Effects of the Antimicrobial Sulfamethoxazole on Groundwater Bacterial Enrichment

Jennifer C. Underwood,[†] Ronald W. Harvey,[*,†] David W. Metge,[†] Deborah A. Repert,[†] Laura K. Baumgartner,[‡] Richard L. Smith,[†] Timberly M. Roane,[§] and Larry B. Barber[†]

[†]United States Geological Survey, 3215 Marine Street, Boulder, Colorado 80303

[‡]Molecular, Cellular and Developmental Biology, University of Colorado—Boulder, UCB 347, Boulder, Colorado 80309

[§]Department of Integrative Biology, University of Colorado—Denver, Campus Box 171, Denver, Colorado 80204

ABSTRACT: The effects of "trace" (environmentally relevant) concentrations of the antimicrobial agent sulfamethoxazole (SMX) on the growth, nitrate reduction activity, and bacterial composition of an enrichment culture prepared with groundwater from a pristine zone of a sandy drinking-water aquifer on Cape Cod, MA, were assessed by laboratory incubations. When the enrichments were grown under heterotrophic denitrifying conditions and exposed to SMX, noticeable differences from the control (no SMX) were observed. Exposure to SMX in concentrations as low as 0.005 μM delayed the initiation of cell growth by up to 1 day and decreased nitrate reduction potential (total amount of nitrate reduced after 19 days) by 47% ($p = 0.02$). Exposure to 1 μM SMX, a concentration below those prescribed for clinical applications but higher than concentrations typically detected in aqueous environments, resulted in additional inhibitions: reduced growth rates ($p = 5 \times 10^{-6}$), lower nitrate reduction rate potentials ($p = 0.01$), and decreased overall representation of 16S rRNA gene sequences belonging to the genus *Pseudomonas*. The reduced abundance of *Pseudomonas* sequences in the libraries was replaced by sequences representing the genus *Variovorax*. Results of these growth and nitrate reduction experiments collectively suggest that subtherapeutic concentrations of SMX altered the composition of the enriched nitrate-reducing microcosms and inhibited nitrate reduction capabilities.
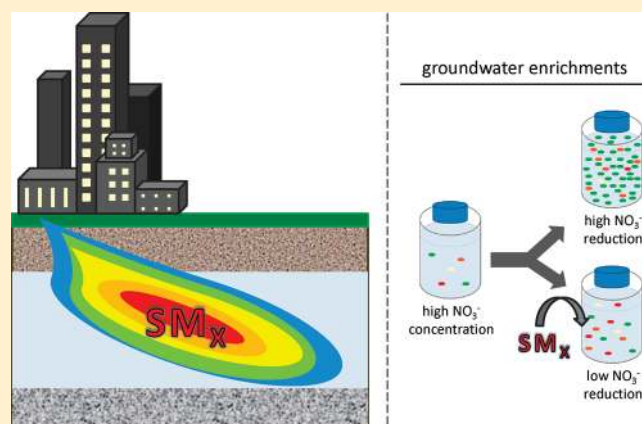
INTRODUCTION

Sulfamethoxazole (SMX), a sulfonamide antimicrobial, was among the most prevalent antimicrobial contaminants detected in a nationwide groundwater survey conducted by the United States Geological Survey (USGS).[1] SMX was detected in 23% ($n = 47$) of the samples with a peak concentration of 0.004 μM (1.11 μg/L).[1] SMX has also been frequently observed worldwide in contaminated wastewaters (0.005 μM and 0.27 μM mean and maximum concentrations, respectively; $n = 177$), and natural waters (0.001 μM and 0.02 μM mean and maximum concentrations, respectively; $n = 115$).[2] Although SMX has been shown to be degradable in surface waters,[3,4] groundwater may be particularly vulnerable to the persistence of SMX as natural attenuation appears to be constrained, due to the absence of photodegradation by sunlight.[5−7] Groundwater is predominantly exposed to SMX contamination caused by the incomplete removal of SMX and other contaminants during wastewater treatment at municipal wastewater treatment facilities or from residential septic tanks.[2] The ability of SMX to persist in groundwater is a concern because it has an unknown potential to cause adverse effects to aquifer bacteria and, by extension, ecosystem function.

