, the best BLAST (Altschul *et al.*, 1997) hit of the OTU's representative (most abundant) sequence to the 16S rRNA gene of a cultured organism is shown in parentheses. Candidate phylum- and class-level groups, as identified in the Hugenholtz phylogeny on Greengenes (DeSantis *et al.*, 2006), include: (a) *Chloroflexi* group TK17; archaeal groups, (b) GBS\_L2\_E12 and (c) pSL4; and bacterial phylum-level groups GAL35, EM19, (d) OctSpA1-106, (e) OP10, (f) SR1 and (g,h) EM3.

well as an OTU (1.7% of sequences) in SSW water (Fig. 5). The representative sequences from these OTUs were identical, and shared 99.5% identity with the 16S rRNA gene of 'Ca. N. yellowstonii' in a 231-nucleotide region spanning the V4 region (Baker *et al.*, 2003). Mixed cultures of close relatives of 'Ca. N. yellowstonii' (> 99% 16S rRNA gene identity) were recently obtained from enrichments for ammonia oxidizers with bicarbonate as a sole C source with GBS sediment as inoculum (J.R. de la Torre and H.M. Gray, pers. comm.). It is therefore likely this OTU representing 'Ca. N. yellowstonii' relatives is responsible for the ammonia oxidation rates measured in GBS and SSW sediments (Fig. 1).

Microbial groups potentially responsible for denitrification or DNRA were less conspicuous in the pyrosequencing data, but could possibly include members of the genera *Hydrogenobacter*, *Sulfurihydrogenibium*, *Anoxybacillus* and *Thermus*. The microbial communities in the spring water fractions were each dominated by a single, distinct OTU representing members of the bacterial phylum *Aquificae*. In GBS, the OTU's representative sequence was 97% and 96% identical to the 16S rRNA genes of several *Hydrogenobacter* spp. and *Thermocrinis albus*, respectively, members of the family *Aquificaceae*. An identical sequence represented an OTU in GBS sediment, which comprised 11.6% of the total sequences in that environment. In SSW, the representative sequence was 97% identical to several *Sulfurihydrogenibium* spp., including *S. yellowstonii*, members of the *Hydrogenothemaceae*. One of the three described species of *Hydrogenobacter*, *H. thermophilus* TK-6, has been shown to be capable of denitrification (Suzuki *et al.*, 2001). No *Thermocrinis* spp. are known to use nitrate as an electron acceptor (Huber *et al.*, 1998; Eder and Huber, 2002) and only one of the five described *Sulfurihydrogenibium* spp., *S. subterraneum*, can use nitrate, but not nitrite, as a terminal electron acceptor for denitrification (Takai *et al.*, 2003). One OTU in SSW water (1.7% of sequences) was closely related to several *Anoxybacillus* spp., many of which are capable of nitrate reduction (Poli *et al.*, 2009), although gas production from nitrate (full denitrification to N<sub>2</sub>) has not been reported. It is possible that some N<sub>2</sub>O production could be due to nitrifier denitrification (Wrage *et al.*, 2001) by the 'Ca. N. yellowstonii' relatives, although it is not known if this process occurs in AOA, and would not explain the N<sub>2</sub>O production under acetylene blocked conditions or the stimulation by NO<sub>3</sub><sup>-</sup>.

Other than 'Ca. N. yellowstonii' and *Aquificaceae* relatives, the OTUs representing > 1% of total sequences in GBS sediment either were classified as candidate phylum- or class-level groups with no cultured representatives, or were only distantly related (85–86% 16S rRNA gene identity) to cultured bacteria; thus, it is difficult to predict their potential role in nitrogen cycling in GBS.

While the temperature of SSW water during sampling was 79°C, above the upper temperature limit of photosynthesis (Brock, 1967), the second most abundant OTU was closely related to several *Cyanobacteria* (100% 16S rRNA gene identity). These were all members of a group of highly thermophilic *Synechococcus* isolates (*Synechococcus* Group IV) with an optimal growth temperature near 70°C (Miller and Castenholz, 2000). It is likely that these represent dead or inactive cyanobacterial cells carried in from the fringes of the spring pool, where the temperature is low enough to allow photosynthesis and obvious green- and yellow- pigmented microbial growth is present. One OTU each in SSW water and sediment were also closely related to non-thermophilic *Stenotrophomonas* spp., *Gammaproteobacteria* that are present in many natural environments, often as endophytes or otherwise in association with plants (Ryan *et al.*, 2009). Although it is possible that these are the result of contamination, it is likely that they also were transported in from lower-temperature environments, as there is abundant plant growth up to the edges of the SSW source pool. These results suggest that influx of allochthonous biomass from non-thermal environments may represent a possible source of fixed carbon to microbial communities in SSW and other springs.

