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Antibiotic Resistance and Substrate Utilization by Bacteria Affi liated with Cave Streams at Diff erent Levels of Mammoth Cave

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Introduction

Located in south-central Kentucky, Mammoth Cave is one of the most unique National Parks in the United States. The surface landscape includes complex relationships between the flora and fauna along with human influences. However, the primary ecological focus is concealed below ground. Over four-hundred miles of cave passages, created by flowing groundwater over millions of years, host a variety of macro and micro organisms. The Green River has cut into the limestone formation over geologic time, creating a complex network of passages that are stacked, one below the other, with the newer levels of cave lying near the bottom. Palmer (2007, 1987) describes 4 main levels of cave passages in the Mammoth Cave system. A detailed discussion of the geology and conditions that formed the cave levels can be found in several reports (Palmer, 1987; Palmer 1989; White and White, 1989; Granger, et al, 2001). Precipitation continues to provide water that traverses from the surface, through the unsaturated vadose levels of the cave, and down to the water table in the lower level. Water enters the cave system through direct recharge at sinkholes and through diffuse percolation. The rapid infiltration of stormwater often exceeds the carrying capacity of the upper cave passages and excess water is pushed into void pore-spaces near the top of bedrock. This stored water is slowly released and provides base-flow to cave streams that replenish the pools and streams in the lowest level of the cave (Ryan and Meimen, 1996). These perennial cave streams carry many of the organic compounds that provide energy to the cave ecosystem (Barr, 1976).

During May 2011 to August 2012, the Park endeavored to prevent the spread of *Geomyces destructans* spores by using mats that were saturated with a quaternary ammonia compound (QAC) solution to disinfect the footwear of everyone who entered the cave. QAC residue on the ground near the disinfection stations was visibly evident during prime tourist season. This heavy use of QACs raised concern about the potential for the disinfectant to be carried into the cave via storm runoff. Also, there was concern about QACs in accidental leaks associated with the recreational vehicle wastewater disposal (Diehl and others, 2012). These potential QAC sources were all within the small River Styx watershed boundary. One potential consequence of QAC in the River Styx watershed was that it could lead to selection of QAC-resistance in the microbial community. Previous research found that bacteria resistant to QACs were likely to be resistant to other antibiotics (Chapman, 2003). Accordingly, it was possible that repeated exposure to QACs could also lead to resistance of medical antibiotics. There was additional concern that QACs may inadvertently act as microbial signals (Keller and Surette, 2006)

or disrupt natural biogeochemical cycles (Underwood and others, 2011) at sublethal concentrations.

Microorganisms play an essential role in the health of Mammoth Cave's ecosystem, yet the ecology of microbial communities in the cave streams has been largely neglected (Barton and Northup, 2007). Most of the scientific literature concerning cave microbiology addresses microbes that live on the cave walls and sediments (Rusterholtz and Mallory, 1995; Northup and Lavoie, 2001; Barton, and others, 2007) or speleothems (Palmer 2007). This project begins to address the gap in microbial ecology of cave streams.

This collaborative project between the U.S. Geological Survey, Mammoth Cave National Park, Tennessee State University, and Mammoth Cave Learning Center focused on the microbial communities associated with the perennial cave streams in four levels of Mammoth Cave. The objective of this project was to determine the substrate utilization and dose-response to five antibiotic compounds in the microbial communities of cave streams. Sites selected for the study correspond to perennial water from three different levels of the cave within the River Styx Spring basin. The River Styx basin is a small, 1.2 square mile watershed, located beneath the campgrounds to the Visitors Center. Water samples used for microbial analysis were collected during the summer of 2012. Base-flow samples were used for most of the analysis since that would reflect the ambient condition in the cave streams. It should be noted that Central Kentucky experienced a severe drought during the summer of 2012, which also affected our ability to collect storm samples.

