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Potential role of *Thermus thermophilus* and *T. oshimai* in high rates of nitrous oxide (N_2O) production in ~80 °C hot springs in the US Great Basin

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SUMMARY

Ambient nitrous oxide (N_2O) emissions from Great Boiling Spring (GBS) in the US Great Basin depended on temperature, with the highest flux, 67.8 ± 2.6 μmol N_2O-N m^{-2} day^{-1}, occurring in the large source pool at 82 °C. This rate of N_2O production contrasted with negligible production from nearby soils and was similar to rates from soils and sediments impacted with agricultural fertilizers. To investigate the source of N_2O, a variety of approaches were used to enrich and isolate heterotrophic micro-organisms, and isolates were screened for nitrate reduction ability. Nitrate-respiring isolates were identified by 16S rRNA gene sequencing as *Thermus thermophilus* (31 isolates) and *T. oshimai* (three isolates). All isolates reduced nitrate to N_2O but not to dinitrogen and were unable to grow with N_2O as a terminal electron acceptor. Representative *T. thermophilus* and *T. oshimai* strains contained genes with 96–98% and 93% DNA identity, respectively, to the nitrate reductase catalytic subunit gene (*narG*) of *T. thermophilus* HB8. These data implicate *T. thermophilus* and *T. oshimai* in high flux of N_2O in GBS and raise questions about the genetic basis of the incomplete denitrification pathway in these organisms and on the fate of biogenic N_2O in geothermal environments.

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INTRODUCTION

Nitrous oxide (N_2O) is a powerful greenhouse gas that has become a major focus of climate change research in recent decades. Measurements of N_2O flux across soil/air and water/air interfaces from a wide variety of terrestrial and marine environments have documented dramatic increases in emissions owing to anthropogenic sources of fixed N, more than 70% of which can be linked with the use of agricultural fertilizers (Seitzinger *et al.*, 2000, 2006; EPA, 2010). In soils and both freshwater and coastal marine sediments, N_2O emissions can be elevated several orders of magnitude owing to the direct application of fertilizers or runoff (Matson *et al.*, 1998). Biological nitrification and denitrification are the two major sources of N_2O, with denitrification likely to be the dominant source (Zumft & Kroneck, 2007). N_2O is an intermediate in typical denitrification pathways in which dinitrogen gas (N_2) is the terminal product. N_2O typically constitutes a trace emission from denitrifying microbial communities (e.g., <5% of N_2O + N_2), and globally N_2O is estimated to comprise ~2.6–3.9% of the total denitrification flux (Seitzinger, 1988; Schlesinger, 2009).

A wide diversity of bacteria and archaea (>60 genera) and even some eukaryotes are capable of denitrification, the vast majority of which catalyze complete denitrification with N_2 as the final product (Zumft & Kroneck, 2007). Micro-organisms with truncated denitrification pathways that terminate with N_2O have been known for several decades (Hart *et al.*, 1965), and genomics has revealed that this phenotype can result from mutations in the N_2O reductase (*nos*) genes or from the complete absence of *nos* genes (Zumft & Kroneck, 2007). In geothermal environments, a wide variety of thermophiles can respire nitrate or nitrite, including both *Crenarchaeota* (Völkl *et al.*, 1993; Blöchl *et al.*, 1997; Afshar *et al.*, 1998) and

Euryarchaeota (Hafenbradl *et al.*, 1996; Vorholt *et al.*, 1997) and members of several phyla of *bacteria*, such as the *Aquificae* (Eder & Huber, 2002; Gotz *et al.*, 2002; Takai *et al.*, 2003), *Firmicutes* (Huber *et al.*, 1996; Mishima *et al.*, 2009; Poli *et al.*, 2009), and *Thermales*. The *Thermales* include some of the best-studied nitrate- and nitrite-reducing thermophiles. Some isolates of *Thermus thermophilus* are capable of denitrification to N_2 (Cava *et al.*, 2008); however, most nitrate-reducing *Thermales* can only reduce nitrate to nitrite, including some isolates of *T. thermophilus* (Cava *et al.*, 2008) and all facultative anaerobic species of *Meiothermus*, *Oceanithermus*, and *Vulcanithermus* (Miroshnichenko *et al.*, 2003a,b; Pires *et al.*, 2005; Albuquerque *et al.*, 2009, 2010; Zhang *et al.*, 2010). Some strains of *T. oshimai* and *T. brockianus* are reported denitrifiers, but the denitrification phenotype has not been described in detail (da Costa *et al.*, 2001).

Considerable research has been carried out to elucidate the genetic and biochemical basis of nitrate reduction pathways in *T. thermophilus*. Genes encoding the ability to reduce nitrate to nitrite in *T. thermophilus* HB8 and NAR1 comprise three adjacent operons, *nar*, *nrc*, and *dnr* that are located on a megaplasmid, termed the nitrate conjugative element (NCE). The NCE megaplasmid can be transferred among *T. thermophilus* strains by conjugation (Ramirez-Arcos *et al.*, 1998). The *nar* operon encodes the membrane-bound nitrate reductase (*narG,H,I*), among other genes necessary for nitrate respiration (Ramirez *et al.*, 2000; Zafra *et al.*, 2002). The nitrate reductase alpha subunit, *narG*, is the highly conserved catalytic subunit and is commonly used as a functional biomarker for dissimilatory nitrate-reducing bacteria in molecular ecology studies (Philippot *et al.*, 2002).