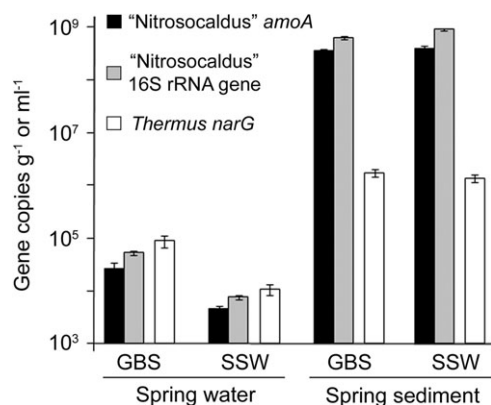
It is notable that the pyrosequencing data revealed distinct microbial populations in the water and sediment because very different conclusions might be drawn about microbial community structure and function based on analysis of either environment on its own. Most molecular microbial censuses in hot spring environments to date have focused exclusively on sediment or biofilm communities. The predominance of *Aquificales* in GBS and SSW water is consistent with other studies of terrestrial hot spring environments, and their abundance in these systems has led to the suggestion that oxidation of H<sub>2</sub> or reduced sulfur compounds may be key energy sources driving primary production and microbial metabolism in these communities (Reysenbach *et al.*, 2005; Spear *et al.*, 2005; D'Imperio *et al.*, 2008). The abundance of 'Ca. N. yellowstonii' spp. and the associated ammonia oxidation rates in GBS and SSW sediments, however, suggest that ammonia oxidation may also be an important energy source in these springs. In general, the relative importance of sediment and waterborne communities might be tied to the flow rate and size of the spring source pool. In outflows or source pools with a short residence time relative to the minimum known generation time for microorganisms, such as SSW (~5 min), waterborne cells likely originate from the continuous release of cells from sediment or biofilm communities in the subsurface or surface pool rather than from growth in the water column. In contrast, for springs with a relatively long residence time, such as GBS (~1–2 days), waterborne cells may represent genuine planktonic communities that are active



and growing within the water column. Consistent with this interpretation, cell counts in spring water obtained by flow cytometry of samples taken on June 2008 were approximately  $1 \times 10^5$  in SSW and  $2 \times 10^6$  in GBS (J.A. Dodsworth and B.P. Hedlund, unpublished) and the planktonic community in GBS described here was extremely uneven compared with that in SSW (Fig. 5). Particularly in springs with long residence times, such as GBS, consideration of both planktonic and sediment or substrate-attached microbiota may be necessary to fully understand the ecology of the surface pool.

#### Quantification of biomarkers for putative AOA and denitrifiers

Because of the apparent relative abundance of '*Ca. N. yellowstonii*' relatives in GBS and SSW sediments, qPCR was used to quantify *amoA* and 16S rRNA gene copies using primers specific to '*Ca. N. yellowstonii*' and sequences amplified from GBS and SSW (Dodsworth *et al.*, 2011). '*Candidatus N. yellowstonii*' *amoA* and 16S rRNA genes were present at  $3.5\text{--}3.9 \times 10^8$  and  $6.4\text{--}9.0 \times 10^8$  copies  $\text{g}^{-1}$  sediment respectively (Fig. 6). When converted to copies per g sediment dry weight (gdw), the abundance of archaeal *amoA* genes in GBS and SSW,  $1.6\text{--}1.8 \times 10^9$   $\text{gdw}^{-1}$ , is high in comparison with both archaeal and bacterial *amoA* gene levels in natural non-thermal environments, including soils [ $10^5\text{--}10^8$   $\text{gdw}^{-1}$  (Leininger *et al.*, 2006)] and salt marsh sediments [ $2 \times 10^4\text{--}5.8 \times 10^9$   $\text{gdw}^{-1}$  (Moin *et al.*, 2009)]. To relate these to the total microbial population, a rough approxi-



