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Effects on Groundwater Microbial Communities of an Engineered 30-Day In Situ Exposure to the Antibiotic Sulfamethoxazole

Sheridan K. Haack,*[,†] David W. Metge,[§] Lisa R. Fogarty,[†] Michael T. Meyer,[‡] Larry B. Barber,[§] Ronald W. Harvey,[§] Denis R. LeBlanc,[∥] and Dana W. Kolpin[⊥]

[†]Michigan Water Science Center, U.S. Geological Survey, Lansing, Michigan

[‡]Kansas Water Science Center, U.S. Geological Survey, Lawrence, Kansas

[§]National Research Program, U.S. Geological Survey, Boulder, Colorado

[∥]Massachusetts Water Science Center, U.S. Geological Survey, Northborough, Massachusetts

[⊥]Iowa Water Science Center, U.S. Geological Survey, Iowa City, Iowa

Supporting Information

ABSTRACT: Effects upon microbial communities from environmental exposure to concentrations of antibiotics in the μg L^{-1} range remain poorly understood. Microbial communities from an oligotrophic aquifer (estimated doubling rates of only once per week) that were previously acclimated (AC) or unacclimated (UAC) to historical sulfamethoxazole (SMX) contamination, and a laboratory-grown *Pseudomonas stutzeri* strain, were exposed to 240−520 μg L^{-1} SMX for 30 days in situ using filter chambers allowing exposure to ambient groundwater, but not to ambient microorganisms. SMX-exposed UAC bacterial communities displayed the greatest mortality and impairment (viable stain assays), the greatest change in sensitivity to SMX (dose−response assays), and the greatest change in community composition (Terminal Restriction Fragment Length Polymorphism; T-RFLP). The *sul1* gene, encoding resistance to SMX at clinically relevant levels, and an element of Class I integrons, was not detected in any community. Changes in microbial community structure and SMX resistance over a short experimental period in previously nonexposed, slow-growing aquifer communities suggest concentrations of antibiotics 2−3 orders of magnitude less than those used in clinical applications may influence ecological function through changes in community composition, and could promote antibiotic resistance through selection of naturally resistant bacteria.

INTRODUCTION

Numerous reports document antibiotic presence in wastewater effluents globally,[1−5] with frequent detections occurring in aquifers near effluent inputs.[6,7] After infiltration through the vadose zone near contaminant sources, antibiotic degradation may be limited and parent compounds may persist long enough to reach groundwater.[7,8] Sulfamethoxazole (SMX) is one of the most frequently detected antibiotics in the environment.[1,9] Previous research has documented that SMX undergoes subsurface transport similar to conservative, nonreactive tracers such as bromide.[7] Commonly detected concentrations of SMX in groundwater range up to 1.1 μg L^{-1}.[6]

Sulfonamide-resistant bacteria and genes responsible for resistance occur within surface waters, groundwaters, and fecal sources such as animal waste lagoons and human wastewater.[10,11] The relative importance of ambient exposure to subclinical antibiotic levels, migration and establishment of already-resistant bacteria from sources and exchange of resistance genes, novel resistance mechanisms or inherent physiological tolerance,[12] and changes in community composition on the development, maintenance, and significance of antibiotic resistance in the environment is poorly understood. Some studies have established that longer-term exposure to subtherapeutic (e.g., prophylactic) antibiotic concentrations,[10,11] especially over multiple generations,[13] leads to increased antibiotic resistance in microbial populations. However, growth rates in the environment are generally much lower than those in clinical, in vivo, or wastewater settings. Using laboratory microcosms and microbial communities taken from the study site reported herein, Underwood et al.[14] demonstrated that SMX concentrations as low as 1.3 μg L^{-1} inhibited nitrate-reduction rates, and at >13 μg L^{-1} altered microbial community structure.

Sulfonamide antibiotics were among the first antimicrobial groups prescribed in human medicine (~1930s), and act by inhibiting bacterial folate synthesis. In clinical settings, the minimum inhibitory concentrations for SMX range from 64−

