AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Implications of Limited Thermophilicity of Nitrite Reduction for Control of Sulfide Production in Oil Reservoirs

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ABSTRACT

Nitrate reduction to nitrite in oil fields appears to be more thermophilic than the subsequent reduction of nitrite. Concentrated microbial consortia from oil fields reduced both nitrate and nitrite at 40 and 45°C but only nitrate at and above 50°C. The abundance of the *nirS* gene correlated with mesophilic nitrite reduction activity. *Thauera* and *Pseudomonas* were the dominant mesophilic nitrate-reducing bacteria (mNRB), whereas *Petrobacter* and *Geobacillus* were the dominant thermophilic NRB (tNRB) in these consortia. The mNRB *Thauera* sp. strain TK001, isolated in this study, reduced nitrate and nitrite at 40 and 45°C but not at 50°C, whereas the tNRB *Petrobacter* sp. strain TK002 and *Geobacillus* sp. strain TK003 reduced nitrate to nitrite but did not reduce nitrite further from 50 to 70°C. Testing of 12 deposited pure cultures of tNRB with 4 electron donors indicated reduction of nitrate in 40 of 48 and reduction of nitrite in only 9 of 48 incubations. Nitrate is injected into high-temperature oil fields to prevent sulfide formation (souring) by sulfate-reducing bacteria (SRB), which are strongly inhibited by nitrite. Injection of cold seawater to produce oil creates mesothermic zones. Our results suggest that preventing the temperature of these zones from dropping below 50°C will limit the reduction of nitrite, allowing more effective souring control.

IMPORTANCE

Nitrite can accumulate at temperatures of 50 to 70°C, because nitrate reduction extends to higher temperatures than the subsequent reduction of nitrite. This is important for understanding the fundamentals of thermophilicity and for the control of souring in oil fields catalyzed by SRB, which are strongly inhibited by nitrite.

Deep oil fields harbor anaerobic, thermophilic microbial communities, which include sulfate-reducing, fermenting, and methanogenic *Archaea* and *Bacteria* (1–3). Although the temperature limit for microbial life is above 110°C, there is good geochemical evidence to suggest that microbial activity in oil fields stops at 80 to 90°C (4). Hence, deep oil fields with a resident temperature above 90°C are likely to be sterile. However, such fields may gain lower-temperature habitats once oil is produced by water injection. For example, in the Terra Nova field, offshore from Newfoundland, Canada, continuous injection of cold seawater (4°C) into a 95°C oil-bearing zone located at 3,200 to 3,700 m below the sea floor gives rise to a near-injection wellbore region (NIWR) in which the temperature increases from 35 to 95°C. Hence, the NIWR comprises successive mesothermic (35 to 45°C), thermogenic (45 to 85°C), and abiotic (85 to 95°C) zones, as indicated in Fig. 1A. Because seawater has a high sulfate concentration, its injection will stimulate the growth of mesophilic sulfate-reducing bacteria (mSRB), thermophilic sulfate-reducing bacteria (tSRB), and thermophilic sulfate-reducing *Archaea* (tSRA), causing the production of sulfide in the NIWR, a process referred to as souring (3, 5, 6). Since souring increases corrosion risk (7, 8), the sulfide concentrations in produced fluids are carefully monitored, with remedial measures being taken when these exceed a defined threshold. Measures include the use of biocides (9–11) or the injection of nitrate (3, 12–14).

Although nitrate is not typically present as an electron acceptor in oil fields, its large-scale injection to control souring will induce growth of mesophilic nitrate-reducing bacteria (mNRB) and thermophilic nitrate-reducing bacteria (tNRB). Several mechanisms act in the control of souring by the activity of NRB, including the reduction of nitrate to nitrite, which is a strong inhibitor of dissimilatory sulfite reductase (Dsr), the enzyme that produces sulfide (15). However, many NRB reduce nitrite further through denitrification to N_2 or through dissimilatory nitrate reduction to ammonium (DNRA) (16–19), causing the inhibition of SRB by nitrite to be only transient.

Although oil field tSRB and tSRA isolates can grow at temperatures of up to 105°C under laboratory conditions, oil field tNRB are less thermophilic, with maximal growth temperatures of up to 70°C. In a seminal bioreactor study on control of souring by tSRB consortia from hot Alaskan oil reservoirs with nitrate at 60°C, Reinsel et al. (13) found that nitrate was reduced to nitrite but that nitrite was not reduced further. As a result, souring control was observed with a low concentration of continuously injected nitrate (0.71 mM) under these conditions. Likewise, tNRB enrichments from the Barrancas field in Argentina, grown on nitrate and volatile fatty acids (VFA) (a mixture of acetate, propionate, and butyrate), accumulated nitrite at 60 but not at 37°C (2). These

Received 23 February 2016 **Accepted** 28 April 2016

Accepted manuscript posted online 6 May 2016

Citation Fida TT, Chen C, Okpala G, Voordouw G. 2016. Implications of limited thermophilicity of nitrite reduction for control of sulfide production in oil reservoirs. Appl Environ Microbiol 82:4190–4199. doi:10.1128/AEM.00599-16.

Editor: F. E. Löffler, University of Tennessee and Oak Ridge National Laboratory

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00599-16.