**Fig. 6.** Quantification of genes associated with potential nitrifiers ('*Ca. N. yellowstonii*') and denitrifiers (*Thermus* spp.) by qPCR. Template DNA was extracted from either spring sediment or material retained on a 0.2  $\mu\text{m}$  filter after passage of 300 ml of spring water, collected on the October 2008 sampling trip. Primers in the qPCR targeted putative ammonia monooxygenase genes (*amoA*) and 16S rRNA genes associated with '*Ca. N. yellowstonii*' spp., and nitrate reductase genes (*narG*) from *T. thermophilus* and *Thermus* spp. isolated from GBS. Data are expressed as gene copies per gram of sediment (wet weight) or per millilitre of water. Error bars represent the standard error of the mean ( $n = 3$ ).

mation of the total number of genomes present was made based on the amount of DNA (2 and  $1.3 \mu\text{g g}^{-1}$  in GBS and SSW respectively) and an average genome size (1.5–3 Mbp, based on a typical range of thermophile genomes on the JGI IMG database; <http://img.jgi.doe.gov>), which yielded  $6\text{--}12 \times 10^8$  and  $4\text{--}8 \times 10^8$  genomes  $\text{g}^{-1}$  in GBS and SSW extracts respectively. If a single copy of *amoA* per genome is assumed, which is the case for the only cultivated AOA genome sequenced to date, *Nitrosopumilus maritimus* (Walker *et al.*, 2010), and if the relative proportion of genomes in the DNA extracts is representative of their host organisms *in situ*, the qPCR data indicate that '*Ca. N. yellowstonii*' relatives comprise 30–60% of the total population in GBS sediments and 50–99% of the total population in SSW. These percentages are similar to the results obtained from pyrosequencing (Fig. 5). By assuming one *amoA* copy per genome and two genomes per cell (Bernander and Poplawski, 1997), qPCR data can be combined with  $\text{NO}_3^-$  pool dilution data (Fig. 1) to yield per-cell ammonia oxidation rates of 0.008–0.01  $\text{fmol N cell}^{-1} \text{h}^{-1}$ , which are low in comparison with those for non-thermophilic AOA and ammonia-oxidizing bacteria [0.03–43  $\text{fmol cell}^{-1} \text{h}^{-1}$  (Coskuner *et al.*, 2005; Wuchter *et al.*, 2006)]. Because of the assumptions involved, these relative abundances and per-cell activities should be considered only provisional. However, the data collectively indicate that close relatives of '*Ca. N. yellowstonii*' are prominent members of the microbial communities in GBS and SSW sediments.

Lower amounts of '*Ca. N. yellowstonii*' *amoA* and 16S rRNA gene copies were detected in GBS and SSW water (Fig. 6). If cell counts on the October 2008 sampling trip were similar to those in June 2008 described above, then these data indicate that '*Ca. N. yellowstonii*' relatives make up a minor proportion of the microbial community in the spring water (~1% or less), consistent with the pyrosequencing data and the low or undetectable rates of ammonia oxidation in the spring water (Fig. 1B). Because of the low abundance of '*Ca. N. yellowstonii*' in spring water in comparison with the sediment and the fact that the  $\text{NO}_3^-$  pool dilution technique employed in this study would not be sensitive to such low ammonia oxidation rates, our data do not address whether AOA in the water are less active on a per-cell basis than those in the sediment; the lack of significant activity in spring water could be due solely to the relatively low abundance of AOA.

Close relatives of *Thermus thermophilus* represented the single most abundant group of isolates obtained in enrichments for heterotrophic denitrifiers done using as inoculum GBS and SSW sediment collected on October 2008 and several other sampling dates (B.P. Hedlund, A.I. McDonald and J. Lam, unpubl. results). To determine the abundance of denitrifying *Thermus* spp., qPCR was performed using primers specific for *narG*, designed using

sequences amplified from these isolates and the sequence of the *T. thermophilus* HB8 *narG* (Dodsworth *et al.*, 2011). While more prevalent than 'Ca. N. yellowstonii' *amoA* in GBS and SSW water, *Thermus* spp. *narG* copies were ~200-fold less abundant than 'Ca. N. yellowstonii' in the sediments of these springs where denitrification rates were high (Fig. 6). In the pyrosequence data sets, OTUs representing *Thermus* spp. represented 0.9% of the amplicons in GBS sediment, but were < 0.1% of amplicons present in GBS and SSW water, and were not observed in SSW sediment. The extent to which *Thermus* spp. quantitatively play a role in denitrification in GBS and SSW is therefore unclear.