Despite a strong foundation based on a large body of research in nonthermal environments and biochemistry of nitrate reduction pathways in *T. thermophilus*, very few studies have addressed denitrification in terrestrial geothermal environments (Burr *et al.*, 2005). A recent study reported coordinated measurements of gross ammonia oxidation, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), microbial community composition, and abundance and distribution of ammonia monooxygenase large subunit genes (*amoA*) and *narG* in two ~80 °C springs in the US Great Basin, Great Boiling Spring (GBS) and Sandy's Spring West (SSW) (Dodsworth *et al.*, 2011). The study revealed an extremely active N-cycle, including abundant populations of ammonia-oxidizing archaea related to '*Ca*. Nitrosocaldus yellowstonii' that are likely responsible for the oxidation of source water ammonia to nitrite at rates comparable to those in nonthermal aquatic sediments. Denitrification rates were high and could be stimulated ~10-fold by the addition of nitrate and a complex organic mixture, implying an important role of heterotrophic denitrifiers, whereas possible electron donors for chemolithotrophy failed to stimulate denitrification. However, the identities and activities of heterotrophic denitrifiers in the springs remained speculative.

Here, we report a substantial flux of N_2O from GBS and describe the isolation and characterization of a large number of heterotrophic nitrate-reducing micro-organisms from GBS and SSW. We show that all isolates produce N_2O as the terminal nitrate reduction product, regardless of sampling date, location, or isolation strategy, which is surprising in light of the absence of previous reports of any member of the *Thermales* with this phenotype. The potential implications of these results on the fate of microbially produced N_2O in terrestrial geothermal ecosystems are discussed.

MATERIALS AND METHODS

Sample sites, field chemistry, and measurement of *in situ* gas flux

The aqueous chemistry, mineralogy, and sediment and planktonic microbial communities inhabiting source pools of GBS and SSW have been described in detail previously (Costa *et al.*, 2009; Dodsworth *et al.*, 2011). Physical and limited chemical data collected on four additional sampling trips are summarized in Table 1. In the field, nitrate and nitrite concentrations were measured by diazotization with sulfanilamide and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride with or without cadmium reduction, respectively (LaMotte, Chestertown, MD, USA). Total ammonia was measured by the nesslerization method using a commercially available kit (LaMotte). For the measurement of dissolved N_2O, triplicate 40 mL water samples were collected in 160-mL serum bottles, cooled to ambient temperature (~25 °C), and shaken three times for 30 s at intervals of 3 min to equilibrate N_2O with the headspace. Gas samples were stored, transported, and analyzed for N_2O by GC-ECD as described in detail below for the measurements of N_2O flux.

On April 7–9, 2009, net fluxes of N_2O, methane (CH_4), and carbon dioxide (CO_2) at the air–water interface were measured using floating static chambers (MacIntyre *et al.*, 1995). In GBS, gas fluxes were measured at three locations, the center of the large source pool (Fig. 1; 82 °C), a shallow shelf

Table 1 Spring temperature, pH, and bulk water inorganic N chemistry (μM)

Site/date	Temp. (°C)	pH	NH_4^+	NO_2^-	NO_3^-
GBS					
Oct. 2008	81	7.20	39	4.2	16
Dec. 2008	83	7.15	70	0.79	1.8
April 2009	82	6.90	28	10.2	7.7
June 2009	84.4	6.85	92	1.0	8.8
SSW					
April 2009	79	6.73	43	0.86	<0.1

NH_4^+, ammonia/ammonium; NO_2^-, nitrite; NO_3^-, nitrate.

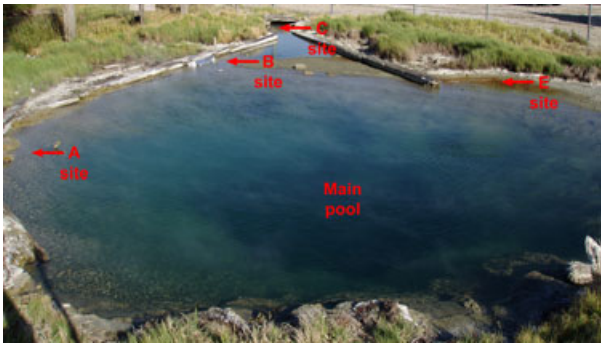

Fig. 1 Photograph of GBS showing sites for measurement of *in situ* gas flux and sampling for microbial enrichments. Gas flux was measured in the center of the spring and sites B and E. Gas flux was also measured in grass-covered soil a few meters from the spring to the right and behind the photographer. Samples for microbial enrichments were collected on the shallow shelf at site B and a neuston biofilm at site C. A foil-covered tube rack with denitrification experiments described by Dodsworth *et al.* (2011) is visible at site B. GBS, Great Boiling Spring.

used for denitrification and DNRA field measurements (Fig. 1 Site B; 82 °C (Dodsworth *et al.*, 2011), and a shallow shelf hosting a thin cyanobacterial mat (Fig. 1 Site E; ~65 °C). Additional samples were collected in a patch of ambient-temperature soil hosting grasses a few meters from GBS. In SSW, samples were collected from the center of the source pool.