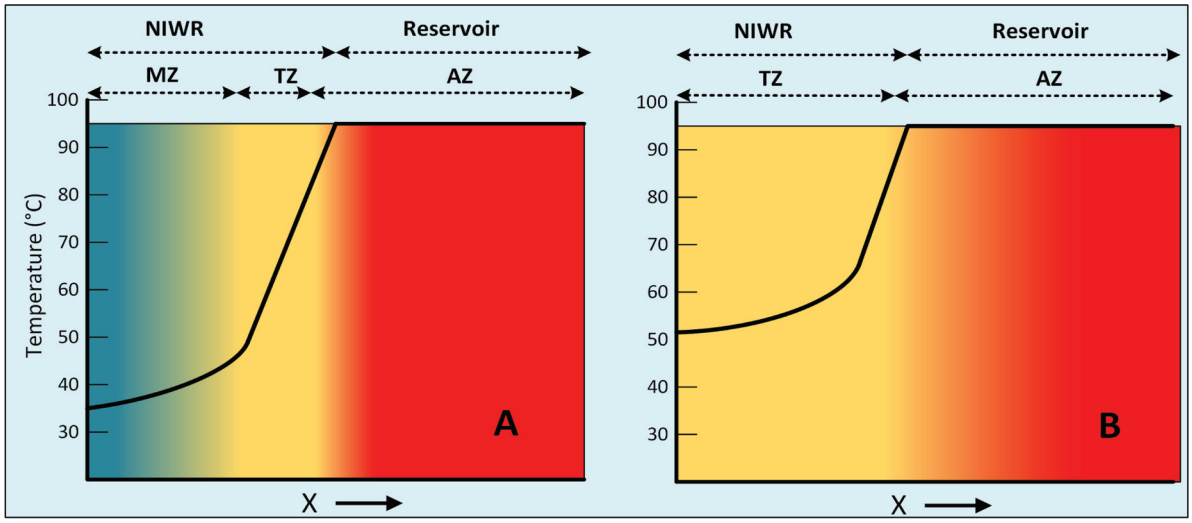

FIG 1 Hypothetical temperature profiles as a function of radial distance *x* from the injection well for a high-temperature oil field, such as Terra Nova, injected with cold (4°C) seawater, which heats up to 35°C during its 3,000-m downward transport (A), and with hot produced water or heated seawater (B). The near-injection wellbore region (NIWR) is the region in which the temperature increases from that of the incoming injection water (35°C) to that of the reservoir (95°C). Mesothermic (MZ) (35 to 45°C), thermogenic (TZ) (45 to 85°C), and abiotic (AZ) (85 to 95°C, the reservoir temperature) zones are indicated.

results suggest that in oil field consortia, reduction of nitrate to nitrite extends to higher temperatures than the subsequent reduction of nitrite to nitrogen or ammonium. We have since found this for other high-temperature oil fields, including for Terra Nova. Activities of tNRB and tSRB from such fields are best characterized by concentrating samples by up to 50-fold prior to their use as inocula. Using this approach we were also able to demonstrate the presence of tNRB in samples from the low-temperature (30°C) Medicine Hat Glauconitic C (MHGC) field in Alberta, Canada, and these were used to delineate the temperature limits of tNRB activities in the present study.

MATERIALS AND METHODS

Study site and sample collection. Samples of injection water (14IW), produced water (18PW), and source water (22SW) were obtained monthly from the MHGC oil field near Medicine Hat, Alberta, Canada. This heavy oil-producing field has been described before (20). The oil in the produced water-oil mixture is separated by treatment with demulsifier at 80°C and transferred to storage tanks, while the deoiled produced water is mixed with source water (also referred to as makeup water) and reinjected as injection water (see Fig. S1 in the supplemental material). Application of nitrate for control of souring has been in operation since 2007 (20). Samples were collected in sterile 1-liter Nalgene bottles filled to the brim to exclude air during transportation. Following arrival at the University of Calgary within half a day from collection, samples were immediately transferred to a Coy anaerobic hood with an atmosphere of 90% N_2 and 10% CO_2 (N_2-CO_2). Methods for chemical analysis of oil field water samples have been described elsewhere (20, 21).

Incubation of concentrated cell suspensions with nitrate and oil organics. Coleville synthetic brine medium K (CSBK) (22) with sodium nitrate (10 mM) as the electron acceptor, 3 mM VFA (3 mM [each] acetate, propionate, and butyrate) as the electron donor, and an N_2-CO_2 headspace was used for cultivation of mNRB and tNRB. For cultivation of tNRB, water samples (20 ml) were concentrated up to 20-fold by centrifugation at 15,000 × *g* for 20 min in an Avanti JE centrifuge (Beckman Coulter). Each pellet was then suspended in 1 ml of the supernatant, whereas the remaining supernatant was filtered through an 0.2-μm nylon membrane filter (Pall Life Science, NY). The nylon filters were then added to the suspended pellets. The combined cell suspensions were added to 60-ml serum bottles closed with butyl rubber stoppers containing 19 ml of CSBK with nitrate and VFA. Duplicate samples were incubated in the dark at 40, 45, 50, 55, 60, 65, 70, and 75°C without shaking. Sterile controls without inoculation were identically treated and incubated at 40 or 70°C. BTEX compounds (benzene [34 mM], toluene [28 mM], ethylbenzene [24 mM], *m*-xylene [24 mM], *o*-xylene [24 mM], and *p*-xylene [24 mM] in 0.5 ml of 2,2,4,4,6,8,8-heptamethylnonane [HMN]) were also used as electron donors at either 30 or 60°C. The concentrations of nitrate and nitrite were determined with high-performance liquid chromatography (HPLC), using a Waters 1515 HPLC instrument equipped with a Waters 2489 UV/visible detector and an IC-PAK Anion HC 4.6- by 150-mm column (Waters, Japan), as described elsewhere (23). The concentrations of ammonium were quantified using the indophenol method (24). Nitrite was also monitored by chemical reaction using the Griess-Ilosvays reagent (Sigma-Aldrich) (25).

Community analysis. DNA was extracted at the end of incubation when no more reduction of nitrate or nitrite was observed. Equal volumes of samples (500 μl) were taken and centrifuged at 17,000 × *g* for 5 min to pellet the cells. DNA was isolated using the FastDNA extraction kit for soil (MP Biomedicals), according to the manufacturer's instructions. Centrifuged samples not subjected to incubation were also analyzed. DNA was quantified with a Qubit fluorimeter (Invitrogen) using the Quant-iT double-stranded DNA (dsDNA) HS assay kit (Invitrogen). 16S rRNA genes were amplified through a two-step PCR using nonbarcoded universal 16S forward primer (926Fw, AAACTYAAAKGAATTGACGG) and reverse primer (1392R, ACGGGCGGTGTGTRC) for 25 cycles followed by amplicon purification and PCR using barcoded primers 454T_RA_X and 454T_FwB, which have 926Fw and 1392R as their 3′ ends, for 10 cycles, as described elsewhere (26). The purified PCR product (100 ng) was subjected to pyrosequencing at the McGill University Genome Quebec Innovation Centre, Montreal, using a Genome Sequencer FLX instrument and a GS FLX titanium series XLR70 kit (Roche Diagnostics Corporation). Sequences were subjected to quality control and bioinformatics analysis using Phoenix 2 (27).

Enumeration of NRB. Aliquots (100 μl) of concentrated MHGC samples were serially diluted in triplicate in CSBK medium (900 μl) containing 10 mM nitrate and 3 mM VFA in 48-well microtiter plates. These were incubated anaerobically at 40°C or 60°C for 7 days in jars flushed with N_2-CO_2. Wells with turbidity due to microbial growth or with color change due to formation of nitrite as assayed with the Griess-Ilosvays

reagent were scored positive. Most probable numbers (MPNs) were derived from the data using appropriate statistical tables (28).