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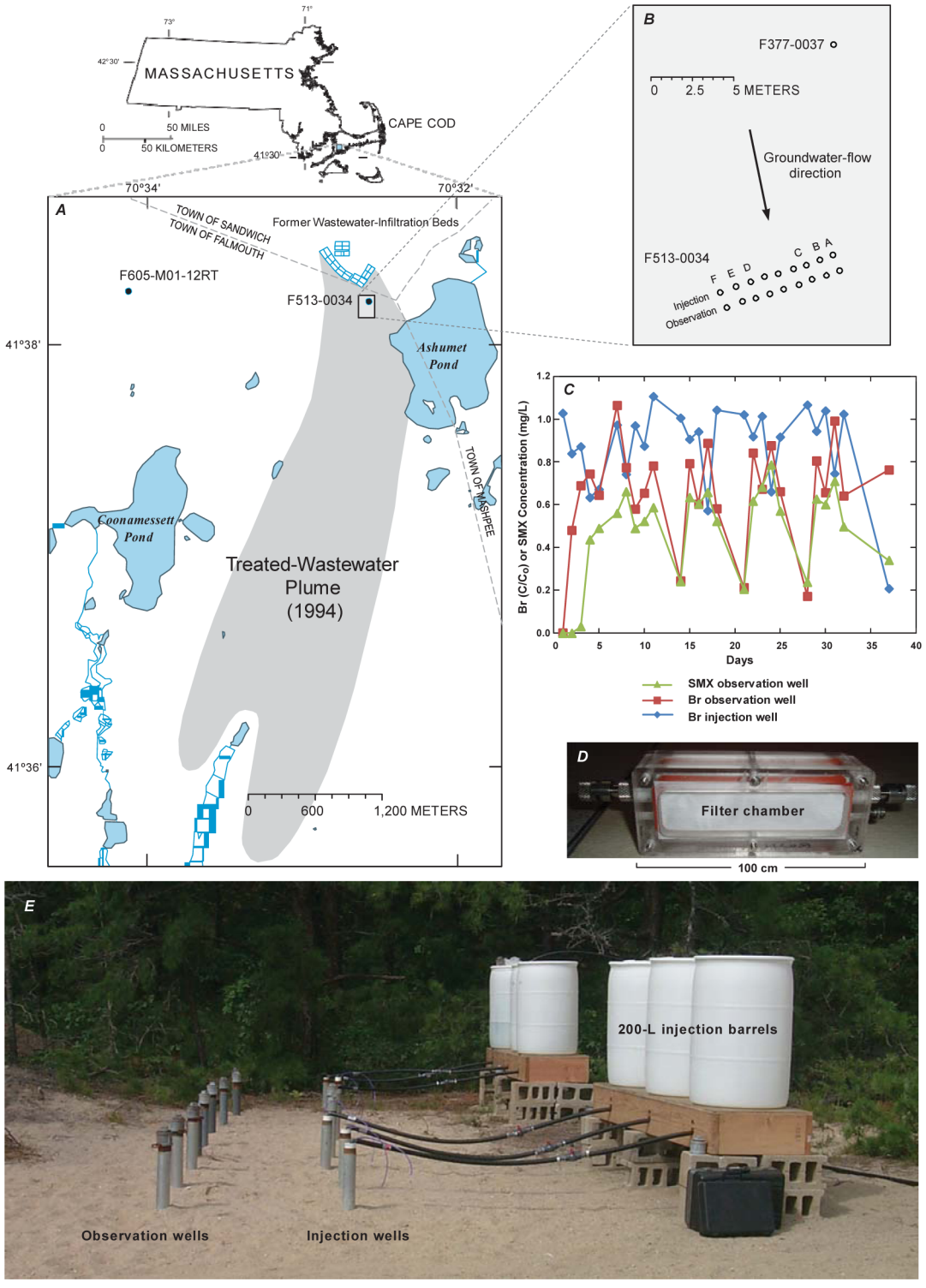

Figure 1. Location of (A) test site, (B) layout of experimental wells, (C) example breakthrough curves from well pairs F, and photographs of (D) filter chamber (shown horizontally; inserted vertically in observation well), and (E) experiment test site.

510 mg L^{-1} for different bacterial genera, mostly in the *Enterobactereaceae*.[15] Acquisition of resistance genes, or mutation, are the most widely studied forms of antibiotic resistance, [16] and the SMX-resistance genes *sul1*, *sul2*, and *sul3* can be transferred among bacteria by Class 1 integrons.[17] However, acquisition of resistance genes is not the only mechanism of antibiotic resistance. Physiological differences in bacterial cell membranes, or metabolism, make some genera inherently less susceptible to various antibiotics.[18] Bacteria in a nongrowing state can be physiologically tolerant of antibiotics.[16]

Since 1983, the United States Geological Survey (USGS) Toxic Substances Hydrology Program research site near the Massachusetts Military Reservation on Cape Cod, Massachusetts has been used to understand hydrological, chemical, and microbial influences on the fate and transport of contaminants

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in groundwater. From 1936 to 1995, a contaminant plume was present resulting from secondary-treated wastewater disposal onto an unconfined glacial outwash aquifer.[19,20] Since 1995, the area of the aquifer impacted by the historic plume has been reverting back to oligotrophic conditions. SMX transport along the path of the former plume was nearly conservative,[7] and because SMX was detected as late as 2005 at concentrations up to 1.5 μg L^{-1}, it can be assumed that indigenous groundwater microbial communities in the study area were exposed historically to SMX, and a mix of other micropollutants commonly found in sewage.

The microbial community in this sole-source, drinking-water aquifer, has been extensively studied. With the exception of the core of the wastewater plume prior to system recovery, the aquifer was oligotrophic. Historically, average doubling times for aquifer bacteria ranged from once per two days immediately downgradient from the loading beds, to several times per year outside the plume.[21,22] Recently developed bacteria isolation chambers[23] have allowed exposure of microbial communities to groundwater conditions but not to indigenous predatory protists, or to ambient groundwater bacteria.

To characterize and understand better the effects of μg L^{-1} SMX exposure upon groundwater microbial communities, we conducted an in situ exposure study within this aquifer. The study was designed to assess how exposure to SMX affects bacterial community composition and/or SMX resistance in the absence of interaction with bacteria from external sources, and involved comparing responses between an acclimated (AC) groundwater microbial community previously exposed to SMX, an unacclimated (UAC) community from a nonimpacted aquifer zone, and a bacterial isolate obtained from a pristine aquifer in New Hampshire. The overarching objective of this unique field study was to characterize the subtle, in situ responses of groundwater microbial communities exposed to SMX concentrations in the μg L^{-1} range. Our major hypothesis was that the UAC community would be more sensitive to SMX exposure than the previously exposed AC community and that we would be able to detect this sensitivity through changes in cell viability, community antibiotic resistance levels, and community composition. This paper describes an in situ, SMX-dosing study that employed aquifer bacteria sequestered in down-well filter-chambers in order to obtain an initial evaluation of this hypothesis.