The static chambers were 1-cm walled PVC tubes, 30 cm diam. and 15 cm height, capped with a circular 1-cm-thick piece of flat PVC. A sample port was located on the top of each chamber and consisted of a stainless steel quick-connect bulkhead valve. A vinyl vent tube (1.3 cm diam. by 15 cm long) passed through the top of the chamber and was attached to the inside wall. The length and diameter of vent tube were calculated to reduce mixing of chamber air with outside air owing to perturbations from wind and sample collection. To measure gas fluxes, chambers were placed in inflatable inner tubes floating on the spring surface, such that the PVC chamber wall descended 3 cm into the water. Immediately after deploying the chamber and after 20 and 40 min, air samples were collected in 100 mL Silonite™ coated stainless steel sample canisters (Entech Instruments, Inc., Simi Valley, CA, USA) equipped with quick disconnect valves and evacuated prior to field sampling. Samples were taken by attaching one end of a sample canister to the chamber bulkhead fitting, such that the pre-evacuated canister immediately collected 100 mL of chamber air. To detect leaks during transport to the laboratory, the canister was then overpressurized as follows: A syringe was attached to the other end of the canister and an additional 60 mL of chamber air withdrawn. The syringe and canister were then together disconnected from the chamber, and the additional 60 mL was injected into the canister from the syringe. Canisters were transported back to the laboratory for the analysis of CO_2, CH_4, and N_2O concentrations. Ten millilitres of gas was removed from each canister by syringe and injected into an Agilent Technologies Model 6890A gas chromatograph (Agilent Technologies, Inc., Palo Alto, California, USA). CO_2 and CH_4 concentrations were measured in He carrier using a flame ionization detector with in-line methanizer, and N_2O was measured using an electron capture detector, with an argon/methane carrier. Gas flux rates were calculated using linear regression of gas concentration over time and (accounting for chamber volume and area) expressed as moles of gas per m^2 per day.

Microbial enrichment and isolation

Information on the identity, source material, and enrichment strategy for each isolate is summarized in Table 2. All samples for enrichments were collected from water or surface sediments (top 1 cm) from a shallow shelf in GBS (Fig. 1; Site B) or a neuston biofilm collected in the outflow channel (Fig. 1; Site C). Samples from SSW were taken from shallows in the small source pool about 5–10 cm below the water–air interface at the east side of the spring pool at the site described (Dodsworth *et al.*, 2011).

Four different enrichment strategies were used, as follows. For strategy 1, sediment was collected in sterile polypropylene tubes and transported to the laboratory and stored in the dark without redox or temperature control. Within a week of sampling, samples were used to inoculate 40 mL of aerobic or anaerobic (N_2-sparged; 2 atm N_2) GBS spring water ultrafiltrate amended with 0.1% peptone and 0.05% yeast extract in stoppered serum bottles (e.g., Wheaton 160 mL bottles, 223748). Anaerobic enrichments were supplemented with 9 mM $NaNO_3$. For strategy 2, spring water or a dilute spring water/sediment slurry was transferred into a serum bottle, fortified with 0.1% peptone and 0.05% yeast extract, and immediately incubated under aluminum foil in the shallows of the spring without allowing the sample to cool. For strategy 3, Castenholz medium D, which contains 9 mM NO_3^- (Castenholz, 1969), in borosilicate screw-capped tubes (3 mL per tube) was amended with a variety of carbon substrates, inoculated with hot, freshly collected samples, and incubated with an air headspace in the shallows of the springs under aluminum foil. Successful enrichments used for microbial isolation shown in Table 2 include lactate (1 mM), an equimolar mixture of formate, lactate, acetate, and propionate (1 mM each), BSA (0.01%), yeast extract and peptone (0.05%), and casamino acids (0.05%). For strategy 4, sediment slurries were transported to the laboratory without temperature or redox control and inoculated into the medium of Allen (Allen, 1959), which was modified to pH 7 and by the addition of 0.01% yeast extract and 20 mM sucrose or 0.5% potato starch. Turbid cultures were plated onto the same medium solidified with gelrite.

All enrichments were incubated for 12–36 h, and contents were plated onto Castenholz medium D supplemented with

Table 2 Summary of source material and enrichment strategy for each nitrate-reducing isolate