### Conclusion

The data reported here demonstrate active ammonia oxidation, denitrification and DNRA in two US Great Basin hot springs, GBS and SSW (Table 1). Activity was contained primarily in the sediments, as opposed to the spring water. The rate measurements for these processes were similar in magnitude to those observed in a variety of non-thermal environments. Denitrification could be stimulated by addition of nitrate and further by addition of complex organic carbon, suggesting an important role for heterotrophs in denitrification. High rates of DNRA suggest this process is important and justifies further investigation of the relative importance of denitrification and DNRA in geothermal ecosystems.

Spring sediment and water microbial communities were distinct. The observed ammonia oxidation activity is likely due to a single phylotype closely related to the autotrophic AOA 'Ca. N. yellowstonii' (de la Torre *et al.*, 2008), which was a prominent member of the sediment communities in both springs. Waterborne communities were dominated by either *Sulfurihydrogenibium* spp. or *Hydrogenobacter/Thermocrinis* spp. and may be controlled by water residence time. Several studies have suggested that members of the *Aquificae* are responsible for the bulk of carbon fixation in terrestrial hot springs, and that oxidation of either hydrogen (Spear *et al.*, 2005) or reduced sulfur compounds (D'Imperio *et al.*, 2008) is the primary energy source for this process. However, the abundance of 'Ca. N. yellowstonii' and the rates of ammonia oxidation in GBS and SSW indicate that this process may also be an important energy source driving carbon fixation and microbial growth in these springs. The detection of putative archaeal *amoA* genes in other terrestrial geothermal systems worldwide (Reigstad *et al.*, 2008; de la Torre *et al.*, 2008; Zhang *et al.*, 2008) and the recent cultivation of thermophilic AOA (Hatzenpichler *et al.*, 2008; de la Torre *et al.*, 2008) suggests that ammonia oxidation may be an important energy source in a variety of terrestrial hot springs.

## Experimental procedures

### Sample sites and general techniques

The hot spring sources GBS and SSW have been described previously (Costa *et al.*, 2009). Sample collection and experiments for rate measurements were conducted on two field trips, 24–26 October 2008 and 7–9 April 2009. Field measurements of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations were made using colorimetric kits and a Smart2 handheld spectrophotometer [LaMotte, Chestertown, MD, USA (Dodsworth *et al.*, 2011)]. For all experiments in SSW, sediment samples were taken in a small area (~10 × 30 cm) about 5–10 cm below the water–air interface at the east side of the spring pool. In GBS, all samples were taken from a shallow area just downstream of the outflow from the main pool except samples for ammonia oxidation rates and DNA extraction on October 2008 which were taken ~1 m up-flow (north) of this site in the main spring pool. Water samples were taken from spring water directly above the sediment sampling sites.

### DNA extraction and quantification

Sediment and water samples were collected for DNA extraction on the October 2008 sampling trip. Sediment from the sediment/water interface (top ~1 cm) was collected in a 50 ml polypropylene tube and shaken vigorously to homogenize. Aliquots were made in 1.5 ml tubes and were immediately frozen on crushed dry ice. For collection of waterborne communities, 300 ml of spring water was passed by syringe through a 0.2 µm Supor filter (Pall Life Sciences, Ann Arbor, MI, USA) contained in an Easy Pressure Syringe Filter Holder (Pall Life Sciences). The filter was removed using sterile forceps, placed in a 1.5 ml tube containing 0.15 ml of 5× TE (50 mM Tris and 5 mM EDTA at pH 8), shaken and immediately frozen on crushed dry ice. Samples were stored on dry ice or at –80°C after transport back to the laboratory. DNA was extracted from frozen sediments or filters/TE using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA was precipitated with 70% ethanol, resuspended in 0.5× TE and quantified using a Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA).