MATERIALS AND METHODS

Study Site. The study was conducted within the zone of the historic wastewater-contamination plume at the Cape Cod site (Figure 1). The AC community .was collected from well F513−0034, located ∼0.3 km downgradient from the historical infiltration beds. The UAC microbial community (no previous SMX exposure) was collected from multilevel sampling well F605-M01-12RT, located ∼1 km west of the AC site (Figure 1). At both collection sites groundwater was aerobic and 10−11 °C.

SMX Exposure Experiment. The 30-day in situ exposure experiment was conducted at the F513-0034 AC site (Figure 1) with the objective of understanding the effects of exposure to ∼500 μg L^{-1} SMX on the slowing-growing groundwater microbial communities. This concentration of SMX is 1−2 orders of magnitude greater than reported environmental (<1−10 μg L^{-1}) levels[6,7] but 2−3 orders of magnitude less than clinical effect levels.[15] Our previous studies of SMX transport in this aquifer[7] indicated that a dosing concentration of 1 mg L^{-1} would result in quantitatively reliable concentrations in the plumes and would achieve a reasonable compromise between the experimental challenges of a field experiment and the approximation of environmental exposures. The experimental system uses natural groundwater flow and hydrodynamic dispersion to generate a dilute SMX concentration field at the observation wells 1 m downgradient from the injection wells. The study involved six pairs of 5-cm-diameter PVC injection and observation wells with slotted screens placed 9.6−10.2 m below land surface (∼5 m below the water table), with 1 m spacing between wells, with a central unused set of injection and observation wells to separate the SMX plumes from the control wells. Each injection well was connected to a 200 L polyethylene reservoir (Figure 1 inset E), which was filled with 200 L of groundwater collected from the injection depth, but 50 m upgradient at well F377-0037. Three injection/observation well pairs used as controls (Control A, B, and C) were dosed with the conservative tracer sodium bromide (Br^-), and three well pairs used for SMX exposure (SMX-Exposure D, E, and F) were dosed with SMX and Br^-. Bromide and SMX concentrations were ∼50 mg L^{-1} and ∼1000 μg L^{-1}, respectively in the injectates, added by gravity feed to the aquifer as pulses three times per week over 30 days. The injectate temperature was 13−16 °C because of warming during the 1−2 h of pumping from the supply well into the barrels, addition of the tracers, and dosing the injection wells. Duplicate groundwater samples were collected in 60 mL amber glass bottles from the injection and observation wells prior to initiation of the experiment and at each injection cycle. Br^- concentration in the injection and observation wells was measured by ion chromatography, and SMX concentration by liquid chromatography/tandem mass spectrometry with multiple-reaction monitoring and electrospray ionization in positive ion mode.[7]

Microbial Community Collection and Isolation. Groundwater microbial communities were obtained from the AC and UAC wells after purging three volumes using a peristaltic pump (0.6−1 L min^{-1}) fitted with Norprene tubing previously rinsed with 200 mL isopropyl alcohol and 1 L filter-sterilized deionized water. An inline filter unit (Pall Gelman 1.2-μm pore size) removed protists that conduct size-selective grazing in this aquifer and therefore might influence community composition and complicate interpretation of results.[24] Microbial communities were collected on a Gelman 0.45-μm pore size, 47-mm filter and resuspended in 200 mL of filter-sterilized groundwater from F513−0034 to final concentrations of ∼1 × 10^6 cells mL^{-1}, as determined by epifluorescence microscopy.[25] Approximately 250 to 350 bacteria in 10 reticle fields were counted to ensure statistically valid bacterial concentrations. A groundwater bacterial isolate, *Pseudomonas stutzeri* + *bfp* gene (PS),[26] was grown to ∼1 × 10^9 cells mL^{-1} in a modest nutrient broth (1% tryptic soy, 90% filtered groundwater, 10% soil extract broth amended with 10 mg L^{-1} arabinose and 50 μg mL^{-1} ampicillin). Cells were harvested, centrifuged, and washed 2−3× in filtered groundwater, then suspended in filtered groundwater (1 × 10^6 mL^{-1}) from well F513−0034, 3 days before deployment. PS has been very robust in previous injection-and-recovery transport tests performed at the site.[27] As an isolate from a pristine aquifer with no previous SMX exposure, it was included in the current study to compare the effects of SMX exposure on a single population as opposed to a complex community, and to evaluate the potential effect of physiologic state on experimental outcomes.