Isolation and identification of NRB strains. NRB enrichments obtained at 40 to 70°C were plated on CSBK medium with 3 mM VFA and 10 mM nitrate, solidified with 15 g/liter of agar. The plates were incubated at the same temperature as used for the enrichment in jars flushed with N_2-CO_2. Individual colonies were picked and grown in CSBK medium with 10 mM nitrate and 3 mM VFA. The grown isolates were phylogenetically identified by Sanger sequencing of 1,500-bp 16S rRNA gene amplicons obtained with primers 27F and 1525R (29) at the Core DNA Services Laboratory of the University of Calgary. Duplicate samples from selected isolates were incubated at 20, 30, 40, 45, 50, 55, 60, 65, 70, and 75°C. Samples (1 ml) were periodically withdrawn to monitor growth as optical density at 600 nm (OD_{600}). The concentrations of nitrate and nitrite were quantified in the supernatants obtained by centrifugation at 17,000 × *g* for 5 min.

Quantitative PCR (qPCR). The presence of *nirS* for heme *c*- and heme d_1-containing nitrite reductase and of *nirK* for copper- and heme d_1-containing nitrite reductase in 18PW consortia and in pure isolates was determined by PCR of 0.4 ng of DNA using PCR conditions and primers NirS2F/NirS3R (30) and PCR conditions and primers GnirK2F/GnirK2R (31), respectively. 16S rRNA genes were amplified with primers 16SrRNA-1055F/1392R (32). After confirming the presence of *nirS* and 16S rRNA genes by agarose gel electrophoresis of the PCR products, their abundance was quantified with the SsoFast EvaGreen Supermix kit (Bio-Rad) using a CFX96 real-time PCR detection system C1000 thermal cycler (Bio-Rad) according to the manufacturer's instructions. Calibration curves, made with purified PCR products of target genes serially diluted over a range of 8 orders of magnitude, were used to determine gene copy numbers.

Thermophilic nitrite reduction by deposited strains. Fourteen strains of tNRB and thermophilic nitrate-reducing *Archaea* (tNRA) were obtained from the DSMZ culture collection, Braunschweig, Germany. Following cultivation in the suggested medium at the optimum temperature for each strain, cultures were transferred to CSBK with 10 mM nitrate and either 3 mM VFA, 10 mM acetate, 20 mM succinate, or 20 mM lactate. Incubation was done at the optimum temperature reported for each strain from 55 to 85°C.

Accession number(s). The 16S rRNA gene amplicon sequence reads of samples incubated at 40, 45, 50, 55, 60, 65, and 70°C and prior to incubation were deposited in the Sequence Read Archive (SRA) at NCBI under accession numbers SRX1435999, SRX1436001, SRX1436002, SRX1435957, SRX1435982, SRX1435996, SRX1435997, and SRX1435998, respectively. The 16S rRNA gene sequences of *Thauera* sp. strain TK001, *Petrobacter* sp. strain TK002, and *Geobacillus* sp. strain TK003, isolated in this study, were deposited in GenBank with accession numbers KU057961, KU057962, and KU057963, respectively. Partial *nirK* sequences, representing PCR amplicons of pure cultures, were deposited in GenBank with accession numbers KX139464 to KX139468.

RESULTS

Activity of tNRB in water samples from the MHGC field. The physicochemical properties of water samples obtained from the MHGC field are indicated in Table S1 in the supplemental material. When microbial activities of these samples were analyzed by injecting 5% (vol/vol) into CSBK medium with 10 mM nitrate and 3 mM VFA at 37 or 60°C, activity was observed only for the mesophilic incubations with nitrate (results not shown). However, incubations with 20-fold-concentrated inocula indicated the presence of both mNRB and tNRB activity (Fig. 2; see Fig. S2 in the supplemental material).

Temperature dependence of reduction of nitrate and nitrite by MHGC microbial consortia. Concentrated microbial consortia of 18PW-produced water and of 14IW injection water (18PW and 14IW consortia) showed rapid and complete reduction of 10 mM nitrate with 3 mM VFA as the electron donor within 24 h with transient formation of nitrite at 40 and 45°C (Fig. 2; see Fig. S2 in the supplemental material). Similar to what has been found elsewhere (32), ammonium was not a significant product of nitrite reduction. At 50 to 65°C 18PW samples reduced nitrate to nitrite within 200 h. Nitrite was not reduced further, even with extended incubation times up to 720 h. The use of either 1 or 5 mM nitrate gave the same results, indicating that the lack of nitrite reduction was not caused by nitrite toxicity (results not shown). At 70°C, partial reduction of 7.5 mM nitrate to nitrite was observed, whereas no nitrate reduction was observed at 75°C (Fig. 2). These data indicate that the 18PW consortia had temperature limits of 70°C for reduction of nitrate to nitrite and of 50°C for subsequent reduction of nitrite. Concentrated 14IW consortia, incubated at or above 50°C, reduced nitrate partially at 60°C only (see Fig. S2 in the supplemental material). Neither mNRB nor tNRB activity was observed in the 22SW source water sample. Abiotic reduction of nitrate was not observed at either 40 or 70°C. These results indicate the presence of high mNRB activity in both the produced water and injection water, whereas tNRB activity was higher in the produced water than in the injection water. This was confirmed by determination of MPNs of mNRB at 40°C (5×10^5 ml^{-1} and 3×10^7 ml^{-1} for 18PW and 14IW, respectively) and of tNRB at 60°C (45 ml^{-1} and 20 ml^{-1} for 18PW and 14IW, respectively).

NRB in the MHGC field use low-molecular-weight oil components, especially toluene, as electron donors for nitrate reduction (33, 34). Concentrated 18PW reduced 10 mM nitrate at 40°C with toluene and ethylbenzene, but not with benzene or xylenes, as the electron donor (see Fig. S3 in the supplemental material). With toluene and ethylbenzene, 3.5 and 1.3 mM nitrite remained, respectively (Fig. S3), because insufficient electron donor was added to reduce all nitrate to N_2 (as explained in the legend to Fig. S3). Nitrate reduction was not observed at 60°C with any of these BTEX compounds.

Change in community composition as a function of temperature. Microbial community analysis indicated that mesophilic enrichments (40 and 45°C) of 18PW were dominated by *Proteobacteria*, whereas thermophilic enrichments (50 to 70°C) were dominated by *Firmicutes*. The dominant mesophilic genera were *Thauera* and *Pseudomonas*, whereas the dominant thermophilic genera were *Geobacillus* and *Petrobacter* (Table 1). *Petrobacter* was prominent only at 50°C, whereas *Geobacillus* was strongly represented at all temperatures from 50 to 70°C (Table 1). In addition to *Geobacillus*, *Anoxybacillus* (Table 1, 60°C only) and *Pseudomonas* appeared as significant community members under thermophilic conditions. *Proteobacteria* (*Pseudomonas*) and *Euryarchaeota* (*Methanoculleus*, *Methanolinea*, and *Methanosaeta*) dominated the 18PW community not subjected to incubation. The microbial community composition of 14IW incubated at 40, 45, and 60°C, conditions where nitrate reduction was observed (see Fig. S2 in the supplemental material), was similar to that of 18PW incubated at these same temperatures (see Fig. S4 in the supplemental material). At 60°C, in addition to *Geobacillus*, *Ignavibacteria* were a major community component. Cultured representatives of this class are thermophilic, facultatively anaerobic heterotrophs found in oil fields (35).

