

The role of organic matter in the fate and transport of antibiotic resistance, metals, and nutrients in the karst of Northwest Arkansas

ABSTRACT

Isotopes of dissolved inorganic carbon ($\delta^{13}\text{C-DIC}$), nitrate ($\delta^{15}\text{N-NO}_3$), and dissolved oxygen were used to assess microbial responses to increasing concentrations of acetate, a labile form of dissolved organic carbon (DOC). Acetate is a product of fermentation, which occurs in anaerobic lagoons used for storing concentrated animal feeding operation (CAFO) waste effluent. The waste effluent is a mixture of water, urine, and manure, and is stored for future use as a liquid fertilizer. In the epikarst of Northern Arkansas in the Buffalo River watershed it was hypothesized that additional input of labile DOC derived from waste slurry fertilizers would increase the activity of microorganisms responsible for transforming DOC into DIC. Additionally, microbial processes coupled to DOC-DIC conversion, such as denitrification, would also be positively impacted resulting in more nitrate removal. However, the influx of DOC potentially exposes the epikarst environment to the flux of metals, antibiotics, and other contaminants of both anthropogenic and environmental origins. Toxic metal concentrations, organometallic complexes were hypothesized to be temporarily detrimental to DOC conversion to DIC and NO_3 removal, but as an expression of resistance response, resistant bacteria species to macrolide antibiotics and tetracycline antibiotics would remain present and active while exposed to toxic metals. DOC to DIC conversion was quantified using laboratory microcosms in which isotopic fractionation trends and changes in DIC and NO_3 concentrations were measured to indicate microbial respiration and denitrification activity. The laboratory microcosms were conducted to identify the effect of nutrient and metal species and concentrations on microbial activity and microbial biomass fatty acid methyl ester (FAME) composition. Field studies were used to calibrate the conditions in the laboratory such that they were a best representation of natural environmental conditions. Overall, microbial activity and biomass production was

greatest when DOC was prevalent and NO_3 concentrations were approximately 10 times less than DOC concentration. Under these conditions respiration and denitrification occurred, producing biologically derived DIC and reducing nitrate concentration. Denitrification and respiration activity ceased upon addition of metals, but respiration activity resumed in microcosms with metal concentrations below $10 \mu\text{g/L}$. Biomarkers from FAME analysis gave clear indication of the presence of gram-negative bacteria in biomass samples collected from the spring orifice and microcosms treated with metals; however, biomass collected from microcosms amended with nutrients and DOC displayed indications of predominantly gram-positive bacteria. This finding was critical because it provided evidence that bacteria species transported in spring discharge were resistant to low levels of tetracycline and to a greater extent erythromycin, but also displayed a tolerance for metals. Conceptually, DOC can be an active transport vector of metals, antibiotics, and nutrients; however seasonal variations and periods during fertilizer application will result in seasonal variation in the presence, activity, and community structure of the epikarst microbial population. The evolution of water chemistry in the epikarst will also be affected by the seasonality of DOC, nutrient, and metal fluxes into subsurface flow paths resulting in the transport of more co-selected metal and antibiotic resistant bacteria.

ACKNOWLEDGEMENTS

The path to obtaining this degree has been long, but has been full of emotional high-points and emotional low-points. Throughout my career as a student I've always been able to rely on the support of family and a nucleus of friends to provide the motivation to keep striving to achieve the goals I set before myself. First to my mentors and committee members – Dr. Phillip D. Hays, director of my dissertation committee and a very insightful mentor with the perfect demeanor to inspire thoughtful reflection when the perils of working on this degree personally were getting the best of me, to Dr. Van Brahana for behinding

DEDICATION

This dissertation is dedicated to my wife, Ashley, to my children, Victor and Victoria, to my parents, grandparents, and everyone who has had a stake in my education and or rearing. The community at large has had a stake in my life, and is now a shareholder in the dividends of this achievement.

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CHAPTER 1: INTRODUCTION

Concentrated Animal-Feeding Operations (CAFOs) pose a potential threat to groundwater and surface-water quality. Pharmaceutical, heavy metal, nutrient, and biological constituents entering groundwater, surface-water, and soil environments may present water-quality issues. Elevated nutrients may lead to eutrophic conditions, fish kills, and increased mortality among macroinvertebrates inhabiting or foraging in surface-water and groundwater ecosystems.