Methods and Materials

Site description

The sampling sites were selected to represent different levels in the cave system (Figure 1). The Post Office parking lot was selected as a surface site for storm sampling. Previous storm sampling had found QACs associated with the RV wastewater disposal system (Diehl and others, 2012). A quantitative dye study in December of 2011 found that it took approximately 75 minutes for the tracer from the parking lot storm filter discharge to cascade down Annette's Dome located in the upper part of cave Level B, and is the beginning of Shaler's Brook (Embry and others, 2012). It took another 20 minutes for the dye to reach Lee's Cistern in lower Level B. The tracer study failed to show that water flowed from Lee's Cistern to Charlotte's Dome (Level C) and Charon's Cascade (Level D). The Devil's Cooling Tub, located near the south-east end of Gratz Avenue, cave level B, was also found to be hydraulically connected to the Post Office runoff. However, it took approximately a month for the tracer released at the Post Office to reach the Devil's Cooling Tub. A stagnant pool mid-way along Gratz Avenue (level B) was selected because it represented a different hydrologic setting from Annette's Dome, Lee's Cistern and Devil's Cooling Tub.

Water samples for microbial analysis were collected approximately every 2 to 3 weeks in the summer of 2012. Grab samples were collected during base flow using clean sterile 250 mL bottles. A first flush storm sampler (Diehl, 2008) was also used at the Post Office filter discharge pipe and in Annette's Dome. The water samples were brought back to the lab in Nashville and stored at 5oC until analysis was done.

Analysis included bacteria plate counts using 2% agar containing 10% strength Tryptic Soy nutrients (10% TSA). Previous work by Byl and others (2002) found karst groundwater bacteria grew better on low strength media than full strength TSA. Known concentrations of the antibiotics QAC, tetracycline, gentamicin, kanomycin, and erythromycin, were mixed into the