Identification and physiological properties of isolated NRB strains. Plating of NRB enrichments obtained at 40 to 70°C yielded three different bacterial isolates able to grow and reduce

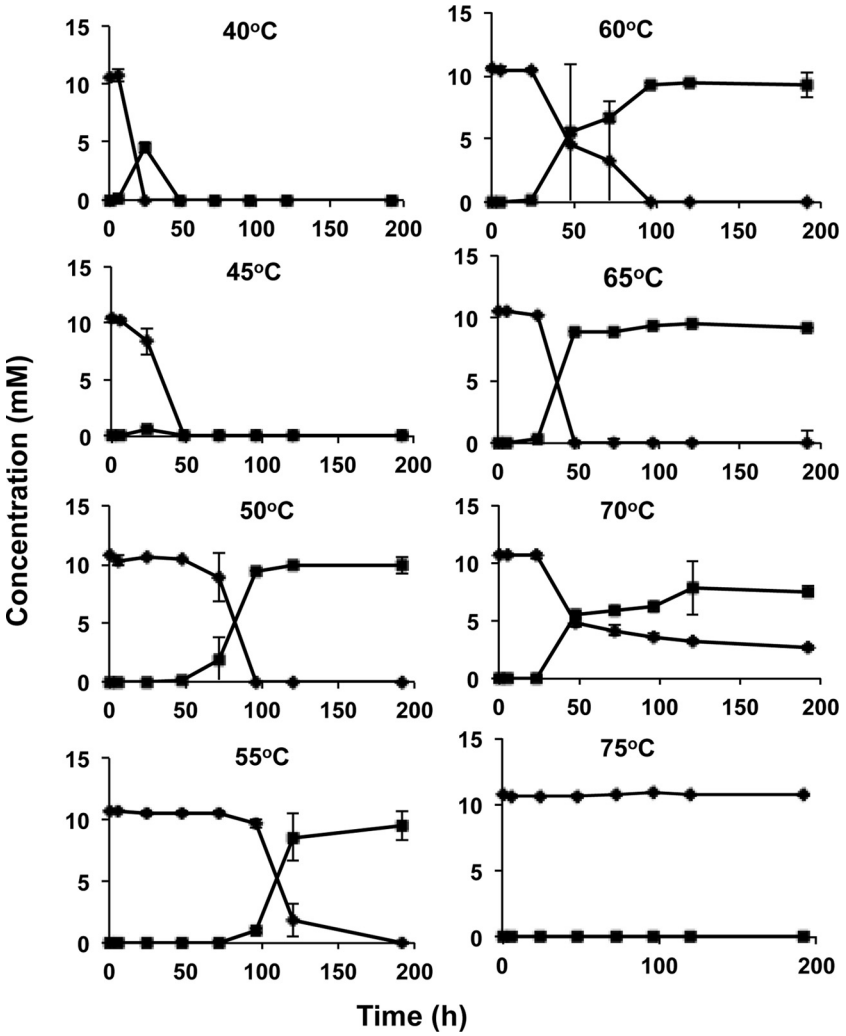

FIG 2 Reduction of nitrate (◆) to nitrite (■) in CSBK medium with VFA as electron donor by concentrated 18PW consortia at different temperatures. Data shown are averages from duplicate incubations. No reduction of nitrate was seen at 40 or 70°C in the absence of inoculation.

nitrate at different temperatures. 16S rRNA gene sequence analysis indicated that isolates TK001 (40°C), TK002 (55°C), and TK003 (70°C) had 99% sequence identity to *Thauera aminoaromatica* S2 (36), 99% to *Petrobacter succinatimandens* 4BON^T (37), and 99% to *Geobacillus kaustophilus* HTA426 (38), respectively.

Thauera sp. TK001 grew and reduced nitrate from 20 to 45°C (Fig. 3A; see Fig. S5 in the supplemental material). The optimum growth temperature for this strain was near 30°C. It did not grow or reduce nitrate at or above 50°C. Nitrite did not accumulate at any growth temperature. *Petrobacter* sp. TK002 grew and reduced nitrate to nitrite from 50 to 60°C, with the optimum growth temperature being 55°C (Fig. 3B; see Fig. S5 in the supplemental material). Growth and nitrate reduction were not observed below 50°C or above 60°C. The accumulated nitrite was not reduced further. *Geobacillus* sp. TK003 grew and reduced nitrate to nitrite within a wider range of temperatures, from 45 to 70°C, with the optimum growth temperature being near 65°C (Fig. 3C; see Fig. S5 in the supplemental material). Similar to the case for *Petrobacter* sp. TK002, the accumulated nitrite was not reduced further. None of the isolates were able to use BTEX compounds as electron donors to reduce nitrate. These results indicated that *Thauera* sp. TK001 can be categorized as an mNRB, whereas *Petrobacter* sp. TK002 and *Geobacillus* sp. TK003 are tNRB, with *Petrobacter* sp. TK002 being a moderate tNRB. The isolated mNRB and tNRB reflected the community property of being able to reduce nitrite at temperatures of 45°C and lower but not at temperatures of 50°C or higher (Fig. 2 and 3; see Fig. S2 in the supplemental material).