Each of the three different microbial communities (AC, UAC, and PS) was aseptically loaded into six separate in situ microbial isolation chambers (18 total). The chambers consist of three Plexiglas plates (100 × 40 mm) held together with stainless steel screws (Figure 1 inset D). The middle of the plates had 80 × 20 mm cutouts (9.6 mL volume) that served as the holding chamber for microbes during the down-well incubations. Rectangular membrane filter strips (0.45-μm-pore-size, Millipore, Bedford, MA) were sandwiched between the middle and outer plates along with protective strips of woven polypropylene mesh (1000 μm openings, Spectra Physics, Houston, TX). These membranes allow for relatively rapid geochemical exchange between chamber contents and ambient groundwater, but not for bacterial exchange as the membrane pore size is well below measured average bacterial diameters (chamber population or ambient free-living aquifer bacteria).[24] The middle plates were fitted at both ends with stainless steel syringe hubs (sampling/loading ports) and with stainless steel eyelets for attachment of monofilament line for deployment and retrieval. Each of the six observation wells (three Controls and three SMX exposures) contained three chambers, each holding either the AC, UAC, or PS microorganisms (18 total). Thus, there were three AC and three UAC Control microbial community samples, three AC and three UAC SMX-exposed community samples, and three Control and three SMX-exposed PS samples for the experiment (18 total). Filter chambers were located adjacent to the screened interval of the well, were retrieved after the 30 day exposure period, and were not sampled during the intervening period. Microbial communities were aseptically removed from each chamber, ~4 mL aliquots were placed into sterilized test tubes, and shipped overnight on ice to USGS laboratories in Lansing, MI and Boulder, CO for microbial analyses described below.

Microbial Viability Staining Procedures. The LIVE/DEAD Cell Viability assays for Gram positive and negative bacteria were performed following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Under epifluorescence microcopy (excitation wavelengths of 470−490 and 560−600 nm), "viable" cells fluoresce green (λ = 520 nm), dead or "nonviable" cells red (λ = 565−617 nm), and metabolically impaired cells greenish-orange (λ = 530−560 nm). Ratios of operationally defined live:dead and live:total bacteria were determined before and after the SMX exposure experiment for the AC, UAC, and PS communities.

Microbial Resistance to SMX. An aliquot of each microbial community (three Control and three SMX-exposed communities) was serially diluted 10^{-1} to 10^{-6} in phosphate buffered saline solution and 100 μL of each dilution was plated onto R2A media (BD Difco, Franklin Lakes, NJ) without SMX, as well as to R2A supplemented with SMX at eight concentrations between 0.2 and 320 mg L^{-1}. R2A is a low-nutrient medium often used to isolate culturable groundwater bacteria. [28] R2A supports typical soil and aquatic bacteria, and genera which include human or plant pathogens (*Pseudomonas*, *Burkholderia*, or *Aeromonas*) for which antibiotic resistance is a concern. Dilutions yielding between 1 and 100 colonies (usually only one plate in the dilution series satisfied this criterion) were counted at each concentration of SMX. If multiple dilutions yielded countable plates, counts were averaged. Percent mortality (colony count at each SMX concentration/colony count on unsupplemented medium) was determined for each community. Probit analysis[29] was used to transform the sigmoidal dose−response curve to a straight line amenable to regression analysis. Probit values of percent mortality (probit of kill), determined using Finney's table,[29] were plotted as a function of logarithmic SMX concentrations. For each community, linear regression (SYSTAT 13, SYSTAT Software Inc., Chicago, IL) was used to determine the estimated SMX concentration and 95% confidence interval at a probit value of 5.0, which estimates the LC_{50}.

Molecular Microbial Community Analyses. Following DNA extraction (outlined below) the presence of one of the most commonly acquired SMX resistance genes (*sul1*) was tested using polymerase chain reaction (PCR) as described in Lévesque et al.[17] The positive control for the reaction was *Escherichia coli* ECOR-3 (*E. coli* reference collection at Michigan State University, East Lansing, MI).

The diversity of each microbial community was determined by terminal restriction fragment length polymorphism (T-RLFP).[30] Approximately 3 mL of each AC or UAC community was filtered onto a 0.22 μm pore-size filter and DNA was extracted using the UltraClean Water DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Following ethanol DNA precipitation, PCR was performed using 27F-Fam labeled forward primer and 1389R reverse primer to obtain bacterial 16S rRNA gene sequences.[30] The amplified products, and subsequent technical replicates, were cut with restriction enzymes *Hha*I and *Msp*I (New England Biolabs, Ipswich, MA), and were analyzed on an ABI PRISM 3100 Genetic Analyzer. T-RFs <50 base pairs in length and <50 fluorescent units (FU) in area were not included in further analyses. Both communities from the D well yielded anomalously low total FUs, and fewer than 3 T-RFs, even on repeated analysis. Because both communities yielded diverse colony morphologies on R2A medium, these results were considered inaccurate and the T-RFLP profiles from the D well were not further analyzed.

Data generated from GeneScan was uploaded into T-Align,[31] to obtain consensus assignments (based on technical replicates) of T-RF base length and percent peak area. In both the *Hha*I and *Msp*I digests, two samples contained higher total FU than the remaining samples. However, within remaining samples little overall association between T-RF and FU levels existed. The high-FU samples were normalized (to average FU of the remaining samples) with peak areas adjusted accordingly.[30] The resulting outputs were evaluated using Fisher's Exact Test (Systat Software, Chicago, IL) and Principal Components Analysis (PCA) of arcsin-transformed data and the correlation matrix with Bionumerics (v. 3.5, Applied Maths, Austin, TX). The web-based Phylogenetic Analysis Tool (PAT)[32] was used to obtain phylogenetic assignments for some of the *Hha*I and *Msp*I fragments.