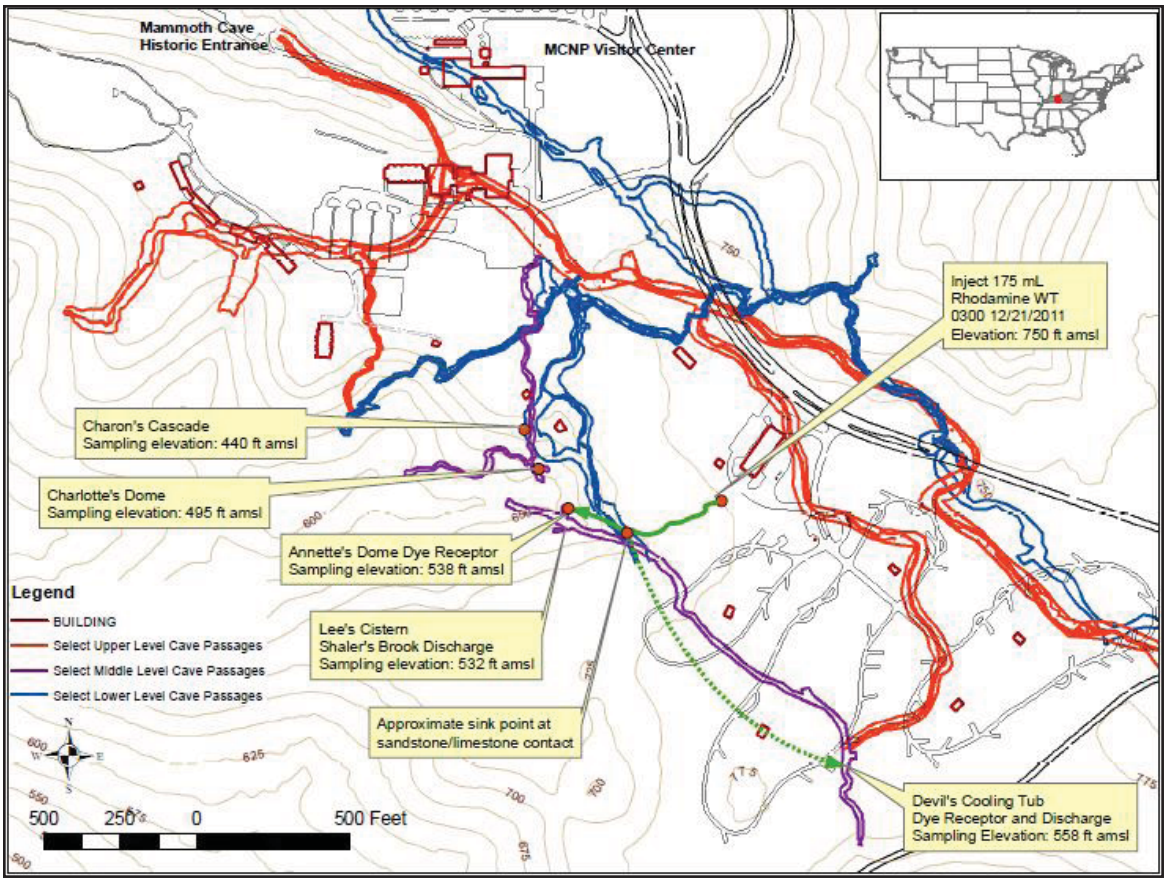

Figure 1: Map depicting sampling sites. Surface water samples were collected at the dye injection point by the Post Office storm filter. Three levels of the cave are represented in this study, levels B (red), C (purple) and D (blue). Flowpath of the Rhodamine-WT tracer is represented in green.

10% TSA just prior to pouring the plates. The cave water samples were hand shaken for a minute to re-suspend the bacteria, and a 10 uL aliquot of raw water was placed on the agar. The cave water containing bacteria was evenly spread over the plate using a sterile bent glass rod. Innoculated plates were inverted and placed in an incubator at 25°C. The bacteria colonies were counted at 1, 2, and 3 days. The results are reported as colony forming units per 10 uL.

The microbial metabolic capabilities were characterized using Biolog's Ecolog™ plates to determine community-level physiological profiles. The plate has 31 different substrates, and three replicates of each substrate (Stefanomicz, 2006). The strength of the bacteria inoculums were normalized by diluting the cave waters with sterile distilled water to a standard turbidity of 1 nephelometric turbidity unit. Standardizing the inoculum, as described in Haack and others (1995), assured that observed differences in community-level physiological profiles were not simply due to differences in bacteria concentration. Readings of the plates were taken at 12, 24, 48, 72, 96, and 120 hours. Analysis included richness, Average Well Color Development (AWCD) (Stefanowicz, 2006), and, Gini coefficient (Harch and others, 1997). Stata™ statistical package was used to calculate the

Gini coefficient. The richness is a measure of how many substrates the bacteria community used. AWCD is an indicator of community metabolic rate. The Gini coefficient is a measure of how evenly the bacteria used the 31 substrates.

Results

The results of this project are split into two subsections, antibiotic resistance and substrate utilization by the microbial communities. Antibiotic resistance evaluations were achieved by running dose-response tests on 10% TSA plates dosed with increasing concentrations of antibiotics. The substrate utilization tests used Ecolog™ plates to quantify community-level physiological profiling by providing 31 potential food substrates for the bacteria communities to consume over a 5 day period.

Antibiotic resistance

The antibiotics used in the antibiotic-resistance tests included QAC, tetracycline, gentamicin, kanamycin, and erythromycin. Bacteria collected from various cave streams and a pool were sensitive to increasing concentrations of QAC (Figure 2a). The QAC effectively inhibited growth of bacteria at concentrations of 0.66 grams QAC per liter and above. Figure 2b shows the microbial growth response to increasing concentrations of the medicinal antibiotic, tetracycline. The bacteria show atypical dose-response curve, with decreasing numbers of colony forming units as the concentration of tetracycline increases. The dose-response pattern was very similar for the antibiotics gentamicin and kanamycin (not shown). Adding the antibiotic erythromycin to the agar media produced a very different dose-response (Figure 2c). Erythromycin actually stimulated the growth of bacteria colonies at doses of 0.01 to 1.0 mg/L. However, 10 mg/L erythromycin inhibited colony formation.