### Ammonia oxidation measurements using $^{15}\text{N-NO}_3^-$ pool dilution

An  $^{15}\text{N-NO}_3^-$  pool dilution technique (Barraclough, 1991) was used to measure nitrification (as ammonia oxidation) rates on the October 2008 and April 2009 sampling trips essentially as described previously (Dodsworth *et al.*, 2011). This technique involves amendment of a sample with a small amount (5–10% of the total *in situ* pool size) of highly enriched  $^{15}\text{N-NO}_3^-$ . Assuming that the label is well mixed throughout the total pool in the sample, that fluxes out of the pool do not significantly affect the at% $^{15}\text{N}$  abundance, and that rates follow zero order kinetics (facilitated by short incubation times), change in the size and at% $^{15}\text{N}$  of the  $\text{NO}_3^-$  pool over time can be used to calculate gross flux of unlabelled N into this pool. As implemented here, the at% $^{15}\text{N}$  and pool sizes are determined for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  combined ( $\text{NO}_x$ ), rather than

separately, and thus indicate gross ammonia oxidation rates specifically, rather than complete nitrification (ammonia oxidation plus nitrite oxidation).

For the October 2008 trip, 25 ml aliquots of spring water or sediment slurry (~1/3 sediment by volume, collected from the top ~1 cm of sediment) were transferred into sterile 160 ml serum vials (Wheaton, Millville, NJ, USA) without allowing the samples to cool. Vials were amended with 25, 40 or 6.25  $\mu\text{l}$  of 98 at%  $^{15}\text{N-KNO}_3$  for GBS water, GBS sediment or SSW samples (both water and sediment), respectively, to achieve ~10 at%  $^{15}\text{N-NO}_3^-$ . These amounts were based on field measurements of  $\text{NO}_3^-$  in spring water or KCl-extracted (see below) sediment slurries. Some vials were additionally amended with 25  $\mu\text{l}$  of 1 M  $\text{NH}_4\text{Cl}$  to a final concentration of 1 mM. Vials were sealed with blue butyl rubber stoppers (BellCo Glass, Vineland, NJ) and aluminium crimps, and incubated in plastic racks suspended in the spring water. Triplicate vials were sampled destructively either immediately (initial time point) or after 2.66, 6 and 12.25 h for GBS sediment slurries, or 2.83, 6.25 and 12.75 h for SSW slurries. Spring-water-only samples were sampled at the initial and end time point. To prevent possible oxygen limitation, the vial headspace was flushed every 2 h with 120 ml of air, added by needle and syringe. Upon removal from the spring, vials were cooled on ice to ~25°C and contents were decanted into sterile 50 ml polypropylene tubes containing 12.5 ml of 3 M KCl. Tubes were shaken at 120 r.p.m. for 1 h in a rotary shaker at ambient temperature for extraction of cations and anions, and subsequently centrifuged at 1500 g for 10 min. The supernatant was passed through a 0.2  $\mu\text{m}$  IC Acrodisc filter (Pall Life Sciences), frozen on dry ice and stored at -80°C until analysis. For samples containing sediment slurry, the dry weight was determined by washing the sediment twice with 45 ml of distilled water to remove KCl, and drying at 65°C to a constant weight. The average sediment dry weight was ~2.5 g per sample.

For the April 2009 sampling, 20 ml aliquots of sediment slurry (collected from the top ~1.5 cm of sediment) were transferred into 120 ml polypropylene specimen cups (Kendall/Tyco Healthcare, Mansfield, MA); 80 ml of spring water was added to each, for a total volume of 100 ml. Cups were amended with 0.1  $\mu\text{mol}$   $^{15}\text{N-KNO}_3^-$ , either with or without 3 mM supplemental  $\text{NH}_4\text{Cl}$ . Specimen cups were capped with plastic tops and incubated in the spring water. Each cup was sequentially subsampled by removing 25 ml of sediment slurry, initially after 30 min and subsequently after 12 h of incubation. The subsamples were immediately extracted in 25 ml of 1 M KCl for 30 min with 1 min of vigorous hand shaking every 5 min. Extractions were allowed to settle for 30 min, and the supernatant was decanted and passed through a 0.2 micron filter. Total sediment mass for each incubation was determined after drying and mathematically correcting for KCl. Average sediment mass was 3.22 g per incubation.