Groundwater ecosystems rely on resident bacterial populations for degradation of contaminants [excess nutrients such as nitrate (NO_3^-) and xenobiotic compounds]. The microbially facilitated transformations of these contaminants are a result of their diverse metabolisms. SMX is a broad-spectrum biostatic drug that inhibits production of folic acid in bacteria, which is a requirement for the production of nucleic acids. Although concentrations of SMX are typically low in aqueous environments affected by human and/or animal waste, consequences for ecological functions of indigenous bacterial communities remain unknown. Denitrification, the dissimilatory reduction of NO_3^- to nitrogen gas (N_2) through a series of intermediates, is an important bacterially facilitated form of nitrate removal that has been well-documented within surficial aquifers such as the sand and gravel aquifer at Cape Cod, MA.[8,9] Pristine environments are generally nitrogen-limited (≤ 142.9 μM NO_3^-)[10]; however, anthropogenic sources of NO_3^-, such as wastewater and agriculture, make NO_3^- one of

the leading forms of nutrient contaminants in groundwater. Occurrence of elevated concentrations of NO_3^- (≥ 285.7 μM NO_3^-) in drinking water is linked to several health disorders, including methemoglobinemia and non-Hodgkin's lymphoma.[11] Antimicrobials such as SMX may co-occur with NO_3^- contamination from wastewater or livestock operations,[5,12] and thus it is important to understand the effects of these antimicrobials on naturally occurring microbiological processes such as denitrification.

There has been limited research on the effect of SMX on cell growth and denitrification by environmental bacteria. Clinical research reports minimal inhibitory concentrations (MIC) for common bacterial pathogens ranging from 0.99 μM SMX in *Neisseria* spp. to 505 μM SMX in Enterobacteriaceae.[13] The environmental bacterium *Pseudomonas putida* was shown to be inhibited by 50% (IC_{50}) when exposed to 1 μM SMX.[14] The effect of exposure to 5 mg/g SMX—penicillin (50:50) on the nitrogen cycle was studied by quantifying changes in the abundance of different nitrogen metabolism genes.[15] These high-level exposures led to the rapid disappearance of the *amoA* gene (nitrification gene) and the *napA* gene (membrane-bound nitrate reductase gene, sugar cane soil only) and resulted in a rapid accumulation of ammonia but did not alter soil NO_3^- concentrations.[15] The *napA* gene was absent from sugar cane soil within 24 h after exposure to the SMX—penicillin mixture but this absence did not result in altered NO_3^- concentrations.[15] However, all of these examined SMX concentrations exceed what has been observed in aquatic environments. The highest groundwater concentration of SMX (0.024 μM) was reported for septic tank effluent,[16] but SMX-contaminated groundwater typically does not exceed 0.006 μM.[5] Because SMX contamination is frequently found in groundwater,[1] elucidation of the potential consequences to groundwater bacterial communities and ecosystem function at environmentally relevant concentrations is needed.

In this study, environmentally relevant concentrations of SMX were tested on bacteria collected from a pristine zone of a contaminated aquifer in Cape Cod, MA using a denitrification assay. Analyses of bacterial growth, nitrate reduction activity, and molecular composition of enriched microcosms were used to understand bacterial functions and genera that might be particularly sensitive (or resistant) to SMX and of interest to ecosystem-level processes.

■ MATERIALS AND METHODS

Study Site. Groundwater bacterial communities were collected from well site F605 within an unconfined sandy aquifer in Cape Cod, MA, on March 2009. Well F605 has never been affected by wastewater disposal or any other known contaminant plume and has no known history of NO_3^- or SMX contamination. The groundwater had a pH of 5.55, 33.3 μM dissolved organic carbon, 302.8 μM dissolved oxygen, 3 μM NO_3^-, 55 μM SO_4^-, 254 μM Cl^-, conductivity of 51.3 $\mu S/cm$, temperature of 10.1 °C, and a bacteria concentration of $(6 \times 10^4) \pm 573$ cells/mL.

Field Sample Collections. Groundwater was collected from a depth of 11.3 m below land surface through a multilevel sampler as described by LeBlanc et al.[17] Water samples were stored in sterile 5-L carboys, packed on ice, and then shipped via overnight delivery to the USGS laboratories in Boulder, CO.

Laboratory Incubations. Anaerobic enrichment cultures were grown in the laboratory in heterotrophic media plus NO_3^- to study changes in bacterial growth, nitrate reduction activity, and bacterial compositions in response to the presence of SMX. Dilute tryptic soy broth (TSB), prepared with groundwater,