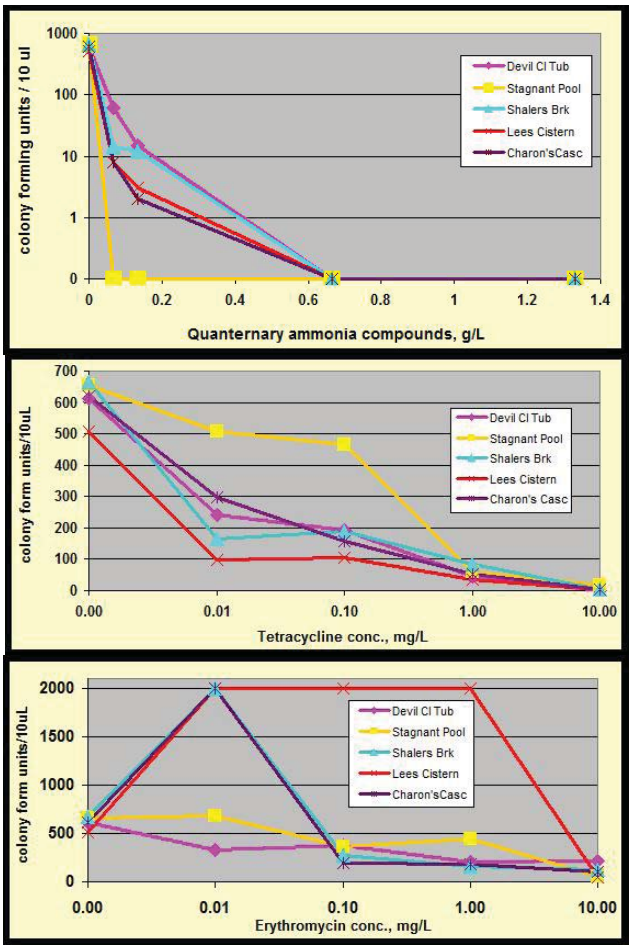

Figure 2a-c (top – bottom): Dose-response of bacteria collected in different waters of Mammoth Cave National Park to QAC, tetracycline, and erythromycin.

The erythromycin results were different from the other antibiotics tested, so a second test was conducted using a new sample collected from Lee's Cistern. This time, there were three replicate plates for each erythromycin concentration to allow a Student T-test, p = 0.05. The results are shown in Figure 3. A similar pattern was observed, where 0.01 to 1 mg/L erythromycin stimulated the number of bacteria colonies. There was a significant decrease in bacteria colonies grown on media with 10 mg/L at 24 and 48 hours. There were significantly more bacteria colonies on media containing 0.1 and 1.0 mg/L erythromycin after 48

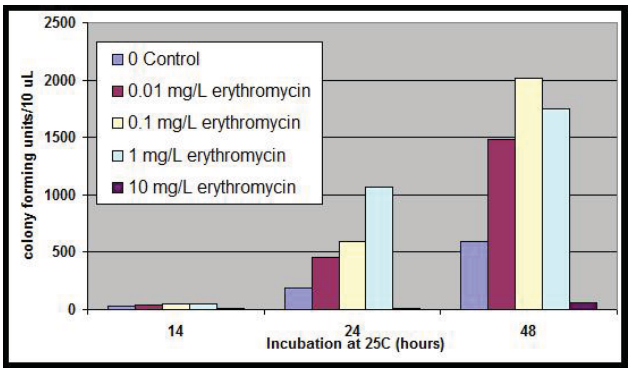

Figure 3: Bacteria from Lee's Cistern were stimulated by low doses of erythromycin. Bars represent average of 3 replicate plates containing increasing concentrations of erythromycin. [* indicates significant difference from controls at p=0.05]

hours. Thus it appears erythromycin has a stimulatory effect on the cave bacteria at low concentrations and a toxic threshold between 1 and 10 mg/L.