In the lab, samples were thawed and concentrations of  $\text{NH}_4^+$  and  $\text{NO}_x$  were determined by automated colorimetry (US-EPA, 1993a,b). The at%  $^{15}\text{N}$  of the  $\text{NO}_x$  pool was determined by conversion to  $\text{N}_2\text{O}$  followed by coupled gas chromatography-isotope ratio mass spectrometry (GC-IRMS) as previously described (Dodsworth *et al.*, 2011).  $\text{NO}_x$  in the sample was biologically converted to  $\text{N}_2\text{O}$  using a cell suspension of

*Pseudomonas aureofaciens* (Sigman *et al.*, 2001). Using the determined  $\text{NO}_x$  pool size and at%  $^{15}\text{N}$  at each time point, the gross ammonia oxidation rate was calculated as described (Dodsworth *et al.*, 2011). Calculations for GBS were performed using a changing  $\text{NO}_x$  pool size and excluded the first time point due to large, initial increases in at%  $^{15}\text{N-NO}_x$ . For SSW a constant  $\text{NO}_x$  pool size at the level of detection (2  $\mu\text{M}$ ) was assumed because levels of  $\text{NO}_x$  in SSW samples throughout the experiment time-course were at or below the level of detection with dilution used for colorimetric quantification.

#### *Denitrification measurements using the acetylene block method*

Prior to field work, an anaerobic medium was prepared from spring water from GBS or SSW, where appropriate, collected on a previous sampling trip as ultrafiltrate from a tangential flow filtration system (Prep/Scale filter with a 30 kDa molecular weight cut-off, Millipore, Billerica, MA). Spring water was sparged for 30 min with  $\text{N}_2$ , transferred to an anaerobic chamber and portioned into 18  $\times$  150 mm serum tubes (Bellco Glass) in 5 ml aliquots. If appropriate, some tubes were amended with 30  $\mu\text{M}$  or 1 mM  $\text{NaNO}_3$ . Tubes were stoppered, sealed, and the headspace was replaced with  $\text{N}_2$  by three cycles of vacuuming and gassing with  $\text{N}_2$  prior to sterilization by autoclaving. In the field, the prepared tubes were pre-warmed and incubated in the spring outflow throughout the following process. The top ~1 cm of sediment was sampled using a cut, plastic 5 ml syringe (Becton, Dickinson and Co., Sparks, MD) as a coring device (1  $\text{cm}^2$  cross-sectional area). Preliminary experiments performed indicated that the majority of  $\text{N}_2\text{O}$  production activity was contained in this surface sediment layer, as opposed to deeper layers (B.P. Hedlund, unpubl. data). Sediment samples were added to the pre-warmed tubes under a stream of  $\text{N}_2$  using the technique of Hungate (1950, 1969). Tubes were immediately stoppered and sealed with aluminium crimps, and tube headspace was flushed for 5 min with  $\text{N}_2$ , using an outflow needle piercing the stopper. For electron donor addition experiments, anaerobic, sterile stocks were added prior to sealing tubes to the following concentrations or amounts: 1 mM dextrose and sodium thiosulfate; 1 mM each of a mix of sodium formate, lactate, acetate and propionate (FLAP); 5 ml of  $\text{CH}_4$  or an 80:20 mix of  $\text{H}_2$  :  $\text{CO}_2$ ; 0.5 g of sulfur; 0.1% and 0.05% of Bacto yeast extract and peptone (Becton, Dickinson and Co.). Where appropriate, tubes were amended with 2.5 ml of acetylene gas, freshly prepared by adding spring water to a sealed, evacuated bottle containing ~1 g of calcium carbide. Tubes were shaken for 30 s and returned to the spring for incubation. Either immediately after preparation or at various time points, triplicate tubes were sampled destructively, cooled to ambient temperature (~25°C) and shaken three times for 30 s at intervals of 3 min to equilibrate  $\text{N}_2\text{O}$  with the tube headspace. Headspace samples from each tube were transferred to stoppered, evacuated 10 ml serum vials using 22-gauge vacutainer needles (#367211, Becton, Dickinson and Co.). Vacuum was relieved from the system by addition of 10 ml of water prior to removal of the serum vial from the vacutainer needle. In the lab,  $\text{N}_2\text{O}$  concentrations in the sample vials were determined by GC-ECD on a GC-2014

Nitrous Oxide Analyser (Shimadzu, Moorpark, CA), and denitrification rates were calculated as described (Dodsworth *et al.*, 2011). To determine the mass of sediment present, the liquid phase was passed through a filter and the filter was dried to constant weight.