was chosen as an undefined, nonselective, heterotrophic growth medium. Serum bottles were prepared, in triplicate, for analyses of bacterial growth and molecular analyses of bacterial composition. Simultaneously, an identical triplicate set of serum bottles were prepared for nitrate reduction analyses. Sterile foil-covered serum bottles (150 mL) containing 100 mL of 0.001× tryptic soy broth (TSB) amended with 2 mM NaNO$_3$ were autoclaved (121 °C for 20 min), cooled, and placed in an anaerobic glovebox containing 85% N$_2$, 5% CO$_2$, and 10% H$_2$ gas mix while warm for ≥ 24 h to remove residual oxygen. Bottles were then capped with rubber stoppers and aluminum crimps and autoclaved again. After cooling, each sample bottle was injected with 99% purity acetylene gas (1 mL of acetylene/6 mL of sample) (Wagner Welding Supply Co.) through a 0.2 μm pore size syringe filter and needle to inhibit the last step of denitrification, nitrous oxide (N$_2$O) reduction to N$_2$ gas.[18] Serial dilutions of SMX dissolved in anoxic deionized water were injected into each bottle in the glovebox by use of sterile needles, syringes, and syringe filters (0.1 μm pore size). The final SMX concentrations were 0, 0.005, 0.01, 0.1, 1, 5, 10, 50, 100, 500, 1000, and 2000 μM. Data are presented only for the 0—50 μM concentrations because the higher SMX concentrations showed no significant growth or nitrate reduction activity within the duration of the experiment. Finally, each serum bottle was inoculated with 7.9— 8.5 mL (variation in order to have the same initial bacterial concentration for all samples) of the bacterial community from the pristine groundwater (F605) that had previously been grown anaerobically in 0.001× TSB amended with 2 mM NaNO$_3$ and incubated at room temperature (21 °C) for 7 days. The initial titer for each serum bottle was 4.89×10^3 cells/mL. All bottles were then incubated with rotation (70 rpm) at ~21 °C.

Sample Collection and Storage. Serum bottles were periodically sampled by use of sterile, helium-flushed syringes to withdraw liquid volumes from 1 to 10 mL for cell growth analyses (depending on estimated cell density), 10 mL for NO_3^- and NO_2^- analyses, and 10 mL for molecular analyses. Bacterial growth samples were vacuum-filtered through 0.22-μm membrane filters atop 0.8-μm backing filters, until ~1 mL of sample remained to be filtered, at which point 0.5 mL of a 10 mg/L solution of nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) was applied. The samples were stained in the dark for ~20 min. After staining, the remaining sample was filtered and the membrane filter was removed and placed on a glass microscope slide. A drop of immersion oil was placed on the filter and then covered with a coverslip. The prepared slide was placed in a slide box and stored in the freezer at −20 °C. Samples for nitrate reduction analyses were filtered through a 0.2 μm pore size syringe filter and the filtrate was frozen at −20 °C. Samples for molecular analyses were stored in sterile centrifuge tubes in the freezer at −20 °C.

Analytical Techniques. *Epifluorescence Microscopy.* An epifluorescent microscope was used to enumerate cell density (number of cells per milliliter) using 630× or 1000× magnification as described by Harvey et al.[19] A minimum of 10 fields of view were counted on each filter that had a cell density of 30— 100 cells/field.

Ion Chromatography. Samples were analyzed with a Dionex DX-120 ion chromatograph using an Ion Pac AS514 column with a detection limit within the matrix of the media of about 2 μM for NO_3^- and NO_2^-. Duplicate measurements were made on 10% of samples and yielded an analytical error of $\leq 5\%$.

DNA Extraction and PCR Amplification of rDNA. Thawed samples were filtered through a 0.2 μm polycarbonate filter (Millipore Isopore), and DNA was extracted via phenol/chloroform

and beadbeating and alcohol-purified as previously described.[20] DNA was quantified with a nanodrop fluorometer via a PicoGreen assay (Quant-iT, Invitrogen). Small subunit rRNA genes were amplified from the DNA extracts via polymerase chain reaction (PCR) with the universal small subunit rRNA gene primer pairs, forward 515F (GTGCCAGCMGCCGCGGTAA) and reverse 1391R (GACGGGCGGTGTGTRCA), which amplify all three domains.[21] PCR reactions were conducted and amplicons were purified as described elsewhere.[20]

Cloning and Sequence Analyses. Purified PCR-amplified rRNA genes were isolated by cloning with Invitrogen TOPO-TA per the manufacturer's instructions, and DNA was prepared and sequenced as previously described.[20] Sequences were base-called, contiged, and BLAST-conducted with Xplorseq.[22] Sequences with short sequence length or poor BLAST identity to the National Center for Biotechnology Information (NCBI) database were excluded from the analysis; of 288 total sequences, 271 were used. Further comparison was conducted via ARB and RaxML,[23] although the major data presented here are from BLAST to the All-Species Living Tree Project Database.[24] Sequences were submitted to GenBank, accession numbers JF326861-JF327131 (clone libraries "0r": control, "005r": 0.005 uM SMx, "1r": 1uM SMx, respectively).

