C.J. Gray and A.S. Engel - "Microbial diversity and impact on carbonate geochemistry across a changing geochemical gradient in a karst aquifer"

Supplementary Materials and Methods

Field sampling, local hydrology, and aqueous geochemistry

For well water sampling, we followed the Standard Operating Procedures of the United States Geological Survey (e.g., Wood, 1981), as part of the National Water Quality Assessment Program (e.g., Koterba *et al.*, 1995; US Geological Survey, http://pubs.water.usgs.gov/twri9A). Prior to sampling a well, all sampling equipment (pump, tubing, water level meter) was precleaned with a dilute solution of 0.005% household chlorine bleach (NaOCl), followed by tap water then DI water rinses. This was especially important because we sampled wells with highly contrasting chemistries (e.g., non-potable saline water versus fresh drinking water). Water in the well casing was purged to the surface to ensure that we obtained a representative sample of aquifer water. Parameters, like temperature, pH, dissolved oxygen, and specific conductance (APHA, 2005), were monitored using standard electrode methods in a flowthrough chamber at the surface during purging. The known volume of water in the casing, which is based on drillers logs and the depth to water in the casing, and pump flow rate were determined to calculate the amount of time required to purge roughly three well volumes. We used solid, high-density Teflon tubing and Grunfos Redi-Flo2 pumps, which are submersible, low-flow, and portable pumps that operate by positive displacement with >95% recovery of volatile organic compounds at reportedly <100 ppb levels.

Once aquifer conditions stabilized, field parameters were recorded and dissolved sulfide, if present, was measured spectrophotometrically using the methylene blue method (CHEMetrics Inc., VA; APHA, 2005). Other unstable dissolved constituents, such as dissolved oxygen, NH_4^+ or Fe^{2+}, were also measured using colorimetric CHEMetrics methods. Tubing was connected to a precleaned, rinsed with ethanol or methanol, filter holder loaded (with sterilized tweezers) with a Whatman glass microfiber (GF/F) pre-baked at 500 ^oC and autoclaved 0.2-µm PVDF (polyvinylidene fluoride) filter to collect water quality samples in separate, precleaned HDPE bottles for anion, cation, and alkalinity measurements. For cation analyses, bottles were also acid-washed with HCl and filtered samples were acidified with trace metal grade nitric acid. Baked amber glass vials were also used for total organic carbon (TOC) and total nitrogen (TN) samples. Depending on pump rate, between 13.5 and 18 L of water were filtered.

Water samples from a freshwater spring at Comal Springs were collected by using a peristaltic pump with precleaned or new Teflon tubing. Parameters, like temperature, pH, dissolved oxygen, and specific conductance, were also monitored in a flowthrough chamber, and once conditions stabilized, the tubing was connected to a precleaned filter holder loaded with a GF/F and 0.2-µm PVDF filter to collect water quality samples in separate, precleaned HDPE bottles or amber glass vials (for TOC and TN), following the same procedures for well water collection. The spring was chosen to represent the freshwater end-member of our well transect because spring flow at Comal Springs, the largest discharging freshwater spring complex for the Edwards Aquifer, has long been recognized to be directly related to the water levels in the aquifer. Based on the regional and local groundwater flow, general movement in the aquifer indicates that most (as much as ¾) of the flow at Comal Springs comes from water along/within the downthrown side of the Comal Springs Fault, which has been determined to be an artesian fault block with both fresh and saline water ~4 miles (~6.4 km) wide in the vicinity of the springs

(Maclay and Land, 1988; Johnson and Schindel, 2008). Our transect wells are situated within the larger artesian fault block, on the downthrown side of the Comal Springs fault, although our wells penetrate smaller fault blocks with minimal offset but that dissect the large block (Supplementary Figure S1). Based on the study of Johnson and Schindel (2008), the adjacent fault blocks are in hydraulic communication with each other. The spring that we sampled has been traced to a proximal monitoring well on the downthrown side of the Comal Springs fault block, which has been determined to be within the same artesian fault block as the wells we sampled. The aqueous geochemistry of the Comal Springs water and the freshwater wells (Girl Scout and LCRA) in our study are statistically similar, supporting this relationship.