Nitrogen contamination poses human health risk through infant methemoglobinemia (blue baby syndrome: Comly, 1945; Addiscott, 2005) and digestive track cancers (Forman et al., 1985; National Academy of Sciences, 1981); however, this issue is of great debate (Powlson et al., 2008). Karst groundwater flow systems provide habitats for specially adapted species of cavefish, cave crawfish, and many unique macro-invertebrates that have evolved to the unique environment of karst (Graening & Brown, 2003). High concentrations of dissolved organic matter, nutrients, trace elements, pharmaceuticals, and pathogens are detrimental to specialized cave species, and provide a source of contamination to groundwater, drinking-water wells, and in some cases surface-water .

This dissertation assessed the role of carbon and nutrient cycling in the nature of antibiotic resistance in karst groundwater. The study will use a multiple parameter approach targeting biofilms in karst flowpaths as reservoirs of antibiotic resistance and as active mechanisms in the cycling of carbon and nutrients.

Groundwater and surface-water quality and CAFOs

Over the last three decades the mechanization of agricultural industries has led to unprecedented growth due to increased operational efficacy with particular emphasis placed on meat production. Between 1950 and 2010 pork production in the United States increased more than

580% (EPI, 2012a). According to the Earth Policy Institute, since 1908 meat consumption in the United States has increased drastically to from 9.8 billion pounds annually, to approximately 52.2 billion pounds in 2012 (2012b). The emergence of CAFOs has been critical to the growth of meat production and meeting increasing public demand; however, the negative impacts of large CAFOs has also been the topic of passionate debate in social, political and academic forums. The history of CAFOs in the United States has been marked with degraded water-quality (Field, 2012, Funkhouser et al. 1999, Hobza et al. 2005, Jarvie et al., 2013, Lerch, 2011, Marshall et al. 1998, Panno, 2006, Quinlan, 1989, Varnel & Brahana, 2003). CAFO associated water quality problems include, but is not limited to organic matter (OM), nutrients (e.g. nitrate, ammonia, phosphate), bacterial contamination, veterinary pharmaceuticals, and heavy metals. Leaking lagoons, large episodic rain events, human error, and the lack of appropriate oversight often lead to dangerous environmental outfall with serious implications for the environment and human health. Regions with karst topography are particularly vulnerable to contamination from CAFOs due to extensive surface and groundwater exchange via dissolution features and fracturing. Bacterial contamination of karst is well documented in literature. Butscher et al. (2011) described the breakthrough of fecal bacteria in karst springs using a numerical modeling approach to verify the vulnerability of karst to bacterial contamination. Because of heterogeneity and associated inherent variability of karst hydrology, identifying sources of bacterial contamination is difficult. Chemical, biological, and genetic methods have been used to identify sources of biological contamination in karst. Weidhass et al. (2013) used genetic markers to identify fecal bacteria contamination from derived from poultry litter applied to fields, and concluded that a multi-parameter approach to identifying fecal bacteria contamination was a more robust method than common single-parameter methods such as monthly or quarterly

biological testing of surface-water and groundwater. Seasonality also affects chemical and biological water-quality in streams due to precipitation frequency and spatial distribution, type and intensity, contribution of groundwater, anthropogenic activity, and annual changes in land-cover. A study conducted in the Ozark Highlands found bacterial constituents, namely fecal coliform and *E. coli* in coves and open-water areas in Lake of the Ozarks, Missouri fluctuated with seasonal hydrology (O’Hearn, 2009). Organic matter (OM) loading is an important component of water chemistry with seasonally varying concentrations. Understanding the seasonal variation of OM loading in groundwater and surface-water is key because OM has broad implications for the fate and transport of other solutes such as endocrine-disrupting compounds (Yamamoto et al., 2003) and metals (Seiler and Berendonk et al., 2012). The proposed study is particularly focused on determining the impact of DOM concentration and species on the cycling of carbon and nutrients in biofilms and characterizing the role of DOM as a transport mechanism for metals and antibiotic compounds. Formation of organo-metallic complexes and adsorption of metals by particulate organic matter occur commonly in aquatic and soil environments. The species of organic matter also impacts the transport of antibiotic compounds and metals. The broader implications of these for the activity in biofilms suggests that biofilm activity and composition will change in response to changes in the OM carried in groundwater and surface water.