#### DNRA measurements using an $^{15}\text{N-NO}_3^-$ tracer technique

Sealed containers of anaerobic spring water were prepared as described above for the acetylene block, except that 160 ml serum vials were used, each containing 60 ml of spring water amended to  $33\ \mu\text{M KNO}_3$  at 10 at%  $^{15}\text{N}$ . Sediment cores were added using Hungate technique as described above, and triplicate vials were sampled destructively after 0, 1, 2, 4 and 16 h of incubation. Vials were cooled and pressurized with  $\text{N}_2$  to 2 atm overpressure, and vials were shaken three times for 30 s at 3 min intervals to equilibrate  $\text{N}_2\text{O}$  with the headspace. Headspace samples were removed with a 60 ml syringe and transferred to an evacuated container. The liquid phase was extracted with KCl, centrifuged, filtered and frozen as described above for the  $^{15}\text{N-NO}_3^-$  pool dilution technique. At%  $^{15}\text{N}$  of  $\text{NO}_x$  and  $\text{NH}_4^+$  were determined separately using a diffusion technique (Dodsworth *et al.*, 2011) followed by Dumas' combustion coupled to GC-IRMS as described (Brooks *et al.*, 1989; Stark and Hart, 1996).  $\text{NO}_x$  and  $\text{NH}_4^+$  pool sizes were determined by automated colorimetry as described above. Rate of flux into the  $\text{NH}_4^+$  pool were calculated as the product of the at% excess  $^{15}\text{N}$  and size of the  $\text{NH}_4^+$  pool divided by the at% excess  $^{15}\text{N}$  of the  $\text{NO}_x$  pool, normalized per g sediment dry weight, determined as described above.

#### 16S rRNA gene amplification, pyrosequencing and analysis

Prior to preparative amplification, the 16S rRNA gene copy number in each DNA extract was determined by qPCR with the universal Taqman scheme (Zhang *et al.*, 2003; White *et al.*, 2009) using primers 515F-UPL, 515F and 1391R [Table S2 (Lane, 1991)], the FAM-labelled, locked nucleic acid probe #149 from the Universal ProbeLibrary Expansion Set (Roche), and the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Amplification was performed using an MX3005-P thermocycler (Stratagene/Agilent Technologies, Santa Clara, CA) with a 10 min hot start (95°C) followed by 40 cycles of 95°C for 30 s and 60°C for 60 s. Serially diluted *Escherichia coli* genomic DNA was used to generate a standard curve.

Based on this quantification, a quantity of the extracted DNA equivalent to ~1 000 000 16S rRNA gene copies was used as template for a preparative PCR to generate adapted and barcoded 16S rRNA gene amplicons for pyrosequencing. Primers 515F and 1391R (Lane, 1991), modified with Titanium pyrosequencing adaptors A (515F) and B (1391R) and a 10-nucleotide barcode (515F; sequences in Table S2), were used to amplify 16S rRNA genes from *Bacteria* and *Archaea*. With the exception of the *Korarchaeota* and *Nanoarchaeota*, these two primers have relatively broad coverage within the *Bacteria* and *Archaea*,

including thermophiles (Baker *et al.*, 2003; Baker and Cowan, 2004). The reactions were performed in 50  $\mu\text{l}$  total volume using Platinum HiFi Master Mix (Invitrogen, Carlsbad, CA) on an MJ PTC-100 thermocycler (MJ research/Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 94°C for 5 min; 24 cycles of 92°C for 20 s, 50°C for 30 s, 65°C for 60 s, 75°C for 60 s, 70°C for 60 s; and a final 10 min extension step at 70°C. Products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA), followed by purification by Solid Phase Reversible Immobilization and size-selection step on calibrated Agentcourt AMPure magnetic beads (Beckman Coulter Genomics, Danvers, MA) according to the Roche/454 method. Purified libraries were quantified using the previously described digital PCR method (White *et al.*, 2009), and sequencing of the 16S rRNA gene amplicons was performed on a Roche 454 Genome Sequencer FLX System using Titanium chemistry according to the manufacturers protocols (454 Life Sciences, Branford, CT). Emulsion PCR was carried out with DNA : bead ratios between 0.08:1 and 0.3:1.