Strain*	Source	Enrichment/isolation (strategy†)
Thermus thermophilus		
SSW-01	SSW sediment (10/2008)	Lab, aerobic, YE/Pept, 75 °C (1)
SSW-06 (JL-6)*	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-10	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-11	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-14	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-18b (JL-18)*	SSW water (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-19	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-20	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-21	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-02	GBS sediment (10/2008)	Laboratory, aerobic, YE/Pept, 75 °C (1)
GBS-03	GBS water (10/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-04	GBS water (10/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-05	GBS water (10/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-08	GBS sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-09	GBS sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-10	GBS sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-12	GBS sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
GBS-27	GBS sediment (06/2009)	Field, aerobic, YE/Pept, 70 °C (3)
GBS-32	GBS sediment (06/2009)	Field, anaerobic, YE/Pept, 70 °C (3)
GBS-33	GBS sediment (06/2009)	Field, aerobic, YE/Pept, 70 °C (3)
GBS-34	GBS sediment (06/2009)	Field, aerobic, Casamino acids, 70 °C (3)
GBS-36	GBS sediment (06/2009)	Field, aerobic, Casamino acids, 70 °C (3)
GBS-40	GBS neuston (06/2009)	Field, aerobic, Lactate, 70 °C (3)
GBS-44	GBS sediment (06/2009)	Field, aerobic, YE/Pept, 70 °C (3)
GBS-45	GBS sediment (06/2009)	Field, aerobic, BSA, 70 °C (3)
GBS-49	GBS sediment (06/2009)	Field, aerobic, FLAP, 70 °C (3)
GBSBH-03	GBS sediment (12/2008)	Laboratory, anaerobic, YE/Pept, 75 °C (1)
GBSBH-05	GBS sediment (12/2008)	Laboratory, anaerobic, YE/Pept, 75 °C (1)
GBSBH-09	GBS sediment (12/2008)	Laboratory, anaerobic, YE/Pept, 75 °C (1)
PS1	GBS sediment (3/08)	Laboratory, aerobic, starch, 76 °C (4)
S1	GBS sediment (3/08)	Laboratory, aerobic, sucrose, 76 °C (4)
T. oshimai		
SSW-02 (JL-2)*	SSW sediment (10/2008)	Laboratory, aerobic, YE/Pept, 75 °C (1)
SSW-03	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)
SSW-04 (JL-4)*	SSW sediment (12/2008)	Field, aerobic, YE/Pept, 75 °C (2)

GBS, great boiling spring.

*Strains used for detailed nitrate reduction stoichiometry experiments. Isolate synonyms in parenthesis are used in Fig. 2.

†The four enrichment strategies are described in detail in the Materials and Methods. Carbon substrate abbreviations are as follows: YE/Pept, yeast extract, and peptone (0.05% each); BSA, bovine serum albumin (0.01%); FLAP, formate, lactate, acetate, and propionate (1 mM each).

0.1% yeast extract and 0.1% tryptone (Castenholz, 1969) solidified with 2% agar or modified Allen medium (strategy 4 only, described earlier). Anaerobic cultures were plated in a Coy Type B anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) containing an atmosphere of N_2 (~90%), CO_2 (~5%), and H_2 (~5%) and incubated under an atmosphere of N_2 (2 atm) in aluminum pressure cups (model 51-600; C.A. Technologies, Boulder, CO, USA) sealed with viton o-rings (#9464K673; McMaster Carr, Robbinsville, NJ, USA). Pressure cups were modified for use as anaerobic growth chambers by sealing all ports with high temperature gasket material (#1967; NEA products Co., Belleville, NJ, USA) and silicone sealant (#81160; Permatex, Solon, OH, USA). For all enrichments, several colonies representing each colony morphology type were picked and streaked onto the same medium until axenic cultures were obtained (3–5× streak plates). Pure cultures were immediately used to make −80 °C freezer stocks in media supplemented with 15% glycerol. Each strain was revived from the freezer stocks, streaked to check purity, and used without delay for nitrate reduction experiments described below.

DNA isolation, 16S rRNA and narG PCR and DNA sequencing

DNA was extracted from isolates using a colony lysis buffer (Johnson *et al.*, 2001), and 16S rRNA genes were PCR-amplified using primers specific for bacteria: 9bF (Eder *et al.*, 1999) and 1512uR (Eder *et al.*, 2001). The 25 μL PCR mixture contained 1 μL DNA extract, 200 nM of each primer,

200 μM each dNTP (Promega, Madison, WI, USA), 0.65 U of GoTaq DNA polymerase (Promega), and 5 μL of 5× GoTaq buffer (Promega). Cycling conditions were denaturation at 96 °C for 4 min followed by 35 cycles of denaturation (30 s at 94 °C), primer annealing (30 s at 55 °C), and elongation (1.5 min at 72 °C), with a final elongation step (10 min at 72 °C). PCR for *narG* was carried out using the primers narG1960f and narG2650r and using the reagents described above and the cycling conditions described by Phillipot (Philippot *et al.*, 2002), except that the final extension was shortened to 10 min. For both 16S rRNA genes and *narG*, PCR products were sequenced using the Sanger method at Functional Biosciences, Madison, WI, using the forward PCR primer. All sequences had >700 base pairs with Phred score >20. Sequences were identified using BLASTn (Altschul *et al.*, 1990). A section of *narG* corresponding to the *T. thermophilus* HB8 amino acid position 581–753 was aligned with related *narG* and deduced NarG sequences using default parameters of ClustalW within BioEdit (Hall, 2005). All alignments were checked manually. 16S rRNA and *narG* sequences have been deposited to GenBank with accession numbers JN115057–JN115090 and JN115091–JN115096, respectively.