In the laboratory, concentrations of major anions were determined on unpreserved filtered samples and major cations were determined on acidified filtered samples using a dual column Dionex ICS-3000 Reagent-free ion chromatograph. Standards checks were accurate within ±2 standard deviations, except fluoride, sodium, and calcium (±3). TOC and TN concentrations were measured on filtered replicate purged aquifer water samples using a TOC-V organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). TOC was determined by difference from total carbon and total inorganic carbon (TIC). Calibration standards were sodium phthalate for total carbon, sodium bicarbonate and sodium carbonate for TIC, and potassium nitrate for TN. Alkalinity as total titratable bases, and representing bicarbonate (HCO_3^-) concentration based on the pH and concentration of titratable bases in the fluids was determined from 0.2 µm-filtered water by end-point titration using 0.1 N sulfuric acid to pH 4.3 (APHA, 2005), which took into account the possibility that some organic acid anions contributed to alkalinity (Lozovik, 2005). This was a necessary precaution because acetate had been found in high concentrations from some of the saline waters with exceptionally high TDS (e.g., Groscehen and Buszka, 1997). However, it should be noted that those wells with considerable acetate have TDS values at least 1 to 2 orders of magnitude greater than the TDS in our study wells. Moreover, from the beginning to the end of the microcosm experiments, only bicarbonate concentration as measured by alkalinity, and dissolved oxygen and sulfide concentrations varied in the well casings. Bicarbonate differed by as much as 5.5% for the Paradise Alley Deep well, and by 1.2% on average for other wells. Differences in dissolved sulfide and oxygen concentrations were likely due to a combination of outgassing, mixing, and microbial activities within the well casing. Geochemical conditions from pre-experiment analyses was used for further analyses.

For the routine quality control of water inorganic and organic geochemical analyses, trip, laboratory, and field DI water blanks were also prepared; several field DI water blanks were collected throughout the sampling period with and without the decontaminated equipment to verify that decontamination was adequate. Trip blanks ensured that the handling and storage of samples did not result in contamination or cross-contamination of samples. In the laboratory, these blanks were analyzed with the real samples to evaluate possible contamination or sample carry-over from dirty equipment or cross-contamination. Replicate samples were also collected to assess combined effects of field and laboratory procedures on measurement variability. For this study, on the basis of the blank and replicate sample concentration data, (i) there was no evidence that any samples were contaminated during or after their collection, (ii) that field decontamination procedures were adequate, and (iii) that replicate data for selected analytes were within analytical and procedural error.

Geochemical modeling

Using methods of Engel and Randall (2011), geochemical results were modeled using PHREEQC 2.15.0 (Parkhurst and Appelo, 1999; http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqci) to determine aqueous speciation and mineral and complex saturation indices. The program corrects for temperature and ionic strength, and uses the Debye-Hückel equation to calculate ion activity coefficients. This equation is valid for waters with ≤ 0.1 ionic strengths, and all samples in this study had ionic strengths ≤ 0.042. Saturation indices of various minerals were calculated based on saturation index (SI) = $log(IAP/K_{sp})$, where IAP is the ion activity product and K_{sp} is a mineral thermodynamic equilibrium constant.

Molecular analyses

DNA extraction

At the end of filtering water from the wells or spring, the filter holder was opened in the field, and the GF/F filter was transferred aseptically with tweezers to a sterile Whirl-bag. The PVDF filter was also aseptically transferred to a separate sterile Whirl-bag. Water sample bottles and filters were placed on ice for transport, then in 4^{o} C or -20^{o} C, respectively, until analyses. Filter bags were not opened until use in the laboratory, and all work was done in a NuAire Class II Type A2 Biological Safety Cabinet after UV sterilization of all internal surfaces and equipment. Nucleic acids were extracted from the filters using procedures described by Fuhrman *et al.* (1988). Simultaneous extraction was also done using blank filters and solutions with no filter to evaluate possible contamination. DNA purity and concentration for each extraction were determined spectrophotometrically using standard OD 260/280 absorbance methods on a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA). Extractions resulted in avg 11.3 ng/µl total nucleic acids. Extractions were stored in TE buffer at -20°C until use. Each sample sequenced, either by Sanger or 454, represented the composite of three replicate extractions from the filters.