RESULTS AND DISCUSSION

Bromide and SMX. The field-scale SMX exposure experiment was designed to deliver SMX dosages in the μg L^{-1} range at the observation wells via a series of 200-L injection pulses, each generating a tracer cloud with a diameter of ~1 m around the injection well. As the tracer cloud is transported under natural flow (0.33 m d^{-1}) conditions it develops an elliptical shape due to dispersive mixing at the leading and tailing edges, resulting in decreased concentrations at the downgradient observation wells. The average dimensionless (C/C_o) Br^- concentrations in wells A-F were 0.61 ± 0.26 (±1 standard deviation), 0.78 ± 0.16, 0.87 ± 0.15, 0.25 ± 0.12, 0.56

± 0.26, and 0.69 ± 0.21, respectively. These variable results indicate that the centers of mass of the tracer clouds did not intersect all the observation wells to the same extent due to aquifer heterogeneities and variations in well alignments. The average SMX concentrations in the observation wells were 240 ± 150 μg L^{-1} for well D, 440 ± 220 μg L^{-1} for well E, and 520 ± 150 μg L^{-1} for well F. Concentration histories for SMX closely tracked those for Br^- (Figure 1). SMX concentrations suggested no overlap among plumes. The conservative transport behavior of SMX found in this and previous studies at the Cape Cod site[7] underscores the importance of recognizing how microbial communities may be affected by long-term SMX exposure.

Cell Viability. Figure 2 shows changes in abundances of "viable" bacteria as determined by differential viability staining

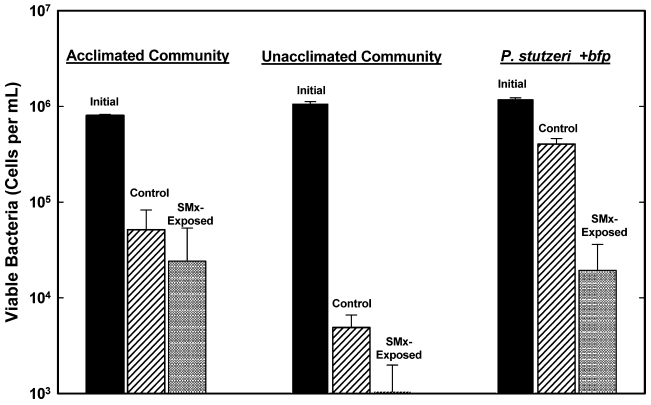

Figure 2. Comparison of field study control and SMX-exposure communities by viability staining. Relative concentrations of live bacteria determined by viable staining in the acclimated (AC; left), unacclimated (UAC; middle), and *Pseudomonas stutzeri* + *bfp* (PS; right) bacterial communities before and after the 30-day in situ sulfamethoxazole (240 to 520 μg L^{-1}) exposure experiments. Error bars reflect the standard error of the initial viability counts or of the average viable cell count for the triplicate Control or SMX-exposed experimental chambers.

during the incubation in the aquifer in the presence and absence of SMX. Our previous work indicates that the typical groundwater bacterial abundance in the vicinity of the test wells is 1×10^5 cells $mL,^{-1}$ therefore the initial loading of 10^6 bacteria mL^{-1} exceeds the aquifer carrying capacity, and a decrease in viable cell count of ~1 log unit was anticipated (as observed for AC and PS controls). The UAC control communities experienced the greatest decline, suggesting greatest sensitivity to experimental conditions.

In a similar fashion, following SMX exposure, the ratio of operationally defined live:dead bacteria decreased for the UAC community by ~6150 fold, for PS by ~1760-fold, but only ~200 fold for the AC community (Table 1). For control wells, live:dead ratios decreased as follows: UAC community ~192 fold; AC community ~127 fold; and PS ~62 fold. The UAC community was, therefore, slightly more sensitive to experimental conditions than either the AC community or the PS strain, but was much more sensitive to SMX exposure, as was hypothesized. Although PS was harvested for the experiment following growth in the laboratory, it was suspended in filtered groundwater prior to the experiment, and was likely in stationary phase or a maintenance state by the end of the experimental exposure. PS exhibits strong survival behavior in the Cape Cod aquifer, even when coinjected with organic contaminants. [27] The AC community appeared slightly more durable than PS to SMX exposure, and similar to the laboratory-grown PS with respect to experimental conditions (controls). Growth conditions in the former sewage-impacted zone (AC) may have been more favorable than in the pristine aquifer (UAC) when the two communities were collected. In addition, the AC community may contain genera more resistant to SMX than PS, either due to intrinsic resistance, or due to prior selection by exposure to SMX, detergents, or other sewage-micropollutants with antimicrobial properties.

Viability stains resulted in moderately fewer (6.8−26%) overall counts relative to DAPI, consistent with observations from other studies.[33] Coefficients of variation for direct counting ranged from 2.9 to 7.4% for DAPI and from 3.9 to 22% for viability stains. Previous research at this site indicated low microbial growth rates.[21,22] Viability staining, an indirect measure of bacterial growth potential, can be problematic if substantive fractions of the SMX-exposed populations remain in viable but nonculturable (VBNC) or metabolically impaired states.[34] Other studies suggest that viability assays which include VBNC cells can be considered growth independent and that metabolically impaired or VBNC cells can reproduce under the right conditions.[35] Indeed, Haznedaroglu et al. (2012)[36] recently showed that, when exposed to various antibiotics in the 1−100 μgL^{-1} range in artificially prepared groundwater, viability of the human pathogen *Salmonella enterica* serovar Typhimurium decreased as much as 88%, and some cells entered a VBNC state, yet survivors showed increased virulence against human epithelial cells or nematodes.