Analysis of the resulting sequence data was performed using PyroTagger [<http://pyrotagger.jgi-psf.org> (Kunin and Hugenholtz, 2010)]. A quality filter of 10% was used for all data sets except for the GBS sediment, where a 99% filter was used due to the lower quality of this sequencing run. Sequences were trimmed from the 3' end to 250 nucleotides in length and OTUs (grouped at 97% sequence identity) were determined using the pyroclust algorithm (Kunin and Hugenholtz, 2010). All pyrotags have been submitted to the NCBI Short Read Archive under Accession No. SRA026491.2. In addition, PyroTagger output including OTU designations and sequences representing each OTU are included in *Supporting information*.

#### qPCR for '*Ca. N. yellowstonii*' amoA and 16S rRNA genes and *Thermus narG* genes

Quantitative PCR was performed on template DNA extracted from GBS and SSW with the following primer sets, as described in Dodsworth and colleagues (2011): CNYamoA-F and CNYamoA-R, specific for the *amoA* of '*Ca. N. yellowstonii*' and closely related sequences in 'Thermophilic Cluster IV' (de la Torre *et al.*, 2008); CNY16S-F and CNY16S-R, specific for the 16S rRNA gene of '*Ca. N. yellowstonii*' and close relatives; and TnarG-F and TnarG-R, specific for the *narG* (encoding nitrate reductase) of *T. thermophilus*. Primer sequences are shown in Table S2. An iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for qPCR. Reactions contained 12.5  $\mu\text{l}$  of 2 $\times$  PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD, USA), 2.5  $\mu\text{l}$  of template DNA (~5 ng) and 400 nM of each forward and reverse primer in 25  $\mu\text{l}$  total volume. Cycling conditions were as follows: an initial melting step of 95°C for 3 min, followed by 45 cycles of 94°C for 15 s, 58°C for 15 s and 72°C for 45 s, with data collection using a SYBR-490 filter (Bio-Rad) enabled during the 72°C step for each cycle. After amplification, melting curves for the products were generated by increasing temperature from 55°C to 95°C by 0.5°C increments for 10 s each. Ten-fold dilutions, ranging from  $10^2$  to  $10^7$  copies per

reaction, of linearized plasmid appropriate for each primer set were used to create standard curves. For the primer set CNY16S-F and -R, plasmid pCR2.1-TOPO containing the clone SSE\_L4\_B03 (Costa *et al.*, 2009) was used. To obtain positive controls and standards for *amoA* and *narG*, 'Ca. N. yellowstonii' *amoA* was PCR-amplified from GBS sediment using primers DegAamoA-F and DegAamoA-R (Dodsworth *et al.*, 2011), and *Thermus* spp. *narG* was PCR-amplified from DNA of a *T. thermophilus* strain isolated from GBS using primers narG1960f and narG2650r (Philippot *et al.*, 2002). These *amoA* and *narG* PCR products were cloned in the vector pCR2.1-TOPO (using the manufacturer's instructions; Invitrogen), linearized, and used to prepare standard curves with primer sets CNY amoA-F/CNY amoA-R and TnarG-F/TnarG-R respectively. Threshold cycles were calculated using the maximum correlation coefficient approach and data analysis was performed using version 3.1 of the iCycler iQ Optical System Software (Bio-Rad). Amplification was log-linear with respect to target concentration from  $10^3$  to  $10^7$  copies per reaction, and amplification efficiencies and correlation coefficients ( $r^2$ ) for regressions ranged from 63.5% to 70.4% and from 0.996 to 0.999 respectively.

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### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Spring temperature, pH and inorganic N chemistry.

**Table S2.** Primers used in this study.

**Supplementary material 1.** Spreadsheet containing Pyrotagger output for GBS and SSW pyrotag datasets.

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