Quantitative PCR. Quantitative PCR (Q-PCR) was performed to determine the abundance of rRNA and nitrate reductase genes from isolated DNA. Q-PCR analyses of the genes for membrane-bound and periplasmic nitrate reductase (*napA* and *narG*, respectively) were amplified with the proteobacterial primers narG-F (TCGCCSATYCCGGCSATGTC) and narG-R (GAGTTGTACCAGTCRGCSGAYTCSG) and napA3-F [TGGAC(A/C/G)ATGG(C/T)TT(C/T)AA(C/T)C)] and napA4-R [AC(C/T)TC(A/G)CG(A/C/T)GC(A/C/G)GT-(A/G)CC(A/G)CA], respectively, via previously described amplification protocols.[25] Q-PCR was conducted in 30 μL reaction volumes containing 2× Sybr Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO), 1.5 μL of each primer (1 μg/μL), 11 μL of H$_2$O, and 1 μL of template DNA (0.12−98.35 ng/mL). Calibration standard curves for positive controls were generated as previously described for *napA* and *narG* genes.[25] For baseline quantitation of bacteria, Q-PCR analyses of rRNA genes were amplified with the bacterially conserved primers 8F (AGAGTTTGATCCTGGCTCAG) and 338R (CTGCTG-CCTCCCGTAGGAGT).[20] Cycling conditions included denaturation at 95 °C for 5 min; then 40 cycles of 95 °C for 15 s, 56 °C for 15 s, 60 °C for 30 s, and 70 °C for 1 s; a fluorescence read, then 1 s at 80 °C, and then a second plate read. Q-PCR reactions were performed in triplicate for each sample and primer set tested. A melting curve was used in all assays to ensure specificity of amplification. Data from the second plate reads were used for quantitation. All assays were performed on a DNA Engine Opticon System (MJ Research).

Statistical Analyses. All p-values were calculated by a two-tailed t test. Both NO$_3^-$ and NO$_2^-$ values were used to report NO$_3^-$ reduction activity because the final concentration of NO$_3^-$ + NO$_2^-$ equaled an average of 97.7% ± 0.4% of initial NO$_3^-$ concentrations, indicating that minimal amounts of NO$_3^-$ was lost to other processes.

■ RESULTS AND DISCUSSION

Microbial Growth. Due to the direct relationship between microbial growth/cell division and metabolic rates,[26,27] it is important to determine what concentration of SMX affects growth and nitrate reduction rates of denitrifying bacteria
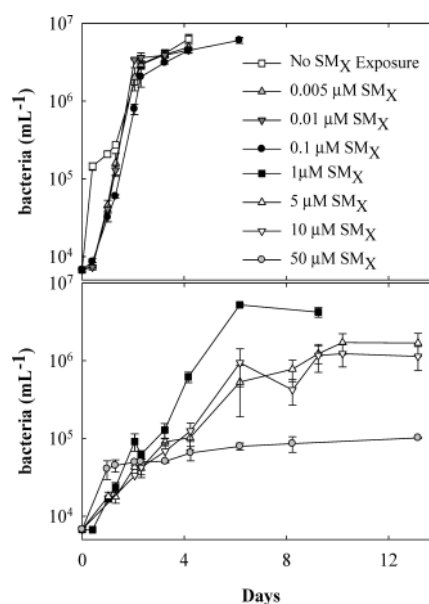
Figure 1. Comparison of growth curves of groundwater bacteria taken from a pristine aquifer on Cape Cod, MA, and incubated under anaerobic denitrifying conditions with or without exposure to the antimicrobial SMX. Error bars represent standard error of triplicate samples.
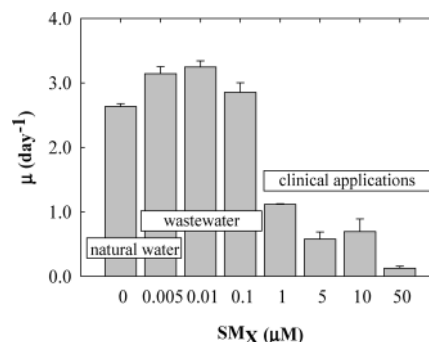
Figure 2. Effect of increasing concentrations of SMX on growth rates during exponential phase of groundwater bacteria from Cape Cod, MA, enriched under denitrifying conditions. Selected concentration ranges of SMX are shown, representing observed concentrations in natural water, wastewater, and reported minimal inhibitory concentrations for clinical bacterial isolates. μ = (ln 2)/generation time. Error bars represent standard error of triplicate samples.

obtained from a pristine aquifer. Simply put, it would be expected that bacterial enrichments that grow at slower rates in the presence of SMX would also have slower oxidation (of TSB) and reduction (of NO$_3^-$) rates. A wide range of SMX concentrations (0−2000 μM), including environmentally relevant concentrations of 0.005 and 0.01 μM, were used to observe the effect on growth rates of groundwater bacteria. Strong effects from SMX exposure were observed at comparably low concentrations, so only data for concentrations 0−50 μM are reported here.