Screen for nitrate reduction phenotype

Isolates were initially screened for nitrate reduction phenotype by testing the ability to grow in anaerobic Castenholz medium D with 0.1% yeast extract and 0.1% tryptone under N_2 with 9 mM nitrate as the sole terminal electron acceptor in Balch tubes (Bellco 2048-00150) sealed with butyl rubber stoppers (Bellco 2048-18150) and aluminum seals (Bellco 2048-11020). The ability to produce N_2O was determined by GC-ECD analysis of headspace gas after 72 h, as described in detail below. The ability to reduce N_2O to N_2 was tested in the same medium under a 100% He atmosphere by testing their ability to produce N_2 as assessed by production of gas in Durham vials and by GC-TCD, as described in detail below. In addition, late-exponential phase (72 h) cells grown anaerobically with 9 mM nitrate were tested for their ability to grow or produce gas on the same medium but with 100% N_2O (2 atm) as the sole terminal electron acceptor.

Measurement of cell density, nitrate, and denitrification products during anaerobic growth

For all nitrate reduction experiments, isolates were grown in Castenholz medium D with 0.1% yeast extract and 0.1% tryptone that was sparged for 45 min with N_2 and dispensed in the anaerobic chamber described earlier. The headspace was changed prior to autoclaving by 10 cycles of evacuation (1 min) and filling to 2 atm with 99.999% helium.

Cultures were revived from freezer stocks by plating onto Castenholz medium D with 0.1% yeast extract and 0.1% tryptone. Colonies were resuspended in small volumes of anaerobic medium and transferred to serum bottles containing a 100% helium atmosphere (2 atm) prepared as described earlier. Cells were grown and passed using He-rinsed syringes and a 1:50 inoculum into the same prewarmed medium twice prior to the experiment to dilute contaminating N_2 and ensure nitrate reduction pathways were active. Cultures were incubated at 75 °C with rotary shaking at 100 rpm with serum bottles secured in a horizontal position to maximize gas equilibration.

Aqueous medium (0.3 mL) and headspace gas (2.5 mL) were collected at each time point using He-flushed syringes for the measurement of cell density, aqueous nitrate, nitrite, and ammonia concentrations, and concentrations of headspace N_2O and N_2. Cells were counted using a Petroff-Hausser chamber and an Olympus BX-51 phase-contrast microscope with brightness and contrast optimized by using PictureFrame software (Optronics, Goleta, CA, USA). Nitrate concentration was measured by nitration of 2-hydroxybenzoic acid at acidic pH, which does not suffer from nitrite interference (Bhandari & Simlot, 1986). Nitrite and total ammonia concentrations were measured by miniaturization of commercially available kits using methods described above for field measurements (LaMotte). Headspace N_2O concentration was determined by GC-ECD by the injection of a 2.0-mL sample into a GC-2014 Nitrous Oxide Analyzer (Shimadzu, Moorpark, CA, USA), modified and operated as described (Dodsworth *et al.*, 2010). At N_2O concentrations >100 ppm, a nonlinear equation was applied to generate and optimize a standard curve using the open-source curve fitting tools hosted at zunzun.com. Headspace N_2 concentration was determined by GC-TCD by the injection of a 0.5-mL sample into a GC-2014 gas chromatograph (Shimadzu) using 99.999% argon at 40 mL min^{-1} as the carrier gas and a Supelco 80/100 mesh Molecular Sieve column (Sigma-Aldrich, St. Louis, MO, USA). Injector, column, and detector temperatures were 150, 80, and 210 °C, respectively. For both gases, headspace concentrations were used to calculate aqueous gas concentrations and subsequently the total molar amount of each gas by using Henry's Law and constants modified for high temperature (Wilhelm *et al.*, 1977).

RESULTS AND DISCUSSION

In situ N chemistry and gas flux

Consistent with the previous reports (Costa *et al.*, 2009; Dodsworth *et al.*, 2011), ammonia concentrations were higher than concentrations of nitrate plus nitrite in GBS and SSW at all sampling dates, particularly in SSW (Table 1). This is consistent with a model in which the common subterranean reservoir that sources these springs is reduced, with essentially no nitrate or nitrite. Because SSW has a short water residence time (5–15 min.), oxidized products of chemolithotrophic

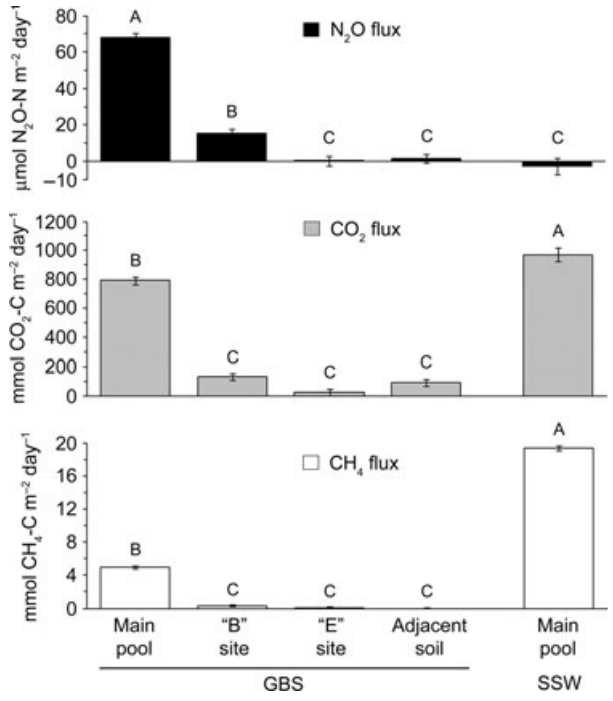

Fig. 2 Gas flux measured in April 2009 at GBS the center of the spring (main pool; 82 °C), site B (82 °C), site E (~65 °C), adjacent grass-covered soil, and in the main pool of SSW. Bars represent the mean and standard error (n = 6–7 for GBS and soil locations; n = 2 for SSW). Letter designations above bars denote levels that are significantly different from each other using one-way ANOVA. GBS, Great Boiling Spring; SSW, Sandy's Spring West.

ammonia oxidation do not accumulate in bulk spring water. In contrast, the higher levels of nitrate and nitrite in GBS are owing to a much longer water residence time (>1 day) and consistent with the high rates of ammonia oxidation recently reported (Dodsworth *et al.*, 2011).