Substrate utilization tests

The Biolog™ results from the Post Office storm drain (surface), Shaler's Brook (upper level B), Lee's Cistern (lower level B), Charlotte's Dome (level C), and Charon's Cascade (level D) are provided in this section. The data analyses include substrate diversity (substrate richness), an indicator of metabolic rate (Average Well Color Development), and substrate evenness (Gini coefficient).

The samples collected on the surface and in the upper levels of the cave had the greatest initial substrate richness values (Figure 4). Charlotte's Dome (level C) was an exception and had relatively high richness. However, the rate at which the microbial community from Charlotte's Dome used the substrates was slow (Figure 5). After 72 hours, the richness values for all the sites tested were similar (Figure 4). This indicates there was an initial preference for certain substrates, but given time, the bacteria communities can utilize almost all 31 substrates to some extent.

Substrate richness and AWCD are useful measures of microbial ecology, but do not provide information about how evenly the microbial community utilized the 31 substrates. The Gini coefficient is a statistical measure of evenness in a population, ranging from 0 to 1, with 1 being the most uneven and 0 being perfectly equal use of all the substrates. The Gini coefficient values decreased in the first 72 hours, indicating that substrate utilization became more even (Figure 6). However, the Gini coefficient from samples collected from deeper in the cave (Charlotte's Dome, level C, and Charon's Cascade, level D) leveled off after 72 hours, indicating that a steady state had been reached. After 120 hours incubation, it was evident that the microbial communities from or near the surface used the substrates more evenly than the communities deeper in the cave.

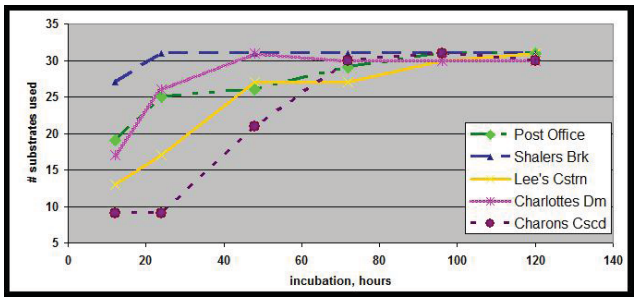

Figure 4: Substrate richness for the different bacteria communities through time.

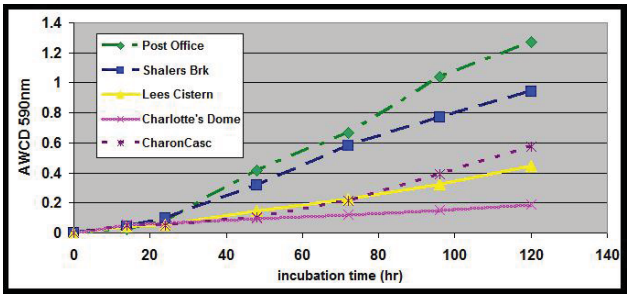

Figure 5: The Average Well Color Development (AWCD) is an indicator of metabolic rates; the steeper the slope, the faster the metabolic rate of the microbial community.

Summary and Conclusion

This project looked at the response of cave microbial communities to five antibiotics and their substrate utilization patterns. The cave bacteria appear to be sensitive to QACs, with a slight resistance at the upper cave levels. The bacteria communities in the cave have varying levels of natural resistance to the other four antibiotics tested. Low doses of erythromycin stimulated bacteria growth. Further studies are needed to determine if erythromycin occurs in this environment to determine if it is a microbial messenger in this environment. Using the Ecolog substrate utilization plates, we found that substrate richness, metabolic rates and evenness of substrate-use tend to decrease in communities deeper in the cave, with some exceptions. There is a shift in microbial physiological capabilities associated with the different levels in the cave system. It should be noted that since the sampling took place during a particularly hard drought, the distinct microbial community patterns described here may vary under different weather or seasonal conditions.

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