There was a substantial difference in bacterial growth between the control (no SMX) and all SMX-treated microcosms, including the lowest environmentally relevant SMX concentration (0.005 μM) (Figure 1). For the control, growth was initiated almost immediately and reached exponential phase within the
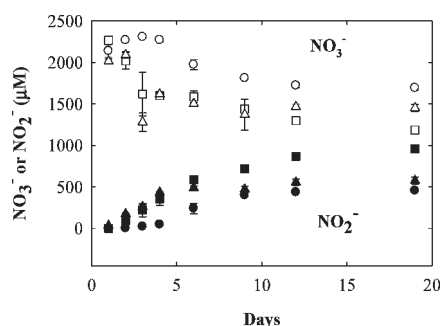
Figure 3. Comparison of NO_3^- (open symbols) and NO_2^- (closed symbols) concentrations over time in groundwater enrichment cultures and incubated under denitrifying conditions without SMX (squares) or in the presence of 0.005 μM SMX (triangles; wastewater relevant) or 1 μM SMX (circles; clinically relevant). Error bars represent standard error of triplicate samples.
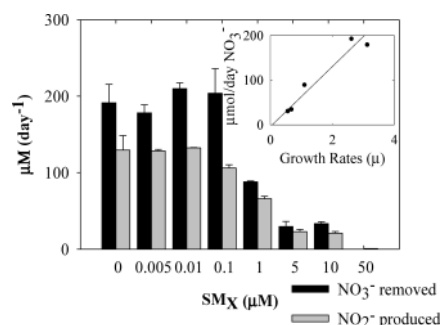
Figure 4. Comparison of NO_3^- removal and NO_2^- production rates during exponential growth for groundwater bacteria enriched under denitrifying conditions with or without exposure to SMX. Error bars represent standard error of triplicate samples. (Inset) Linear relationship ($y = 4.43\mu - 0.9$, $r^2 = 0.96$) between growth rates (Figure 2) and NO_2^- production rates during exponential growth.

first day. In contrast, for all SMX-treated microcosms there was a noticeable lag and exponential growth was not observed until after 24 h postinoculum. Despite the extended lag phase ($>10\,h$, $\leq 24\,h$) as a result of SMX exposure, overall growth rates, as estimated from the exponential part of growth curves, were not significantly inhibited with exposure to SMX at concentrations below 1 μM (Figure 2). Exposure to 1 μM SMX resulted in 58% percent lower growth rate as compared to the control ($p = 5 \times 10^{-6}$). This result is in similar agreement with the effects seen for *P. putida*, a common soil bacterium, by Al-Ahmad et al. [14]

Nitrate Reduction Activity. Results from the concurrent nitrate reduction assay were in close agreement with observed cell growth rates. Nitrate reduction rate potentials were measured by the decrease of NO_3^- and the production of NO_2^- during exponential growth [from 1 to 4 days for low SMX treatments ($0-0.1\ \mu$M) and up to $6-19$ days for higher SMX treatments ($10-50\ \mu$M)] and were not significantly inhibited until exposure to at least 1 μM SMX (Figures 3 and 4). The production of N_2O was observed in separately prepared 37 mL serum bottles; however, a minimal amount of production ($\leq 41\ \mu$M $N-N_2O$) was observed in comparison, so those data are not presented here. Accumulation of NO_2^- has been reported in other studies with enrichments and digestors when NO_3^- concentrations and growth rates are high or other conditions are present (such as high pH, high acetate,
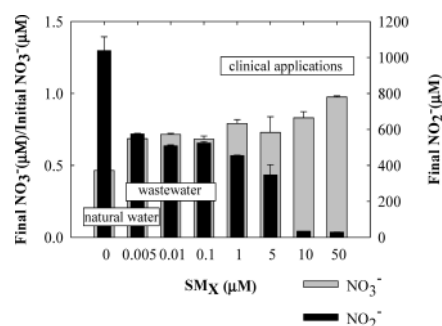
Figure 5. Comparison of final/initial NO_3^- and final NO_2^- values for SMX treatments $0-50\ \mu$M. Results are for 19 day incubations. Final/initial NO_3^- values were used to normalize the data due to variability in initial NO_3^- concentrations between samples. There was no detectable NO_2^- in any sample at the onset of incubation. Error bars represent standard error of triplicate samples, except 0.01 and 50 μM, which are duplicate samples.

or propionate concentrations).[28-30] Figure 3 illustrates the NO_3^- removal and NO_2^- production curves for the control and microcosms exposed to 0.005 μM and 1 μM SMX. Microcosms exposed to $\geq 1\ \mu$M SMX demonstrated delayed onset of nitrate reduction, similar to the longer lag phases observed in the microcosms exposed to $\geq 0.005\ \mu$M SMX (Figures 1 and 3). Exposure to 1 μM SMX resulted in an average decrease in NO_3^- removal rates of 54% and an average decrease in NO_2^- production rates of 49% compared to the control (Figure 4). For comparison, growth rates decreased by 58% on average at this concentration, indicating a linear correlation ($r^2 = 0.97$) between nitrate reduction rate potentials and bacterial growth rates (Figure 4, inset).