SMX Resistance. Following the 30 day field experiment, the SMX-exposed UAC community exhibited the highest LC_{50}. Within the UAC Control or SMX-exposed communities, responses to SMX were consistent among replicates (Figure 3). The 95% confidence intervals (CIs) about the regression lines were narrow and most points fall within the CIs. The CIs for the Control and SMX-exposed regression lines do not overlap until the higher SMX concentrations and the LC_{50} values were significantly different. The regression gave an LC_{50} of 7.49 μg L^{-1} (95% CI: 3.65 to 12.5 μg L^{-1}) for the UAC

Table 1. Initial Bacterial Concentrations Loaded to Chambers and Mean Ratios of Live Cells to Dead Cells in Control (Unexposed) and Sulfamethoxazole-Exposed (240−520 μg L^{-1}) Isolation Chambers

microbial community[a]	initial concentration (cell mL^{-1})	initial live:dead ratio	live:dead ratio smx exposure	live:dead ratio control
P. stutzeri + *bfp*	1.2×10^6	74:1 (±1.50)[b]	1:24 (±0.30)	1:0.83 (±2.05)
acclimated community	0.8×10^6	8:1 (±0.22)	1:25 (±0.26)	1:16(±0.50)
unacclimated community	1.2×10^6	8:1 (±0.14)	1:762 (±0.33)	1:24 (±0.69)

[a]*bfp* gene codes for the production of blue fluorescing protein; Acclimated community, previously exposed to sulfamethoxazole; Unacclimated community, previously not exposed to sulfamethoxazole. [b]Standard error of mean values are provided for live:dead ratio calculations in parentheses. Initial live:total ratio *Ps. stutzeri* + bfp =0.96, Acclimated community = 0.78, Unacclimated community = 0.75.

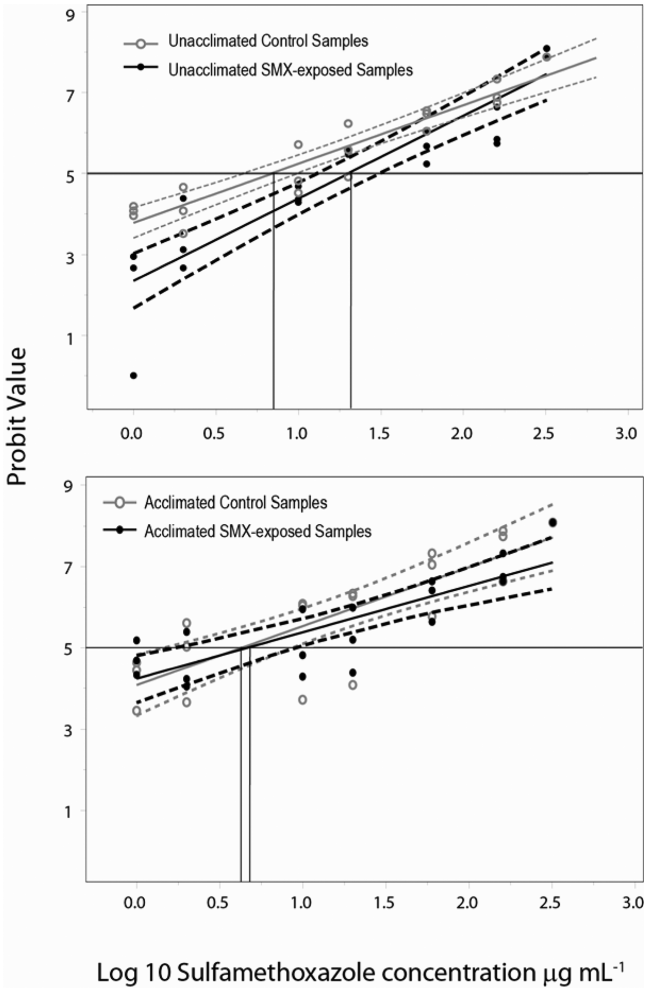

Figure 3. Determination of LC_{50} for each community by probit analysis. Each community sample (three replicates) was challenged against seven concentrations of sulfamethoxazole (SMX), and the percent mortality at each dose was calculated. Percent mortality was converted to probit values.[33] This figure shows least-squares regression of the logarithm of the SMX concentration against probit values. Solid lines (probit regression lines); dashed lines (95% confidence intervals); circles (responses of each sample at test concentrations of SMX). A probit value of 5 represents 50% mortality. Extrapolation to the *x*-axis gives the LC_{50} (concentration resulting in 50% mortality).

Control community and 20.5 $\mu g\ L^{-1}$ (CI: 14.1 to 29.7 $\mu g\ L^{-1}$) for the UAC SMX-exposed community. The UAC Control response was similar to that found by Underwood et al.[14] who tested in the laboratory a similar groundwater microbial community from the same aquifer, and found no growth inhibition (although there was an extended lag phase) at SMX concentrations <0.25 $\mu g\ L^{-1}$, growth inhibition between 0.25 and 13 $\mu g\ L^{-1}$, and no growth above 13 $\mu g\ L^{-1}$.