Antibiotics: Resistance, Fate, and Transport

Antibiotics are released and transported in the environment by human and animal excretion, urban runoff, agricultural runoff, natural fungal and microbial secretions, leaching from agricultural fields, and leaking from agricultural waste lagoons, septic systems, and in discharge from wastewater treatment plants (Katz et al., 2011; Nikolaou et al., 2007; Arikan et al., 2008; Lin et al., 2008; Pal et al., 2010; Poynton and Vulpe, 2009; Stuart et al., 2012; Rizzo et al.,

2013). Studies have detected that as much as 80%-90% of parent antibiotic compounds are excreted from animals treated with antibiotics (Bound and Voulvoulis, 2004; Kümmerer, 2009; Yan et al., 2013). Waste is then moved to lagoons where several antibiotics are commonly detected at much higher levels than detected in adjacent groundwater and surface water. Several studies have detected antibiotics in surface and groundwater associated with wastewater and runoff from agricultural feedlots (Nikolaou et al., 2007; Ternes and Hirsch, 2000; Watkinson et al., 2009). Veterinarian antibiotics lincomycin, ractopamine, sulfamethazine, sulfathiazole, erythromycin, tiamulin and sulfadimethoxine were detected in wastewater from concentrated-animal feeding operations (CAFOs) (Watanabe et al. 2010; Watanabe et al. 2008; Bartelt-Hunt et al. 2011). Antibiotics and other emerging contaminants generally are thought to occur more frequently and in greater concentrations in surface-water than in groundwater because of direct inputs from waste sources, limited attenuation and dilution capacity, and short residence times (Barnes et al., 2008; Lapworth et al. 2012), and significant concentrations of anthropogenic contamination are thought to occur in groundwater due to preferential flow paths rather than by diffuse downward migration that lends itself to more attenuation processes (Lapworth et al., 2012).

Soils have been observed to be a key environmental reservoir for antibiotics and metabolites, particularly agricultural soils. The application of solid manure or manure slurries introduces ARB and ARGs to top soils, which may potentially impact terrestrial organisms directly (Figure 1). Antibiotic concentrations within soil profiles vary depending on a number of factors, but two major controls on soil antibiotic concentrations are the chemical characteristics of the antibiotic of interest and the textural and compositional characteristics of the soil. In the soil environment many antibiotics are readily sorbed to fine clay and silt particles in the soil matrix (Thiele-Bruhn

et al., 2004; Heuer et al., 2008). Adsorption of tetracycline and sulfonamide class antibiotics has been reported three times greater in loamy soils than in sandy soils (Bruhn and Beck, 2005). Residual antibiotics and metabolites accumulate in soils leading to increasing antibiotic concentrations with depth and subsequent leaching into groundwater (Hamscher et al., 2002). Water leaching from the soil profile also carries dissolved and particulate organic matter species that may be integral in the transport of antibiotic compounds and their metabolites, sorbed metals, bacteria, and various other dissolved components. Bacteria transported and growing in receiving groundwater are then selected with pressures evolving from the characteristics of soil-water, surface-water, and in-situ groundwater. Microbial biofilms, which act as homes to diverse communities of bacteria, should provide a good test subject to observe changes in response to evolving water-quality in karst.

Biofilms, nutrient cycling, and antibiotic and metal fate

Biofilms are diverse, living communities of bacteria attached to a solid surface. The commonly accepted theory on the morphology of biofilms is represented in (Figure 2). Free bacteria in the water settle and attach to a surface, grow and excrete extracellular polymeric substance (EPS). This substance gives biofilms their slimy consistency, but provides protection and nutritional advantages for the bacterial communities living within the biofilm. In biofilms individual bacteria cells are protected from predatory organism such as protozoa, able to exchange genetic material with nearby bacterial cells allowing selection and propagation of more desirable traits relative to growth environment conditions, and exchange nutrients with neighboring bacteria cells. Water chemistry is a control on the microbial composition of biofilms. Of particular interest to the researchers of this study are the effects of antibiotic species and concentration effects of antibiotics and metals on the compositions of biofilms and activity of bacteria