Although nitrate reduction rate potentials were not significantly inhibited by SMX exposures less than 1 μM, total nitrate reduction potentials (total amount of NO_3^- removed and NO_2^- produced after 19 days) were reduced by exposure to SMX in all SMX-exposed microcosms (Figures 3 and 5). After 12 days of incubation, nitrate reduction appeared to halt in all microcosms, presumably because the supply of electron donors had been exhausted (Figure 3). However, end-point NO_3^- and NO_2^- concentrations differed between microcosms, indicating that stepwise increases in SMX exposure resulted in stepwise decreases in total nitrate reduction potentials (Figure 5). Exposure to 0.005 μM SMX resulted in a 47% reduction in the total amount of NO_3^- removed compared to the control ($p = 0.02$) [36% ($p = 0.01$) reduction for NO_2^- produced] and exposure to 1 μM SMX led to a 70% reduction ($p = 0.01$) [50% ($p = 1.4 \times 10^{-5}$) reduction for NO_2^- produced] (Figures 3 and 5).

Molecular Analysis of Bacterial Enrichments. The small-subunit rRNA genes from three midexponential growth phase (2 days postinoculation) samples (one each from the control, 0.005 μM, and 1 μM SMX-exposed microcosms) were analyzed to provide a semiquantitative snapshot of the composition of the bacterial enrichments and how they may be altered due to SMX exposure. The sequences were identified via BLAST against a database of 16S rRNA gene sequences from the All-Species Living Tree Project Database [24] (Table 1). Further identification was attempted by analyzing the sequences against their closest relatives in the prokMSA database [31] via bootstrapped trees in RAXML (1000 replicates), but no close associations were determined, which may indicate that the 16S rRNA gene does not have species-level resolution for these organisms. Good's

Table 1. Effect of Wastewater or Clinically Relevant Concentrations of SMX on Bacterial Species Composition in Denitrifying Microcosms during Exponential Growth

	0 μM SMX	0.005 μM SMX	1 μM SMX
nitrate removed (μM)	129.56	128.46	65.88
nitrite produced (μM)	191.25	178.23	88.09
napA:narG	1.37	3.45	5.35
sequences in library	86	93	92

top BLAST Hit[b]	% of library[a]			% ID range (mean)[c]
	0 μM SMX	0.005 μM SMX	1 μM SMX	
Pseudomonas gessardii	13	21	16	99
Pseudomonas proteolytica	83	76	54	98−100 (99)
Pseudomonas rhodesiae	1	1		99
Ralstonia insidiosa	2		1	100
Variovorax boronicumulans			23	98
Variovorax paradoxus			1	98
Xanthobacter autotrophicus	1	2	4	91−92 (91)

[a] Percentage of the library BLASTing to the listed species. [b] Top BLAST hit to all 16S rDNA in the All-Species Living Tree Database. [c] Range of BLAST identities to the top BLAST hit listed.

coverage for clone libraries was 98.8%, 96.25%, and 96.64% (for 0, 0005, and 1 μM SMX, respectively), indicating that the majority of the diversity had been sampled. Sequences with high BLAST identity ($\geq$98%) to *Pseudomonas,* mostly *Pseudomonas proteolytica*, dominated all three libraries, but the percentage of the library represented by these sequences decreased with increasing SMX concentration. Additionally, in the 1 μM SMX treatment, sequences with high identity ($\geq$98%) to various Burkholderiales, mostly the comamonad *Variovorax boronicumulans*, composed 25% of the library. Although many *Pseudomonas* and *Variovorax* are capable of reducing nitrate, laboratory tests have shown that *V. boronicumulans* does not reduce nitrate while *P. proteolytica* does.[32,33] Although species-level resolution, isolation, and isolate nitrate reduction assays were not accomplished in this study, it is interesting that the reduction of sequences belonging to the genus *Pseudomonas* correlated with reduced nitrate reduction rates and an increased ratio of *napA* to *narG* genes (Table 1). Although *napA* and *narG* can be involved in redox reactions other than denitrification (i.e., dissimilatory ammonification, assimilatory ammonification, and anaerobic ammonium oxidation), given the conditions provided in this experiment (heterotrophic medium with nonlimiting NO_3^- concentrations) these processes are unlikely.[34] A previous study found that, under carbon-limited, denitrifying conditions, *Escherichia coli* modified to only express *napA* was at a competitive growth disadvantage to *E. coli* modified to only expressing *narG* due to NO_3^--mediated repression of the *napA* operon.[35] However, the number of *napA* and *narG* gene copies for each species in our study is unknown. Overall, addition of SMX correlated with a change in the sequence libraries, a change in the ratio of *napA:narG* genes, and a decrease in total nitrate reduction activity. Additionally, these results may indicate that members of the group Comamonadaceae are equipped to compete under SMX stress although resistance is not specifically known for this group.