N_2O flux across the water/atmosphere interface measured in April 2009 was highest at the center of the GBS source pool (67.8 ± 2.6 μmol N m^{-2} day^{-1}; 82 °C) and lower but significant on a shallow shelf (Fig. 1, B site; 15.3 ± 2.5 μmol N m^{-2} day^{-1}; 82 °C; Fig. 2). In contrast, N_2O flux from a submerged, thin cyanobacterial mat (Fig. 2 E site; ~65 °C), nearby grass-covered soil, and the SSW source pool were low or negligible. This pattern of N_2O production contrasted with fluxes of CO_2 and CH_4, which were highest in the SSW source pool (Fig. 2). CO_2 has been suggested to be geogenic in some springs in the GBS system based on relatively high $\delta^{13}C$ values of free-phase CO_2 and carbonate solids [~−6 per mil (Romanek *et al.*, 2005)]. The relative enrichment of N_2O in the GBS source pool and shallow shelf, when compared with geogenic CO_2 (which were approximately equal in GBS and SSW), is consistent with a biogenic source of N_2O within the source pool and in shallow, high temperature sediments.

Nitrification and denitrification were highly active in GBS in October 2008 and April 2009, so both nitrification and denitrification are possible sources of biogenic N_2O in GBS (Dodsworth *et al.*, 2011). Acetylene block experiments with sediment slurries revealed denitrification rates at the GBS B site of 2,352 ± 240 μmol N m^{-2} day^{-1} in October 2008 and 10,422 ± 92.2 μmol N m^{-2} day^{-1} in April 2009. Similar unblocked experiments suggested a rate of N_2O flux of approximately an order of magnitude lower. Although sediment slurry experiments may not be fully comparable with *in situ* flux measurements, these comparisons suggest that denitrification contributes significantly to biogenic nitric oxide production *in situ* in GBS. The much lower N_2O flux measured in SSW, which has ammonia oxidation and denitrification rates comparable to those in GBS (Dodsworth *et al.*, 2011), may be due to the relatively short water residence time of the SSW spring pool, preventing nitrate and nitrite from accumulating in sediment pore water or allowing N_2O to be removed primarily in aqueous form rather than by exchange with the atmosphere.

Enrichment, isolation, and identification of nitrate-reducing thermophiles

Heterotrophic micro-organisms isolated on four different field trips, using a variety of carbon substrates and four different enrichment strategies, were identified by 16S rRNA gene sequencing as members of the genera *Thermus*, *Anoxybacillus*, or *Geobacillus*. Only *T. thermophilus* (31 isolates) and *T. oshimai* (three isolates) were able to grow with nitrate as the terminal electron acceptor for anaerobic respiration (Table 2). *T. aquaticus* (four isolates) was not able to reduce nitrate, consistent with the description of that species (da Costa *et al.*, 2001). 16S rRNA gene fragments from Great Basin *T. thermophilus* isolates were 99.4–99.7% identical to the sequence from *T. thermophilus* $HB8^T$, differing by two conserved nucleotide substitutions from HB8, and possessing seven single-nucleotide polymorphisms, including a single-nucleotide insertion. Phylogenetic analysis of the *T. thermophilus* isolates showed no pattern with respect to isolation date or strategy (data not shown). *T. oshimai* isolates shared 99.5–99.6% identity with the sequence from *T. oshimai* $SPS\text{-}17^T$, differing by two conserved nucleotide substitutions from $SPS\text{-}17^T$ and two single-nucleotide polymorphisms, including a single-nucleotide insertion.

Thermus thermophilus and *T. aquaticus* were each isolated from both GBS and SSW sediment and water, whereas *T. oshimai* was only isolated from SSW sediments. *T. thermophilus* and *T. aquaticus* were the dominant *Thermales* detected by 16S rRNA gene-based cultivation-independent censuses at GBS and SSW by both conventional clone library analysis (Costa *et al.*, 2009) and pyrosequencing analysis, where sequences representing close relatives of *T. thermophilus* comprised 0.13–2.89% of pyrotags obtained from GBS site B (Dodsworth *et al.*, 2011; J. Guy, J. Peacock, and B. Hedlund, unpublished results). In contrast, *T. oshimai* was not detected

in the cultivation-independent studies, despite sequencing of >550 16S rRNA gene clones and >100 000 16S rRNA gene pyrotags. Thus, both cultivation-dependent and cultivation-independent works suggest *T. oshimai* was less abundant in these spring samples and possibly implies a more important role of *T. thermophilus* in the N-cycle. Previously, quantitative PCR for *T. thermophilus narG* revealed sediment populations of $1.3–1.7 \times 10^6$ copies g^{-1} sediment at the GBS B site in October 2008 (Dodsworth *et al.*, 2011) and 1.5×10^7 copies g^{-1} sediment at the GBS A site in June 2009 (J. Dodsworth and B. Hedlund, unpublished data). Collectively these results suggest that close relatives of *T. thermophilus* can represent significant members of microbial communities in GBS and, because these isolates all produce N_2O as a terminal product of denitrification, that *Thermus* spp. may therefore contribute to the high observed N_2O flux.