Abundance of nitrite reductase as a function of temperature. The ratio of *nirS* to 16S rRNA gene copies was determined for 18PW samples incubated at different temperatures. An increased ratio was observed for samples incubated at 40 and 45°C compared to samples incubated at 50 to 70°C (Fig. 4A). The ratios of *nirS* to 16S rRNA gene copies in samples incubated at 40 and 45°C were 28 and 48%, respectively, whereas they were maximally 3% for samples incubated at higher temperatures. The higher fraction of *nirS* gene copies at 40 and 45°C and the lower fraction at 50 to 70°C correlate with the presence and absence of nitrite reduction at these temperatures (Fig. 2). The fraction of *nirS* gene copies was also low for isolates with no nitrite reductase activity (0.9% for *Petrobacter* sp. TK002 and 1.1% for *Geobacillus* sp. TK003),

TABLE 1 Microbial community composition as the fraction of total quality-controlled pyrosequencing reads of 18PW incubated in CSBK medium with 3 mM VFA and 10 mM nitrate at different temperatures

Taxon (phylum; class; genus)	%[a] at indicated incubation temp[c] (no. of quality-controlled reads):								
	18PW[b] (8,942)	40 (9,374)	45 (9,852)	50 (11,657)	55 (19,878)	60 (13,016)	65 (1,888)	70 (10.941)	Avg
Firmicutes; *Bacilli*; *Geobacillus*	0.16	0	0	54.65	88.96	86.61	89.35	74.17	49.24
Proteobacteria; *Gammaproteobacteria*; *Pseudomonas*	17.68	67.16	53.88	3.64	7.71	3.77	7.79	18.05	22.46
Proteobacteria; *Betaproteobacteria*; *Thauera*	0.17	10.71	40.14	0.05	0.01	0.02	0.11	0.03	6.41
Euryarchaeota; *Methanomicrobia*; *Methanoculleus*	40.01	0.18	0.39	0.58	0.52	0.51	0.21	0.99	5.42
Proteobacteria; *Betaproteobacteria*; *Petrobacter*	0	0	0	38.66	0	0	0	0.02	4.84
Euryarchaeota; *Methanomicrobia*; *Methanolinea*	9.41	0.04	0.02	0.12	0.1	0.06	0.05	0.22	1.25
Euryarchaeota; *Methanomicrobia*; *Methanosaeta*	9.86	0.01	0	0.13	0	0	0	0.01	1.25
Proteobacteria; *Gammaproteobacteria*; *Citrobacter*	1.14	0.13	0.13	0.39	1.42	0.73	1.48	4.52	1.24
Firmicutes; *Mollicutes*; *Acholeplasma*	0.01	8.79	0	0	0.01	0	0	0	1.10
Firmicutes; *Bacilli*; *Anoxybacillus*	0	0	0	0	0	7.23	0	0	0.90
Euryarchaeota; *Methanomicrobia*; *Methanocalculus*	4.21	0.21	0	0.05	0.06	0.06	0	0.06	0.58
Spirochaetes; *Spirochaetes*; *Spirochaeta*	0.07	3.99	0	0	0	0	0	0	0.51
Euryarchaeota; *Methanobacteria*; *Methanobacterium*	1.19	1.59	0.03	0.03	0.01	0.01	0	0.02	0.36
Proteobacteria; *Alphaproteobacteria*; *Novispirillum*	0.55	1.27	0	0.01	0.01	0.01	0	0.07	0.24
Proteobacteria; *Betaproteobacteria*; unidentified	0.01	0.41	1.44	0.01	0	0	0	0	0.23
Euryarchaeota; *Methanomicrobia*; "*Candidatus* Methanoregula"	1.75	0	0	0	0.01	0.03	0	0.06	0.23
Proteobacteria; *Epsilonproteobacteria*; *Arcobacter*	1.05	0.76	0	0.01	0.02	0	0	0	0.23
Proteobacteria; *Gammaproteobacteria*; unidentified	0.16	0.41	0.95	0.1	0.03	0.01	0	0.07	0.22
Firmicutes; *Clostridia*; family XI *incertae sedis*	0	0.15	1.1	0	0	0	0	0	0.16
Firmicutes; *Bacilli*; unidentified	0	0	0.06	0.64	0.1	0.34	0	0.06	0.15
Euryarchaeota; *Methanomicrobia*; unidentified	1.17	0	0	0	0	0	0	0	0.15

[a] Fractions in excess of 1% are shaded.
[b] Community composition prior to incubation.
[c] Temperature shown in °C.

whereas a fraction of 51% of *nirS* to 16S rRNA gene copies was detected for *Thauera* sp. TK001 (Fig. 4B). The low fractions of *nirS* to 16S rRNA genes in TK002 and TK003 indicate that the gene for cytochrome cd_1 type nitrite reductase (NirS) was absent from these pure cultures. No amplification of *nirK* genes with primers GnirK2F and GnirK2R was detected in the TK001, TK002, and TK003 isolates, whereas genes were found in 18PW enrichments at 50 and 55°C only, but not at higher temperature (Fig. 4D and F). The *nirK* genes detected at 50 and 55°C did not contribute to the reduction of nitrite (Fig. 2). Hence, NirS was the dominant nitrite reductase in denitrification at 40 and 45°C (Fig. 4A and C). Its gene was not present at higher temperature, where *nirK* was found but was not active.

Thermophilic nitrite reduction by deposited pure cultures. Because thermophilic nitrite reduction at temperatures of or above 50°C has been described in the literature, we compared the reduction of nitrate and nitrite by 14 deposited strains of tNRB and some thermophilic *Archaea* with that by *Petrobacter* sp. TK002 and *Geobacillus* sp. strain TK003. These were grown at 55, 60, or 65°C, depending on the optimum growth temperature reported for each strain. Because 3 mM VFA (3 mM [each] acetate, propionate, and butyrate), 10 mM acetate, 20 mM succinate, or 20 mM lactate was added, sufficient electron donor was present to reduce 10 mM nitrate to 5 mM N_2 in all cases. A strain was scored positive for nitrite reduction when its concentration reached a maximum and then declined. By this criterion, *G. kaustophilus* DSM7263 reduced nitrate with all four electron donors and nitrite with VFA, succinate, and lactate, but not with acetate (see Fig. S6 in the supplemental material). *Geobacillus* sp. TK003 and *Ammonifex degensii* reduced nitrate but not nitrite with acetate, VFA and lactate, whereas *Petrobacter* sp. TK002 reduced nitrate with all four electron donors, but did not reduce nitrite (see Fig. S6 in the supplemental material), a phenotype shared with *Geobacillus thermoleovorans* DSM5366, *Geobacillus stearothermophilus* DSM22, and *Geobacillus thermocatenulatus* DSM730. *Tepidiphilus succinatimandens* DSM15512 (formerly *Petrobacter succinatimandens*) reduced nitrate and nitrite with VFA or succinate but not with acetate or lactate. Nitrate reduction was not observed for *Thermus thermophilus* DSM579 at 75°C, *Aquifex pyrophilus* DSM6858 at 80°C, *Thermovibrio ruber* DSM14644 at 80°C, and *Pyrobaculum aerophilum* DSM7523 at 95°C (results not shown).