This study was conducted by examining growth of groundwater bacteria in enrichment cultures with dilute heterotrophic media. This approach was selected to provide maximum sensitivity as an initial test of environmentally relevant concentrations of SMX. The results indicate that SMX does have an effect even at environmentally relevant concentrations on bacterial growth, nitrate reduction activity, and community composition. The relevancy of these results to slow-growing or unculturable members of the groundwater microbial community under in situ conditions or to the attached groundwater microbial population is not known. However, the implication is clear; long-term chronic exposure to environmentally relevant concentrations of SMX could be affecting microbial activity and community composition in the subsurface. Clearly further research is warranted, including studies on the transport and degradation of SMX in the groundwater environment.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: (303) 541-3034; e-mail: rwharvey@usgs.gov.

■ DISCLOSURE

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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■ REFERENCES

(1) Barnes, K. K.; Kolpin, D. W.; Furlong, E. T.; Zaugg, S. D.; Meyer, M. T.; Barber, L. B. A national reconnaissance of pharmaceuticals and

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dx.doi.org/10.1021/es103605e |*Environ. Sci. Technol.* XXXX, XXX, 000–000

other organic wastewater contaminants in the United States—I) Groundwater. *Sci. Total Environ.* **2008**, *402* (2−3), 192–200.

(2) Segura, P. A.; François, M.; Gagnon, C.; Sauvé, S. Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. *Environ. Health Perspect.* **2009**, *117* (5), 675–684.

(3) Lam, M. W.; Mabury, S. A. Photodegradation of the pharmaceuticals atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in natural waters. *Aquat. Sci.* **2005**, *67* (2), 177–188.

(4) Drillia, P.; Dokianakis, S. N.; Fountoulakis, M. S.; Kornaros, M.; Stamatelatou, K.; Lyberatos, G. On the occasional biodegradation of pharmaceuticals in the activated sludge process: The example of the antibiotic sulfamethoxazole. *J. Hazard. Mater.* **2005**, *122* (3), 259–265.

(5) Barber, L.; Keefe, S.; LeBlanc, D.; Bradley, P.; Chapelle, F.; Meyer, M.; Lofton, K.; Kolpin, D.; Rubio, F. Fate of sulfamethoxazole, 4-nonylphenol, and 17-estradiol in groundwater contaminated by wastewater treatment plant effluent. *Environ. Sci. Technol.* **2009**, *43* (13), 4843–4850.

(6) Alexy, R.; Kümpel, T.; Kümmerer, K. Assessment of degradation of 18 antibiotics in the closed bottle test. *Chemosphere* **2004**, *57* (6), 505–512.

(7) Lam, M. W.; Young, C. J.; Brain, R. A.; Johnson, D. J.; Hanson, M. A.; Wilson, C. J.; Richards, S. M.; Solomon, K. R.; Mabury, S. A. Aquatic persistence of eight pharmaceuticals in a microcosm study. *Environ. Toxicol. Chem.* **2004**, *23* (6), 1431–1440.

(8) Repert, D. A.; Barber, L. B.; Hess, K. M.; Keefe, S. H.; Kent, D. B.; LeBlanc, D. R.; Smith, R. L. Long-term natural attenuation of carbon and nitrogen within a groundwater plume after removal of the treated wastewater source. *Environ. Sci. Technol.* **2006**, *40* (4), 1154–1162.

(9) Smith, R. L.; Duff, J. H. Denitrification in a sand and gravel aquifer. *Appl. Environ. Microbiol.* **1988**, *54* (5), 1071–1078.

(10) Mueller, D. K.; Hamilton, P. A.; Helsel, D. R.; Hitt, K. J.; Ruddy, B. C. *Nutrients in ground water and surface water of the United States: An analysis of data through 1992*; Report 95−4031; U.S. Geological Survey: Denver, CO, 1995.

(11) Ward, M. H.; Mark, S. D.; Cantor, K. P.; Weisenburger, D. D.; Correa-Villaseñor, A.; Zahm, S. H. Drinking water nitrate and the risk of non-Hodgkin's lymphoma. *Epidemiology* **1996**, *7* (5), 465–471.

(12) Batt, A. L.; Bruce, I. B.; Aga, D. S. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environ. Pollut.* **2006**, *142* (2), 295–302.

(13) Andrews, J. M. Determination of minimum inhibitory concentrations. *Antimicrob. Chemother.* **2001**, *48* (Suppl. 1), 5–16.

(14) Al-Ahmad, A.; Daschner, F. D.; Kümmerer, K. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Arch. Environ. Contam. Toxicol.* **1999**, *37* (2), 158–163.