Determination of nitrate reduction intermediates and final products

All nitrate-reducing *Thermus* isolates produced N_2O as the dominant product and failed to produce N_2 or ammonia within 72 h of cultivation with 9 mM nitrate as the sole terminal electron acceptor (data not shown). Selected isolates were grown for up to 120 h but still failed to produce N_2 or ammonia (Fig. 3; data not shown). In addition, mid-exponential-phase cells grown with nitrate as the terminal electron acceptor were unable to produce gas or grow when transferred into the same medium with N_2O as the sole terminal electron acceptor.

To study the nitrate reduction phenotype in more detail, two isolates each from *Thermus species* were selected for further analysis based on their robust and consistent growth phenotype (Fig. 3). Consistent with the results described earlier, N_2O accumulated in the cultures over time, was the major end product of nitrate reduction, and was not reduced to N_2 during the course of the experiments. The conversion of nitrate to N_2O was nearly stoichiometric, with transient accumulation of nitrite. Production of N_2O commenced in mid- to late-exponential growth phase and continued during stationary phase, suggesting that denitrification can be, but is not necessarily, coupled to an increase in cell number in these strains. The complete removal of nitrate by *T. oshimai* strains suggests a higher affinity nitrate reductase or better maintenance of this activity during stationary phase than in *T. thermophilus*.

These *Thermus* isolates have a denitrification phenotype previously unknown in the *Thermales*. Other thermophiles that denitrify to N_2O as the final product have been described, including *Ferroglobus placidus* (Vorholt *et al.*, 1997), *Hydrogenivirga caldilitoralis* (Nakagawa *et al.*, 2004), *Petrobacter succinatimendens* (Salinas *et al.*, 2004), *Pseudoxanthomonas*

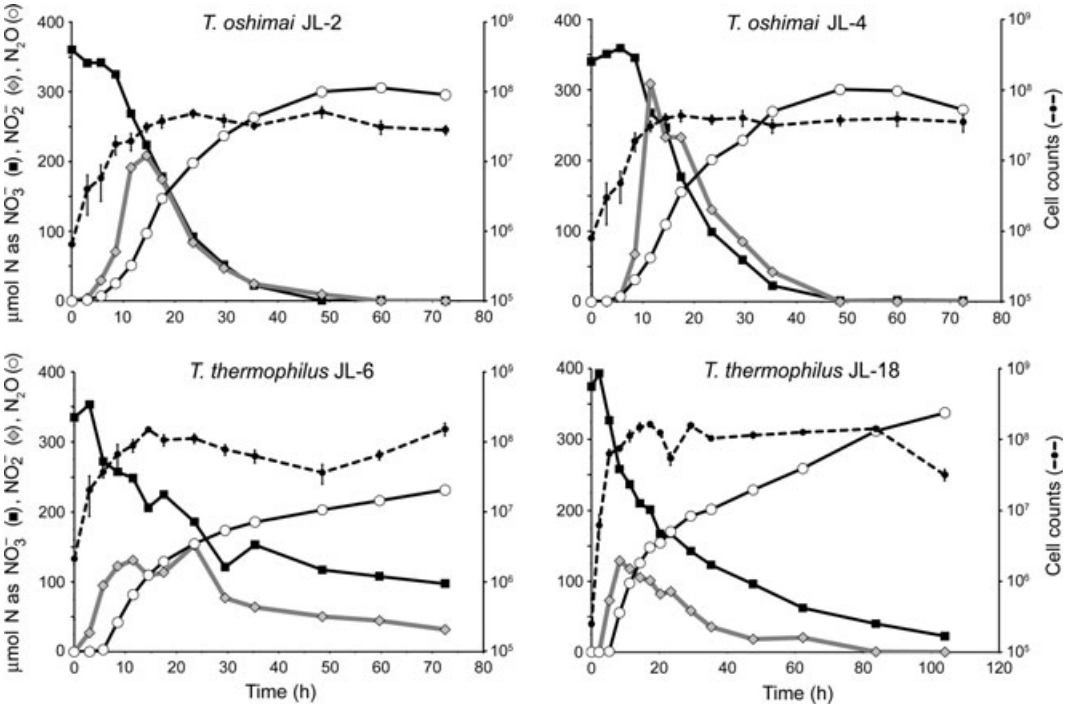

Fig. 3 Near stoichiometric conversion of NO_3^- to N_2O during growth. Cells were grown in *Thermus* medium under a He atmosphere and sampled periodically for quantification of cell density, NO_3^-, and the possible reduction products NO_2^-, N_2O, N_2, and NH_3. Increases in N_2 and NH_3 were never detected (data not shown). Bars for cell counts represent standard deviations. N species were measured once at each time point. Data are representative of two or more identical experiments for each strain.