In contrast to the UAC communities, replicate responses for the AC Control and SMX-exposed communities were variable. Specifically, the AC Control A (lowest open circles in lower panel of Figure 3) response was more like that of the SMX-exposed samples and the AC SMX-exposed E response was more like the Control samples at log SMX concentrations ≤1. The CIs for the two regression lines overlap across all SMX concentrations, and the LC_{50} values were not significantly different at the 95% confidence level. The regression gave an LC_{50} of 6.46 $\mu g\ L^{-1}$ (95% CI: 3.45 to 12.1 $\mu g\ L^{-1}$) for the AC Control and 6.76 $\mu g\ L^{-1}$ (CI: 3.65 to 12.5 $\mu g\ L^{-1}$) for the AC SMX-exposed community.

The finding that the UAC SMX-exposed community had the highest LC_{50} suggests that exposure selected for community members with overall higher resistance levels to SMX. Gene mutation or acquisition of mobile genes such as *sul1*, receives the vast majority of attention in the literature,[16] due to well-documented outcomes in the clinical setting, where it and the similar, but less common, genes *sul2* and *sul3* confer high-level (≥64 mg L^{-1}) resistance to sulfonamide antibiotics to bacteria in the *Enterobactereaceae*, a Gram-negative group. Survival of 160 or 320 mg L^{-1} SMX (log SMX concentration >2.0 in Figure 3) would indicate high-level resistance in the *Enterobactereaceae*, usually due to acquired genes. A probit value of 7.33 indicates 99% mortality, thus Figure 3 indicates the majority of cells were killed at the *Enterobactereaceae* definition for high-level resistance. Because none of the groundwater microbial communities contained evidence of the *sul1* gene, survivors of SMX concentrations >160 mg L^{-1} were likely intrinsically resistant to sulfonamide antibiotics. The *sul1* gene, in addition to conferring SMX resistance, also is typically part of mobile genetic elements called Class 1 integrons.[17] Lack of detection of the *sul1* gene is a small piece of evidence that acquired resistance genes may not be responsible for the apparent increased resistance to SMX of the UAC SMX-exposed community.

A different mechanism contributing to increased antibiotic resistance in the UAC community could be evolution of more-resistant bacteria during the experiment. Other studies have demonstrated increased antibiotic resistance within bacterial populations (single genera) exposed to persistent, subtherapeutic antibiotic concentrations, but these experiments have typically taken place over tens to hundreds of bacterial generations.[13] In this oligotrophic aquifer, and based on prior research,[21,22] it is unlikely that bacteria had doubling times of more than once per week during our 30-day experiment, and evolution of more-resistant bacterial strains is unlikely.

Results from our experiment could have been influenced by the loss of viability in the UAC community. Underwood et al.[14] showed that SMX at concentrations similar to those of this experiment resulted in growth inhibition of a microbial community from the same aquifer. Bacteria that are not growing are completely or largely resistant to antibiotics, a phenomenon termed dormancy. This does not result in increased levels of antibiotic resistance in the surviving cells compared to their resistance before exposure, but provides a reservoir of survivors.[16] For example, Hazendaroglu et al.[36] showed that large percentages of the human pathogen *Salmonella enterica* serovar Typhimurium entered a VBNC state when exposed to antibiotics in the 1−100 ug L^{-1} range in artificially prepared groundwater, and those that were subsequently cultivatable did not express any change in antibiotic susceptibility profile. If some members of the UAC community with greater initial resistance to SMX survived SMX exposure by dormancy, then the UAC community following exposure would have a greater overall resistance to SMX. The T-RFLP results provided insight with regard to community composition.

Microbial Community Composition. T-RFLP patterns for the UAC SMX-exposed E and F communities, and the AC SMX-exposed F community were substantially different from those of the Control communities. As noted previously, T-RFLP profiles from both communities from the D well were

not included in this analysis. Of 50 T-RFs analyzed, 15 present in the control communities were not present in any SMX-exposed community (Supporting Information (SI) Table SI-1), suggesting loss of community members following SMX exposure. In addition, the percentage of 2 T-RFs (*MspI* 52 and *Hha*I 52) was significantly ($p < 0.5$, Fisher's Exact Test) greater in the SMX-exposed, as opposed to control, communities, suggesting increased abundance of some community members following exposure. T-RFs *MspI* 142 and *Hha*I 201, were absent in UAC SMX-exposed communities, but not in controls or SMX-exposed AC communities. T-RF *Hha*I 52 exhibited greater ($p < 0.05$) and *Hha*I 205 lesser percent composition in UAC SMX-exposed as compared to AC SMX-exposed communities. Figure 4 graphically displays these

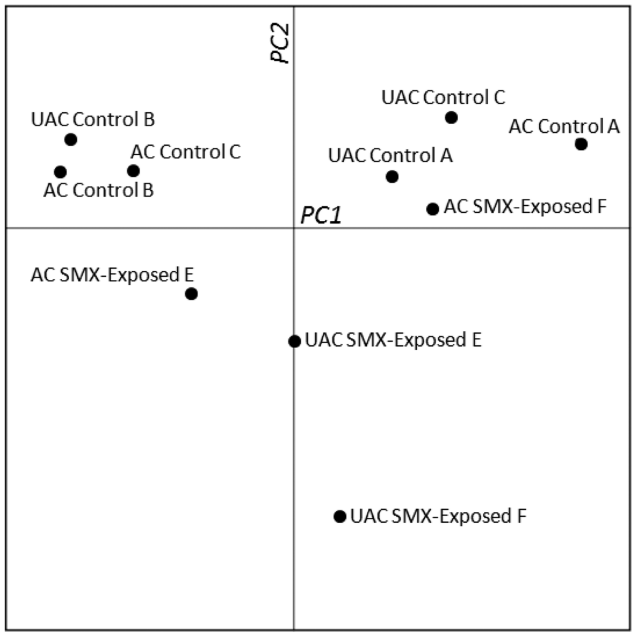

Figure 4. Graphic depicting results of principal components analysis based on the arcsin transformed percent of total area for all terminal restriction fragments (T-RFs) detected in each community. PC1: first principal component (77.6% of variance); PC2: second principal component (32.1% of variance); AC, acclimated community; UAC, unacclimated community; Control, no SMX exposure.