(15) Colloff, M. J.; Wakelin, S. A.; Gomez, D.; Rogers, S. L. Detection of nitrogen cycle genes in soils for measuring the effects of changes in land use and management. *Soil Biol. Biochem.* **2008**, *40* (7), 1637–1645.

(16) Godfrey, E.; Woessner, W. W.; Benotti, M. J. Pharmaceuticals in on-site sewage effluent and ground water, Western Montana. *Ground Water* **2007**, *45* (3), 263–271.

(17) LeBlanc, D. R.; Garabedian, S. P.; Hess, K. M.; Gelhar, L. W.; Quadri, R. D.; Stollenwerk, K. G.; Wood, W. W. Large-scale natural gradient tracer test in sand and gravel, Cape Cod, Massachusetts 1. Experimental design and observed tracer movement. *Water Res.* **1991**, *27* (5), 895–910.

(18) Yoshinari, T.; Hynes, R.; Knowles, R. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. *Soil Biol. Biochem.* **1977**, *9* (3), 177–183.

(19) Harvey, R. W.; Smith, R. L.; George, L. Effect of organic contamination upon microbial distributions and heterotrophic uptake in a Cape Cod, Mass., aquifer. *Appl. Environ. Microbiol.* **1984**, *48* (6), 1197–1202.

(20) Feazel, L. M.; Baumgartner, L. K.; Peterson, K. L.; Frank, D. N.; Harris, J. K.; Pace, N. R. Opportunistic pathogens enriched in showerhead biofilms. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (38), 16393–16399.

(21) Lane, D. J. 16S/23S rRNA sequencing. *Nucleic Acid Tech. Bact. Syst.* **1991**, 115–175.

(22) Frank, D. N. XplorSeq: a software environment for integrated management and phylogenetic analysis of metagenomic sequence data. *BMC Bioinf.* **2008**, *9* (1), 420.

(23) Ludwig, W.; Strunk, O.; Westram, R.; Richter, L.; Meier, H. ARB: a software environment for sequence data. *Nucleic Acids Res.* **2004**, *32* (4), 1363–1371.

(24) Yarza, P.; Richter, M.; Peplies, J.; Euzeby, J.; Amann, R.; Schleifer, K. H.; Ludwig, W.; Glöckner, F. O.; Rosselló-Móra, R. The All-Species Living Tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. *Syst. Appl. Microbiol.* **2008**, *31* (4), 241–250.

(25) Bru, D.; Sarr, A.; Philippot, L. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Appl. Environ. Microbiol.* **2007**, *73* (18), 5971–5974.

(26) Monod, J. The growth of bacterial cultures. *Annu. Rev. Microbiol.* **1949**, *3* (1), 371–394.

(27) Russell, J. B.; Cook, G. M. Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiol. Mol. Biol. Rev.* **1995**, *59* (1), 48–62.

(28) Glass, C.; Silverstein, J. A. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Res.* **1998**, *32* (3), 831–839.

(29) van Rijn, J.; Tal, Y.; Barak, Y. Influence of volatile fatty acids on nitrite accumulation by a *Pseudomonas stutzeri* strain isolated from a denitrifying fluidized bed reactor. *Appl. Environ. Microbiol.* **1996**, *62* (7), 2615–2620.

(30) Her, J. J.; Huang, J. S. Influences of carbon source and C/N ratio on nitrate/nitrite denitrification and carbon breakthrough. *Bioresour. Technol.* **1995**, *54* (1), 45–51.

(31) DeSantis, T. Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E. L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G. L. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **2006**, *72* (7), 5069–5072.

(32) Reddy, G. S. N.; Matsumoto, G. I.; Schumann, P.; Stackebrandt, E.; Shivaji, S. Psychrophilic pseudomonads from Antarctica: *Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov. and *Pseudomonas proteolytica* sp. nov. *Int. Syst. Evol. Microbiol.* **2004**, *54* (3), 713–719.

(33) Miwa, H.; Ahmed, I.; Yoon, J.; Yokota, A.; Fujiwara, T. *Variovorax boronicumulans* sp. nov., a boron-accumulating bacterium isolated from soil. *Int. Syst. Evol. Microbiol.* **2008**, *58* (1), 286–289.

(34) Kraft, B.; Strous, M.; Tegetmeyer, H. E. Microbial nitrate respiration—genes, enzymes and environmental distribution. *J. Biotechnol.* **2011** in press.

(35) Potter, L. C.; Millington, P.; Griffiths, L.; Thomas, G. H.; Cole, J. A. Competition between *Escherichia coli* strains expressing either a periplasmic or a membrane-bound nitrate reductase: does Nap confer a selective advantage during nitrate-limited growth?. *Biochem. J.* **1999**, *344* (Pt 1), 77–84.