populations. Resistance mechanisms include limited diffusion of antibiotics across through biofilms, enzyme-mediated resistance, genetic adaptation, efflux pumps, changing levels of metabolic activity within the biofilm, and cellular and polymer interactions with antibiotic agents within the biofilm matrix (Cloete et al., 2003). Antibiotic resistant genes coding for efflux pump phenotypes are commonly studied resistance mechanisms responsible for providing multiple resistances because their natural physiological function is to extrude toxins from inside bacterial cells (Nies 2003; Alonso et al., 2001; Fernandes et al., 2003). Efflux pumps are compound specific, but they are capable of extruding a range of intracellular toxins. Because efflux pumps can display characteristics of cross-selection or co-selection, microbial resistance activity in contaminated environments (e.g. metals, organic solvents) can be expected to be similar to an antibiotic resistance response in the absence of antibiotics (Alonso et al., 2001). Aquatic environments have been referred to as reservoirs of antibiotic resistance because of the diverse occurrence of antibiotics, antibiotic resistance genes, metals, and other compounds and environmental factors that select for bacteria that have traits that favor their survival.

Groundwater systems receive inputs from both soil and surface environments, so we believe that the composition of biofilms will reflect the diversity of inputs into the system; however, stress from environmental conditions (e.g. less organic matter, fewer nutrients, and toxic levels of antibiotics or metals) should cause changes in the communities of biofilms as observed in biofilms. We hope to contribute data relating to changes in nitrate removal, depending on metal exposure in karst environments with this study. When a biofilm reaches maturity, sloughing occurs, detaching mature portions of the biofilm from the attached younger portions of the biofilm. Sloughing is potentially another transport mechanism not only for microbial communities but also antibiotic resistance genes and substrate for downstream bacteria, but its

contribution to the fate and transport of antibiotic resistance will not be addressed in this study. Aside from preserving the genome of native cave bacteria, biofilms are crucial in the cycling of carbon and nitrogen in groundwater systems.

The vulnerability of karst environments to anthropogenic contamination is well understood. In particular, several detailed studies have been conducted describing the fate and transport of nitrogen in karst flow paths. Similarly detailed studies phosphorus and organic carbon are not documented in the literature (Brown et al. 2008). Nitrogen has been at the center of much scientific literature because of the widespread use of organic and synthetic N fertilizers in agricultural practices. The fate of nitrogen in the environment follows four major pathways; (1) plant uptake, (2) denitrification by bacteria, (3) leaching into groundwater, and (4) assimilation by various organisms (Kendall, 1998). Groundwater associated with agricultural land-use has been shown to have N contamination mainly as NO_3^- (Böhlke, 2002). Several studies have shown that denitrification is the most important process for nitrate attenuation in karst and unconsolidated aquifers (Böhlke, 2002; Einsiedl et al., 2005; Panno et al., 2001; Peterson et al., 2002). Aerobic assimilatory denitrification is a process largely carried out by attached bacteria and limited by the concentration of nitrate in groundwater to approximately 10 mg/L (Bengtsson & Annadotter, 1989). In the Bengtsson & Annadotter experiment, they found that assimilatory nitrate reduction accounted for the removal of 80% - 90% of the nitrate, which was an unexpected result due to the largely accepted belief that denitrification was a largely anaerobic process (1989). A more recent study, Tekaya et al., (2005) describe two strains of aerobic denitrifiers, both of which are capable of removing nitrate while produce relatively small amounts of nitrous oxide, a byproduct of aerobic denitrification and potent greenhouse gas. Tekaya et al.,(2005) also utilized the ^{15}N enrichment approach to quantify gas production

associated with the aerobic denitrification. Studies addressing aerobic denitrification in granular-media aquifers and in wastewater treatment plants outnumber those in karst environments. In this study, nitrogen isotopes will be used to identify biologically mediated process as discussed above as they occur in biofilms.