Overall it appeared that for 12 isolates with four electron do-

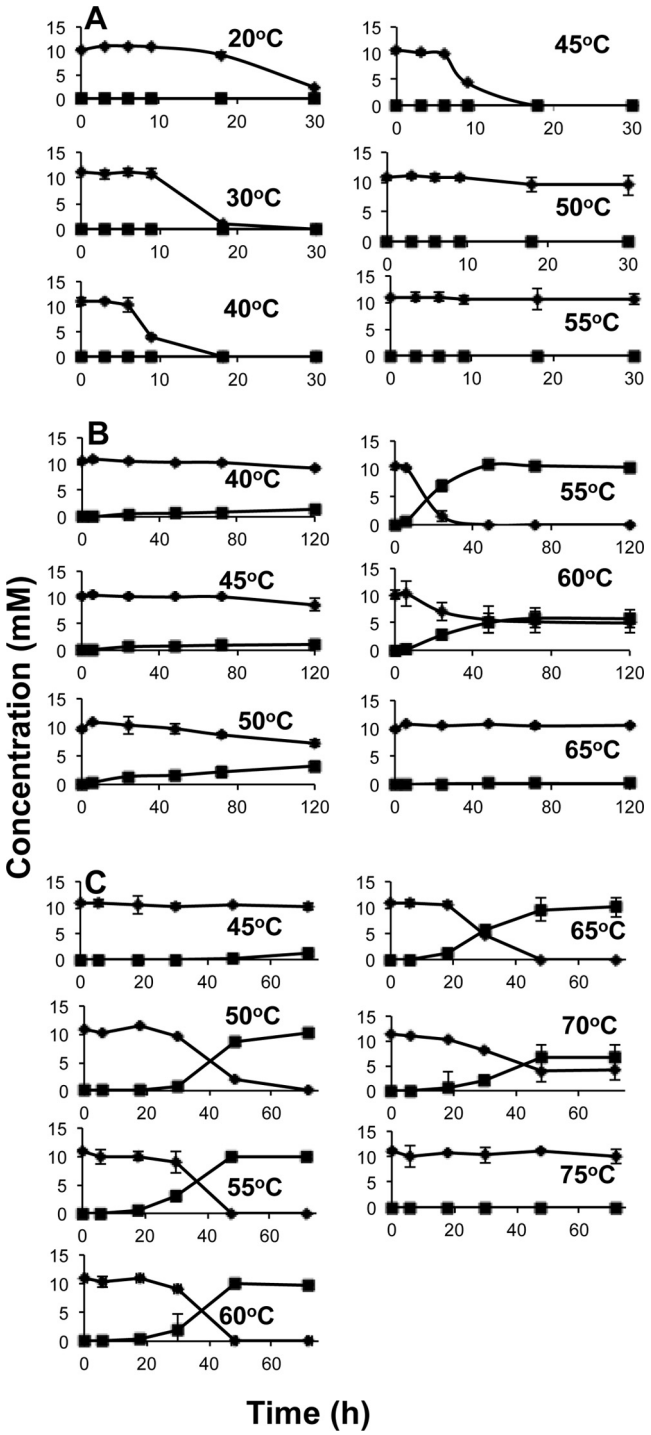

FIG 3 Temperature-dependent reduction of nitrate to nitrite by *Thauera* sp. TK001 (A), *Petrobacter* sp. TK002 (B), and *Geobacillus* sp. TK003 (C) in CSBK medium with 10 mM nitrate and 3 mM VFA. The symbols represent the concentrations of nitrate (◆) and of nitrite (■). Data are averages from duplicate incubations. Data for growth (OD_{600}) of these cultures can be found in Fig. S5 in the supplemental material.

nors, thermophilic nitrate reduction was observed for 40/48 incubations tested, whereas thermophilic nitrite reduction was observed for only 9/48 incubations tested (Table 2). This indicates that thermophilic nitrite reduction in defined media is more the exception than the rule. Thermophilic nitrite reduction with acetate was not observed for any of the 12 isolates. The distribution of the *nirK* gene, found in many *Geobacillus* spp. (31), was determined by PCR (Fig. 4F). Its presence or absence did not correlate well with the reduction of nitrite in defined media; e.g., *G. thermoleovorans*, *G. stearothermophilus*, *G. thermodenitrificans*, and *G. thermocatenulatus* were positive for *nirK* but did not reduce nitrite, whereas *G. kaustophilus* and *G. galactosidasius* were negative for *nirK* but reduced nitrite with at least one of the four electron donors tested. *G. toebii* and *G. subterraneus* were positive for *nirK* and displayed nitrite reduction, whereas *Geobacillus* sp. TK003 was negative for *nirK* and nitrite reduction (Fig. 4E; Table 2). Of the isolates listed in Table 2, *A. degensii* was weakly positive for *nirS* (Fig. 4E, lane 10), whereas the mesophilic *Thauera* sp. TK001 was strongly positive for *nirS* (Fig. 4E, lane 13). Sanger sequencing of PCR products confirmed these to represent *nirK* of *Geobacillus* and *nirS* of *Thauera*, as indicated in Table S2 in the supplemental material.

DISCUSSION

The main result of our study is that the reduction of nitrate to nitrite by tNRB consortia and pure cultures from oil fields is more common than the subsequent reduction of nitrite. This may cause nitrite to accumulate in oil fields injected with nitrate at 50 to 70°C. Our results agree with those reported by Reinsel et al. (13), who demonstrated that tNRB consortia from a high-temperature Alaskan oil field did not reduce nitrite, allowing control of tSRB-mediated H_2S production by injection of low concentrations of nitrate in a bioreactor at 60°C. However, these results may seem at odds with literature on tNRB pure cultures, which are capable of complete denitrification at high temperature (39, 40).

Members of the genus *Geobacillus*, phylum *Firmicutes*, are thermophilic, spore-forming, facultative nitrate reducing anaerobes, which grow from 45 to 75°C (41). Nazina et al. (42), using a medium with 0.2 g liter^{-1} of yeast extract at 60°C, found that of 9 *Geobacillus* spp. capable of reducing nitrate to nitrite, only 4 were able to produce gas from nitrate, indicating reduction of nitrite to N_2 or N_2O. In contrast, Verbaendert et al. (31) found that all of 23 strains of *G. thermodenitrificans*, *G. stearothermophilus*, *G. kaustophilus*, and *G. toebii* had the *nirK* gene and were able to reduce nitrite at 55°C. However, consistent reduction of nitrite was obtained only when minimal medium with nitrate and succinate was amended with 1% Trypticase soy broth (TSB). Using the same primer pair, we found *nirK* to be present in these strains, except in *G. kaustophilus* (Fig. 4F; Table 2). However, nitrite reduction in defined medium without added yeast extract or TSB with succinate as the sole electron donor was found only in incubations with *G. kaustophilus* and *G. toebii* (Table 2). Analysis of the genome of *G. thermodenitrificans* NG80-2 (39) indicated the presence of the complete denitrification pathway with genes for nitrate (*narGHJI*), nitrite (*nirK*), NO (*norZ*), and N_2O (*nosZSYF*) reductase. However, only the *narGHJI* genes were identified in the genome of *G. thermoleovorans* (40), which we found to be positive for *nirK* (Fig. 4F) but to lack the ability to reduce nitrite in defined medium with any of the four electron donors tested (Table 2). Nara et al. (43) demonstrated complete denitrification by *Geobacillus* sp. strain TDN01 in a continuous-culture bioreactor fed with high concentrations (100 mM) of succinate and nitrate. The lack of NirK nitrite reductase activity in defined medium cultures of strains which evidently have the gene (Table 2) and the presence