16S rRNA gene sequence clone libraries

16S rRNA clone libraries were constructed from all of the wells except the Paradise Alley deep well, and clone inserts were Sanger sequenced. Extracted DNA was PCR-amplified for 16S rRNA genes using the universal bacterial primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGTTACCTTGTTACGACTT-3′). Amplifications were done with 5 U/µl Taq DNA polymerase (5Prime, Thermo Fisher Scientific) and executed using a MJ Research Dyad Disciple thermal cycler for 30 cycles under the following conditions after a hot start at 94^{o} C for 4 min: denaturation at 94° C for 60 s, primer annealing at 47° C for 60 s, and chain extension at 72° C for 90 s. Amplified PCR products were purified using 0.7% TAE low-melt agarose gel with a Wizard PCR prep DNA purification kit (Promega Corp., USA), following the manufacturer's instructions. Purified PCR products were ligated into the PCR2.1-TOPO vector with the TOPO Cloning Kit (Invitrogen Corp., USA), according to manufacturer's instructions. Clones were lysed for 10 min at 96° C prior to PCR amplification of products using the M13 primer pair (Invitrogen Corp., USA). Screening for the correct size PCR insert was done by gel electrophoresis. Selected clones were sequenced at the University of Washington's High-Throughput Genomics Unit (Seattle, WA, USA) using capillary sequencers from both M13 primer ends and with internal primers 907R (5'-CCGTCAATTCCTTTRAGTTT-3') and 704F (5'-GTAGCGGTGAAATGCGTAGA-3').

Resulting sequences were assembled using Contig Express, a component of Vector NTI Advance 10.3.0 (Invitrogen Corp., USA), then submitted to Basic Local Alignment Search Tool (BLAST) using GenBank (http://blast.ncbi.nlm.nih.gov) to determine similarity to cultured and uncultured organisms in the database. Sequences were submitted to Greengenes (DeSantis *et al.*, 2006a; DeSantis *et al.*, 2006b) (http://greengenes.lbl.gov/), and 54 sequences were identified as chimera and removed from further analysis. Nucleotide sequences were aligned with ClustalW using the Pasteur Institute Mobyle web portal (Neron *et al.*, 2009) (http://mobyle.pasteur.fr/cgi-bin/portal.py). A distance matrix was created from nucleotide sequences in BioEdit using the F84 model of evolution (Hall, 2005) to evaluate OTU-based coverage and relationships.

Taxonomic classification and statistical analyses

Following tag encoded FLX Titanium amplicon pyrosequencing of 16S rRNA genes, the program mothur (http://www.mothur.org) (Schloss *et al.*, 2009) was used for determining and removing sequences attributed to pyrosequencing errors, sequences with a high probability of being chimera (Haas *et al.*, 2011), and for generating a qualified file for alignment and taxonomy. Taxonomic assignments were completed with RDP Classifier of the aligned 16S rRNA pyrotags from 0% to 20% dissimilarity (e.g., Wang *et al.*, 2007; Cole *et al.*, 2009). An 80% confidence level was chosen for analysis because the number of unclassified pyrotags at the phylum level was reduced from nearly 85% of the full dataset for some samples to about 40% of the full dataset. Operational taxonomic units (OTUs) were determined using mothur, and these clusters were used to generate rarefaction curves (Supplementary Figure S1) and to calculate richness and diversity indexes (Supplementary Table S4).