Use of isotopes to identify biochemical processes has been studied extensively. Residual nitrogen from biological processing will have a unique isotopic composition because of fractionation. However, isotope data alone are not sufficient to determine biological processing. The combination of isotope data, concentration data, and redox data allows a better-informed determination of pertinent biological processes. Nitrogen enrichment studies have been vital in furthering our understanding of the nitrogen cycle in the environment. Tobias et al. (2001) used N enrichment studies to identify the fate of $^{15}\text{NO}_3$ originating from groundwater into a marshy wetland, and concluded that denitrification accounted for the largest loss of $^{15}\text{NO}_3$ followed by long-term immobilization of the nitrate in organic form. The identification of anaerobic ammonia oxidation in anaerobic groundwater by anammox bacteria was observed by the enrichment of $^{15}\text{NH}_4$ and $^{15}\text{NO}_3$ as both contaminants were rapidly degraded along the subsurface flowpath (Clark et al., 2008).

The objective of this research is to document the control of water-quality on microbial biofilm development in a karst environment. Biofilms are thought/hypothesized to play a key role in the cycling of carbon and nutrients in karst environments. More specifically, this research seeks to document the effects of nutrient and organic carbon loading on microbial biofilm growth, nutrient assimilation, and metabolic response to environmental stressors in karst environments using a series of indicators.

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CHAPTER 2: EPIKARST LABILE ORGANIC CARBON TRANSFORMATION AND DENITRIFICATION ACTIVITY IN AN EPIKARST SPRING WATER SAMPLE FROM NORTHERN ARKANSAS, UNITED STATES

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ABSTRACT: Laboratory microcosms were used to assess the effect of labile dissolved organic carbon amendments on microbial activity and nitrate processing. Water and gravel collected from an epikarst spring were amended with phosphate, $\delta^{15}\text{N}$ labeled-nitrate, and $\delta^{13}\text{C}$ labeled-acetate and stored in a dark environment for 13 weeks. Weekly samples collected from the microcosms were analyzed for dissolved inorganic carbon (DIC), dissolved oxygen (O), $\delta^{15}\text{N}$ - NO_3 , and $\delta^{13}\text{C}$ -DIC. Microbial activity decreased rapidly after 3 weeks in all microcosm treatments as observed in $\delta^{13}\text{C}$ -DIC and dissolved oxygen data. The longest sustained microbial activity occurred in microcosms amended with 100 mg/L $\delta^{13}\text{C}$ -acetate. The rate of biological inorganic carbon production in microcosms amended with less than 10 mg/L of acetate was statistically indistinguishable. Treatments of phosphate and nitrate had no statistically significant ($p < 0.05$) effect on biological inorganic carbon production with the only exception given to microcosms with 10 mg/L of both nitrate and phosphate and 100 mg/L acetate. Denitrification along with other processes was observed in the results of $\delta^{15}\text{N}$ analysis as nitrate removal processes. This study is evidence that bacteria living in epikarst are capable of attenuating increasing loads of organic matter, and that nitrate processing changes with water chemistry. As concentrations of labile organic carbon increase the demand for dissolved oxygen increases and production of inorganic carbon, namely CO_2 increases. Substantial quantities of biomass also followed increasing acetate and nutrient concentrations. Increasing biomass is a response and factor in the evolution of surface-water and groundwater chemistry, increased biological oxygen demand, and may have major implications for increasing concentrations of bacteria communities with resistance to multiple antibiotics and metals.

INTRODUCTION

Concentrated animal feeding operations (CAFOs) are sources of organic matter (OM), nutrients, bacteria, and other potentially dangerous products (Wantanabe et al., 2010; Ko et al., 2008; Jarvie et al., 2013; Varnel & Brahana, 2003). Increasing concentrations of OM can have potentially negative ecological and biogeochemical effects, leading to degraded water-quality. Moreover, OM has also been linked to the transport of endocrine disrupting compounds (Yamamoto et al., 2003) and metals (Seiler and Berendonk et al., 2012). In agriculture, manure slurries derived from waste from stock animals, are sold as fertilizers because they are rich in

both nutrients and OM. This practice achieves three major benefits for CAFOs; effective waste management plan, economically profitable, and provides forage for grazing livestock. However, over fertilization, pathogens, veterinary pharmaceuticals, and trace metals are contamination risks for surface and groundwater in the cases of manure slurry retention and manure slurry fertilizer application. CAFO waste effluent in ponds or application to fields poses a threat to groundwater and surface-water in Northern Arkansas. The geologic setting of the region may be characterized as highly fractured and highly weathered, which leads to widespread groundwater and surface water interaction. Due complexity and robust nature of CAFO waste effluent, biological treatment is often preferred over chemical and physical treatment processes because it is as a more cost effective method of managing waste because it occurs naturally.