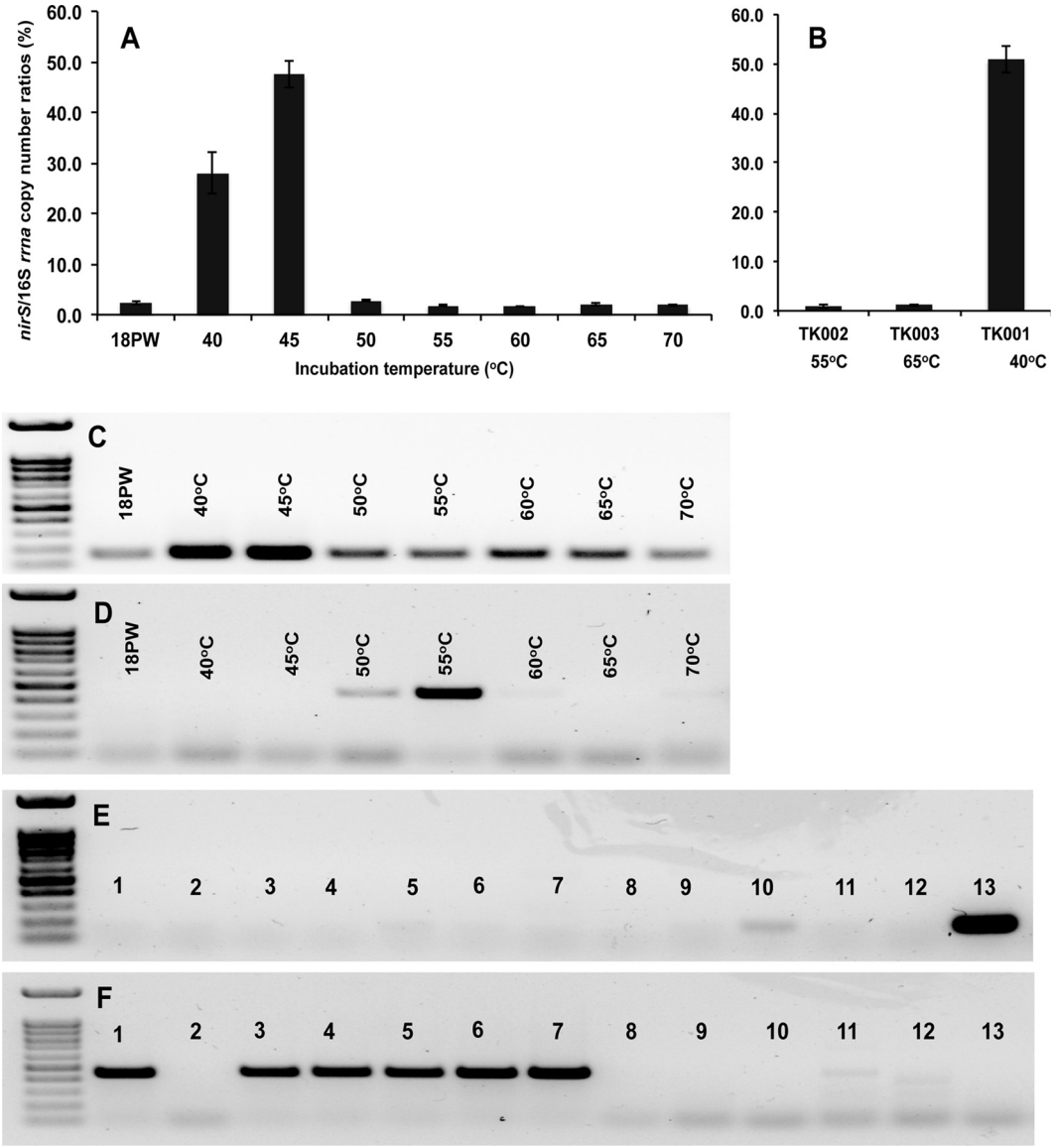

FIG 4 Detection of *nirS* and *nirK* genes in 18PW and pure cultures of tNRB. (A) Ratio of *nirS*/16S rRNA gene copies in incubations with VFA and nitrate at the indicated temperatures of 18PW. (B) Ratio of *nirS*/16S rRNA gene copies in incubations with VFA and nitrate of isolates *Thauera* sp. TK001, *Petrobacter* sp. TK002, and *Geobacillus* sp. TK003 obtained in this study. (C) Detection of *nirS* genes by PCR with primers NirS2F and NirS3R in incubations of 18PW at 40 to 70°C, as indicated; qPCR detection of *nirS* in these incubations is shown in panel A. (D) Detection of *nirK* genes by PCR with primers GnirK2F and GnirK2R in incubations of 18PW as for panel C. (E) Detection of *nirS* genes by PCR with primers NirS2F and NirS3R in the following pure cultures: 1, *Geobacillus thermoleovorans* DSM5366; 2, *G. kaustophilus* DSM7263; 3, *G. stearothermophilus* DSM22; 4, *G. thermodenitrificans* DSM465; 5, *G. toebii* DSM14590; 6, *G. thermocatenulatus* DSM730; 7, *G. subterraneus* DSM13552; 8, *G. galactosidasius* DSM18751; 9, *Tepidiphilus succinatimandens* DSM15512; 10, *Ammonifex degensii* DSM10501; 11, *Petrobacter* (*Tepidiphilus*) sp. strain TK002; 12, *Geobacillus* sp. strain TK003; and 13, *Thauera* sp. TK001. (F) Detection of *nirK* genes by PCR with primers GnirK2F and GnirK2R in pure cultures as for panel E.

of NirK activity in rich media (31) indicate that *nirK* expression may be upregulated by as-yet-unknown rich medium components.