results. The dominant T-RFs positively associated with Principal Component (PC)1 were *Hha*I 205 and *Msp*I 490, found in all communities but in greatest abundance in UAC Controls A and C, and AC Control A. The dominant T-RFs negatively associated with PC1 were *Msp*I 485 and *Hha*I 201, reflecting lesser abundance in the remaining controls. One AC Control and one UAC Control did not group with its replicates. PC2 was positively associated with all 15 T-RFs present exclusively in the control communities, as well as with *Hha*I 205. PC2 was negatively associated with *Hha*I 52 (significantly greater in SMX-exposed communities) and *Msp*I 80 (unique to both F SMX-exposed communities). The UAC SMX-exposed communities had the most negative placement on PC2 due to greater percent composition of *Hha*I 52, less of *Hha*I 205, and absence of *MspI* 142 and *Hha*I 201. As the UAC SMX-exposed communities were most different in T-RF composition from the Control communities, and were, as described previously, the communities having the greatest LC_{50} for SMX, our hypothesis that the UAC community would be most sensitive to SMX exposure appears to be supported by the T-RFLP results, and suggests community change (versus acquired resistance) as the responsible mechanism.

SI Table SI-1 identifies the phylogenetic assignments from the PAT program for the dominant T-RFs contributing to the PCA, or unique to test or control communities. In most cases, each T-RF was given (exclusively or as an alternate possibility) a phylogenetic assignment of widely occurring environmental microorganisms such as *Achromatium*, *Acidovorax*, *Leptothrix*, or *Methylomicrobium*, or classification with an unknown organism. Occasionally, genera were identified (e.g., *Pseudomonas*, *Burkholderia*, *Aeromonas*, or *Clostridium*) that have clinically or agriculturally significant species and for which acquired antibiotic resistance is an issue. Many of the genera identified by the PAT program grow on R2A medium, suggesting some concordance between the communities as tested by T-RFLP and by the probit tests. Underwood et al.[14] showed that exposure in the laboratory to 250 $\mu g\ L^{-1}$ of SMX caused a population shift from dominant *Pseudomonas gessardii* to *Variovorax boronicumulans* in nitrate-reducing enrichments from a community similar to the UAC microbial assemblage. The largest *Hha*I and *Msp*I peaks were assigned as many as 20 identities by the PAT program, an expected result as T-RFs represent multiple fragments of similar size derived from different 16S rRNA genes.[30,32] Although the PAT program suggested identities for many of the fragments, 33% of the *Hha*I peak area and 20% of the *Msp*I peak area remained unmatched.

Our experimental results indicated that, in comparison to the AC community and the PS strain, the UAC community exhibited the greatest loss of viability, the greatest change in SMX resistance, and the greatest change in community composition following 30-day in situ exposure to 240−520 $\mu g\ L^{-1}$ SMX. The UAC community was only slightly more sensitive to experimental conditions (as evidenced by viability in the control wells) than the AC community or PS strain, and the UAC and AC control communities were similar in community composition (T-RFLP analyses), suggesting that the experimental design (handling, chambers, wells, Br^- concentration) did not bias the results. The field deployment was challenging and this initial study was limited to three replicates and 30 days of exposure. Variable results affected our ability to establish statistical significance for some tests. Nevertheless, we employed multiple independent methods, in independent laboratories, to test our hypotheses, and SMX exposure was a consistent factor in all results.

Our findings that continuous in situ exposure to 240−520 $\mu g\ L^{-1}$ concentrations of SMX changed the community composition and SMX resistance of relatively slow growing groundwater microbial communities in a 30 day field study, and in the absence of interaction with externally derived bacteria barred by the membrane from entering the test chambers, remain significant. The occurrence of novel antibiotic-resistance mechanisms in environmental bacteria is widely recognized[37] and our understanding of the sources and means by which antibiotic resistance enters the pool of clinically significant microorganisms remains poorly understood. Therefore, changes in microbial community structure of the type observed in the current study may have an influence on the evolution of antibiotic resistance, and also may affect important microbial community functions.[14] Further and more intensive genetic or genomic analyses would be required to elaborate on our initial findings. This study suggests that longer in situ field-scale trials, coupled with deeper analysis of the microbial community,

might provide new information regarding the mechanism(s) by which microbial communities respond ecologically to persistent SMX exposure at μg L^{-1} concentrations.

■ ASSOCIATED CONTENT

Supporting Information

A table identifying the terminal restriction fragments that were dominant in principal components analysis or were unique to sulfamethoxazole-exposed or control communities, and their phylogenetic assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 517-887-8909; fax: 517-887-8937; e-mail: skhaack@usgs.gov.

Notes

The authors declare no competing financial interest.

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