taiwanensis (Chen *et al.*, 2002), and some strains of *Geobacillus stearothermophilus* (Gokce *et al.*, 1989). In addition, although *Pyrobaculum aerophilum* can reduce N_2O to N_2, nitrous oxide reductase activity is very low, so N_2O accumulates as the major product of denitrification (deVries & Schröder, 2002). Some other thermophiles can denitrify completely to N_2, including *Sulfurihydrogenibium subterraneum* (Takai *et al.*, 2003), *Hydrogenobacter thermophilus* (Suzuki *et al.*, 2001), some strains of *Geobacillus* (Mishima *et al.*, 2009), and some strains of *T. thermophilus* (Cava *et al.*, 2008); however, the kinetics of denitrification in these organisms has not been characterized, so the ratios of N_2O and N_2 produced are not known.

At present, it is difficult to ascertain whether incomplete denitrification is more common among thermophiles and hyperthermophiles than among mesophiles; however, the positive relationship between temperature and N_2O flux measured at GBS hint that incomplete denitrification may be more active at high temperatures. Interestingly, experiments performed at site B of GBS showed ~15- to 20-fold higher rates of N_2O production with an acetylene block, demonstrating that the physiological capacity for N_2O reduction is present (Dodsworth *et al.*, 2011). However, the acetylene block experiments were conducted in a closed system and thus may overestimate *in situ* rates of N_2O reduction. We postulate that in the natural spring environment, the continuous supply of electron donor and nitrate as an electron acceptor favors incomplete denitrification, with N_2O as the dominant end product. It is also possible that complete denitrification does occur under natural conditions through the action of yet-uncultivated complete denitrifiers in these sediments or through the use of released N_2O as a terminal electron acceptor by other organisms.

Analysis of narG fragments

Putative *narG* fragments were amplified from DNA extracts of all denitrifying *T. thermophilus* and *T. oshimai* strains tested. All *narG* fragments were most closely related to the *narG* of *T. thermophilus* HB8 with DNA identities of 96–98% for *T. thermophilus* and 93% for *T. oshimai* and deduced amino acid identities of 96–98% and 95%, respectively. Nucleic acid and deduced amino acid identities to other nitrate-reducing *Thermales*, *Meiothermus silvanus* DSM 9946 and *Oceanithermus profundus* DSM 14977, were 73–83% and 73–86%, respectively. The most closely related *narG* sequences outside the *Thermales* were in the *Deltaproteobacteria* genera *Anaeromyxobacter* and *Geobacter*, with <71% DNA identity and <65% amino acid identity to *Thermus narG*. *T. oshimai* JL-4 and *T. thermophilus* JL-18 each possessed one or more large plasmid, suggesting the possible conservation of the NCE megaplasmid in these organisms (data not shown). However, the close relationship between *narG* within each *Thermus* species, regardless of geographic location, suggests that interspecies or intergenus transfer of the NCE does not occur. The apparent lack of N_2O reductase activity in both *T. thermophilus* and *T. oshimai* from the Great Boiling Spring system suggests a lack of selection for the maintenance of functional *nos* genes, the genetic basis for which will be a subject of future bioinformatic analysis of *T. thermophilus* and *T. oshimai* genomes.

CONCLUSION

The N_2O fluxes measured in the GBS source pool and high temperature shallow sediments (site B) are comparable to rates measured in a variety of aquatic habitats such as coastal marine environments (0–1142 μmol N_2O-N m^{-2} day^{-1} (Seitzinger, 1988; Correador *et al.*, 1999; Chen *et al.*, 2010)), freshwater stream and riparian zone environments (~10–1214 μmol N_2O-N m^{-2} day^{-1} (Seitzinger, 1988; Heftig *et al.*, 2003)), and freshwater lakes [0–19.2 μmol N_2O-N m^{-2} day^{-1} (Seitzinger, 1988; Huttunen *et al.*, 2003)]. In light of the suggestion that more than 90% of N_2O emissions from river and estuary sediments are anthropogenic (Seitzinger *et al.*, 2000), the rates of N_2O flux from GBS are particularly high, as this geothermal system is not impacted by anthropogenic N. The high N_2O flux is, however, in general agreement with high rates of denitrification measured in GBS (Dodsworth *et al.*, 2011). These data suggest a model where the N-cycle in these springs is based on chemolithotrophic oxidation of source water ammonia by ammonia-oxidizing archaea, followed by respiration of nitrate or nitrite to gaseous products (denitrification) or back into ammonia (DNRA). The high flux of N_2O into the atmosphere from GBS may be due to the longer residence time of the main spring pool.

Thermus thermophilus and *T. oshimai* were the dominant nitrate-reducing thermophiles isolated in this study. All isolates produced N_2O as the major product of nitrate reduction, a denitrification phenotype not previously described in the *Thermales,* although known in other thermophiles. Incomplete denitrification to N_2O and respiratory nitrate ammonification may be more common among thermophiles than mesophiles, possibly spelling a different fate for nitrate and nitrite in high temperature geothermal environments. N_2O released by these organisms may be the source of the high N_2O flux from high temperature parts of GBS.

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