In situ microcosm experiments

Reactive and sterile sets of microcosms were modified from Engel and Randall (2011). In this study, microcosms were made from perforated 125 mL HDPE bottles for reactive, or 50 mL tubes for sterile microcosms. All microcosms contained pre-weighed and cleaned fragments (chips) of either Iceland spar calcite (>99% pure) or dolomite (both from Wards Scientific). For the sterile microcosms, chips were placed into dialysis tubing (Spectra/Por 3) with a molecular weight cutoff (MWCO) of 3.5 kD. The dialysis tubing was placed into filter-sterilized (to 0.1µm) $CaCO_3$-equilibrated solutions until microcosm deployment. Chips were sterilized in 100% ethanol prior to insertion in microcosms or dialysis tubing. The total chip mass in each microcosm was weighed three times and ~ 6 g initially were used in reactive microcosms (average of ~11 chips) and 3 g were used initially in each sterile dialysis bag (average of ~6 chips). For all mass measurements, an APX 200 (Denver Instruments) digital balance was used. Precision and accuracy were tested with certified analytical weights, and measurements of ±0.008 mg were obtained.

In the field, it was not possible to place microcosms at the screened intervals due to sedimentation; microcosms were suspended within the larger diameter casing at ~20 to 30 m depth. During microcosm retrieval, some chips (at least one per mineral, per well) from each experimental microcosm were preserved using the hexamethyldisilazane (HMDS) preservation technique, whereby chips were rapidly submerged in cacodylic acid, then submerged in 2.5% glutaraldehyde, and washed in an ethanol series (10 – 100%) for critical point drying (Vandevivere and Bevaye, 1992). Other chips were preserved in 100% ethanol and stored on ice. In the lab, the chips that were HMDS preserved and critical point dried were weighed three times

and then stored in sterile bags. Average changes in microcosm chip weight from pre-deployment to post-retrieval were determined for each microcosm and *P*-values were determined from Student t-tests. A *P*-value <0.05 was considered to be significant, and values between 0.05 and 0.1 were considered to be marginally significant. Values >0.1 were considered to be insignificant. Chips from each reactive and sterile microcosm in all the wells were imaged by scanning electron microscopy (SEM) on a JEOL JSM – 840A scanning electron microscope, using methods and settings described in Engel and Randall (2011). Energy dispersive x-ray analysis (EDAX) was done to determine elemental composition of observed precipitates. Putative microbial cells on mineral surfaces were documented quantitatively by systematically viewing 100 frames in a grid at the same magnification (5000x) for each chip. The number of putative cells was assessed over an area of 0.0625 mm^2 per chip, then extrapolated to the entire surface area of each chip.

Several controls were made to evaluate possible changes in mass as a consequence of the experimental design. After weighing and sterilizing with ethanol, control chips were HMDS preserved and critical point dried. Chips were also weighed, sterilized, and placed into dialysis tubing in filter-sterilized (to 0.1μm) $CaCO_3$-equilibrated solutions as sterile microcosm controls to ensure no changes in weight would occur within the tubing before microcosms were deployed. Some of these chips were also HMDS preserved to assess possible weight changes as a result of the preservation process or possible bacterial contamination from the lab processing. Control chips for each mineral, preserved and non-preserved, were also mounted and gold-coated to observe background surficial textures (non-preserved) and potential preservation effects (preserved). Of the control sets of chips that were made to determine possible changes in chip mass or evidence for cell colonization as a product of the experimental design, the impact of potential contamination was considered to be low. Specifically, of the chips sterilized and weighed, then left in sterile bags and maintained as experimental controls in the lab, there was an insignificant 0.01% weight change for calcite (*P*-value 0.26) and for dolomite (*P*-value 0.35). Unpreserved control chips examined by SEM had smooth, unaltered surface textures, and no cells, secondary precipitates, or dissolution features were observed. Small, sharp pits (averaging < 2μm^2) were observed along cleavage planes or kinks, but they were easily distinguishable as surficial defects. Control chips that were HMDS preserved had a “mottled” texture on the mineral surfaces, and on rare occasion, cells were found on both control and experimental surfaces for chips that were in dialysis bags, although it is unclear how microbes could infiltrate the tubing. Cells on sterile chip surfaces may have resulted from manipulation in the lab prior or during ethanol washing, or were introduced from the dialysis tubing or solution (although manufacturer details ensured sterility). Regardless, based on the changes in masses, and quantified numbers of cells from the 100 frames covering 0.0625 mm^2, significantly more and diverse features were observed on experimental chip surfaces compared to controls (Supplementary Table S4).

Supplementary References

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