Petrobacter succinatimandens 4BON^T, which was recently renamed *Tepidiphilus succinatimandens* comb. nov. (44), is a moderate thermophile (55°C) isolated from a petroleum reservoir (37). Strain 4BON^T reduced nitrate to nitrite and N_2O using succinate as an electron donor. However, *Tepidiphilus thermophilus* reduced nitrate only to nitrite (44). Thermophilic denitrifiers, which reduce nitrate to nitrite only, are thus not at all rare and include *Petrobacter* sp. TK002 and *Geobacillus* sp. TK003, which were isolated from the MHGC field in this study and were found to lack the *nirS* and *nirK* genes, as judged by PCR assays (Fig. 4E and F).

In contrast, mesophilic species like *Thauera* spp. generally have the complete denitrification pathway. However, the pattern of gene expression can differ, with some species expressing all denitrification genes at once (rapid complete onset [RCO]) and others exhibiting progressive onset (PO) of expression of denitrification genes (45). Nitrite transiently accumulates in PO, but not in RCO

TABLE 2 Summary of the results shown in Fig. S6 in the supplemental material for reduction of nitrate and nitrite with acetate, VFA, succinate, or lactate as the electron donor by 12 tNRB

Isolate	DSMZ designation	Reference	*nirK*[a]	Temp (°C)[b]	Reduction[c] with the indicated electron donor							
					Nitrate				Nitrite			
					Acetate	VFA	Succinate	Lactate	Acetate	VFA	Succinate	Lactate
Geobacillus thermoleovorans	DSM5366	40	+	60	+	+	+	+	−	−	−	−
Geobacillus kaustophilus HTA426	DSM7263	58	−	55	+	+	+	+	−	+	+	+
Geobacillus stearothermophilus	DSM22	58	+	55	+	+	+	+	−	−	−	−
Geobacillus thermodenitrificans	DSM465	59	+	60	−	−	+	−	−	−	−	−
Geobacillus toebii	DSM14590	60	+	55	−	+	+	+	−	−	+	+
Geobacillus thermocatenulatus	DSM730	61	+	60	+	+	+	+	−	−	−	−
Geobacillus subterraneus	DSM13552	58	+	55	+	+	+	+	−	−	−	+
Geobacillus galactosidasius	DSM18751	62	−	60	+	+	+	+	−	−	−	+
Tepidiphilus succinatimandens	DSM15512	37	−	55	−	+	+	−	−	+	+	−
Ammonifex degensii AK1	DSM10501	63	−	65	+	+	−	+	−	−	−	−
Petrobacter sp. TK002		This study	−	55	+	+	+	+	−	−	−	−
Geobacillus sp. TK003		This study	−	60	+	+	−	+	−	−	−	−

[a] Presence (+) or absence (−) of the *nirK* gene, as judged by PCR (Fig. 4F).
[b] Optimum temperature for growth used for incubations.
[c] Positive values are shaded.

species. *Thauera* sp. TK001 did not show transient nitrite accumulation, indicating an RCO phenotype (Fig. 3A). However, some mNRB in the 18PW consortium had the PO phenotype, because transient accumulation of nitrite was observed at 40°C (Fig. 2). *Thauera* spp. exist in diverse environments, biodegrading multiple compounds, including aromatic hydrocarbons, such as toluene, benzoate, *p*-cresol, and phenylacetate (36). Strain TK001 grew with VFA but not with toluene. *Thauera* spp. use NirS nitrite reductase (45). Other mNRB include *Pseudomonas* spp. (46). Species of this genus can also be thermophiles, allowing its presence in 18PW consortia from 40 to 70°C with a minimum at 50°C (Table 1).

The higher numbers and activity of tNRB in produced water 18PW than in injection water 14IW (Fig. 2; see Fig. S2 in the supplemental material) suggests that these originated from the subsurface and not from the high-temperature (80°C) oil-water separator (see Fig. S1 in the supplemental material), since higher tNRB activity is expected in 14IW in that case. The tNRB in 18PW may be derived from deeper, hotter subsurface layers connected to the low-temperature MHGC reservoir, which is at 780 m below the surface. The presence of high fractions and activity of denitrifying mNRB in MHGC produced waters has been shown before (33, 47). Toluene and ethylbenzene were also used as electron donors to reduce nitrate (see Fig. S3 in the supplemental material). Although we did not detect nitrate reduction with xylenes, the use of *m*-xylene as an electron donor for nitrate reduction with accumulation of nitrite under mesophilic conditions has been reported previously (34, 48).

A small fraction of the taxon *Geobacillus* was detected in the community composition of 18PW prior to enrichment (Table 1). These thermophiles can survive under lower-temperature conditions, as shown for other cold environments (49–51). This has led to the suggestion that thermophiles in oil fields originated from the low concentrations present in seawater injected to promote oil recovery (1, 52–54) and, conversely, that low numbers of thermophiles in cold marine sediments may have originated from seeps from hot oil fields, where they may be part of the indigenous microbial community (49, 53, 55–57).

Hence, mNRB and tNRB may colonize zones in the NIWR with the appropriate temperature range (Fig. 1A) when this is injected with cold seawater harboring low numbers of these microbes. Unfortunately, when injected seawater is amended with nitrate, a fraction of this will be reduced in the mesophilic zone to N_2 and will not contribute to souring control by inhibition of SRB. Assuming an upper temperature limits of 70°C for reduction of nitrate to nitrite and 50°C for reduction of nitrite to N_2, as found here for oil field environments, we may expect improved performance of nitrate as a souring control agent if the temperature of any part of the NIWR is not allowed to drop below 50°C, as shown in Fig. 1B. This can be achieved, for instance, by reinjection of hot produced water, which is not yet commonly done in offshore seawater flooded reservoirs.

ACKNOWLEDGMENTS

This work was supported through a Natural Sciences and Engineering Research Council (NSERC) Industrial Research Chair awarded to G.V., which was also supported by Baker Hughes, BP, Computer Modeling Group Ltd., ConocoPhillips, Intertek, Dow Microbial Control, Enbridge, Enerplus Corporation, Oil Search Limited, Shell Global Solutions International, Suncor Energy Inc., Yara Norge, and Alberta Innovates—Energy and Environment Solutions (AIEES).

We are grateful to Rhonda Clark for administrative support, to Yin Shen and Dongshan An for technical assistance, and to Baker Hughes and Enerplus Corporation for providing the field samples used in this study. Discussions with Phil Stemler on the Terra Nova field were much appreciated.

FUNDING INFORMATION

This work, including the efforts of Gerrit Voordouw, was funded by Alberta Innovates-Energy and Environment Solutions (AI-EES 2034). This work, including the efforts of Gerrit Voordouw, was funded by Gouvernement du Canada | Natural Sciences and Engineering Research Council of Canada (NSERC) (IRCPJ 358404-11).

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