Manure slurries contain several species of OM in both dissolved and particulate forms. The largest fraction of dissolved organic carbon (DOC) in swine manure slurries are volatile fatty acids. Volatile fatty acids in manure slurries occur as a product of anaerobic microbial degradation of manure e.g. fermentation during storage of liquid manure in storage lagoons (Cooper & Cornforth, 1978; Moore & Holdeman, 1986). Volatile fatty acids account for approximately 80% of DOC in swine manure slurries, and approximately 64% of the DOC pool in swine manure slurries are acetic and propionic acids (Paul & Beauchamp, 1989). The easily degradable nature of acetate makes it a premier energy source from manure slurries for many microbial communities in the soil and epikarst environments and also has implications for other subsurface biogeochemical processes, namely nitrate removal.

Nitrate has been a key contaminant associated with many agricultural installations. Nitrate is readily soluble in water, and easily transported. Chronic nitrate exposure may lead to eutrophication in surface water, gastrointestinal cancers, and methaemoglobinaemia or so-called

“blue baby syndrome” in small children. Recently, the World Health Organization (WHO) released a study citing high levels of nitrate compounds leading to increased cancer risk associated with processed foods. Nitrate is an essential nutrient for plant growth and thus it is used in many fertilizers. Nitrate also occurs as a by-product of microbial ammonia reduction, and in human and animal waste. In the environment nitrate is leached into groundwater, and transported via over-land runoff to surface-waters. Plant uptake, dilution, and microbial oxidation of nitrate are the primary nitrate removal mechanisms in the environment.

Subsurface environments harbor diverse microbiota capable of oxidizing OM to carbon dioxide (CO₂) and in many cases OM oxidation is coupled with other microbial processes such as denitrification, sulfate reduction, or iron reduction. Lovely et al. (1990) describe bacteria in Late-Cretaceous sediments capable of enzymatically oxidizing acetate while at the same time reducing ferric iron. Acetate is also highly regarded as an important electron donor for sulfate reducing bacteria in marine environments (Parkes et al., 1989). Most relevant to this study is the coupled process of acetate oxidation and denitrification.

When favorable conditions are present – anaerobic and pH neutral, denitrification takes place, removing dissolved nitrate from the water column as well as dissolved organic carbon (DOC). Concurrent oxidation of DOC e.g. acetate, increases biological oxygen demand during biological respiration, which provides the metabolic energy and anaerobic conditions necessary for denitrification. The input of residual OM from the soil zone into shallow groundwater in epikarst zones is advantageous because it provides necessary substrate to an environment that is temporally and seasonally energy deprived; however, it may present problems for the chemical and biological quality of shallow groundwater because of the threat of shifting ecology, potential rapid transport of residual nutrients, OM, veterinary pharmaceuticals, and other compounds

mobilized in the soil, and the potential eutrophication of surface-water interacting with groundwater.

This study focuses on the process of denitrification to observe the effects of OC on microbial nitrate removal and biomass production in an epikarst-spring water sample. This study is part of a larger study aimed to assess the role of OM in the transport and fate of antibiotics and antibiotic resistance in karst groundwater. Springs discharging from epikarst are particularly vulnerable because of preferential pathways that connect groundwater and surface water, which allows rapid transport of contaminants. Spring discharge is also important because spring-water represents an integration of water-quality across an entire recharge area with various land-cover and land-uses impacting water-quality. The objectives of the project were; (1) to model changes in microbial metabolic activity based on DOC concentration using laboratory microcosm studies, (2) to model the effect of DOC concentration on nitrate removal, (3) to quantify changes in biomass production under different DOC and nutrient conditions.

METHODOLOGY

Site Description

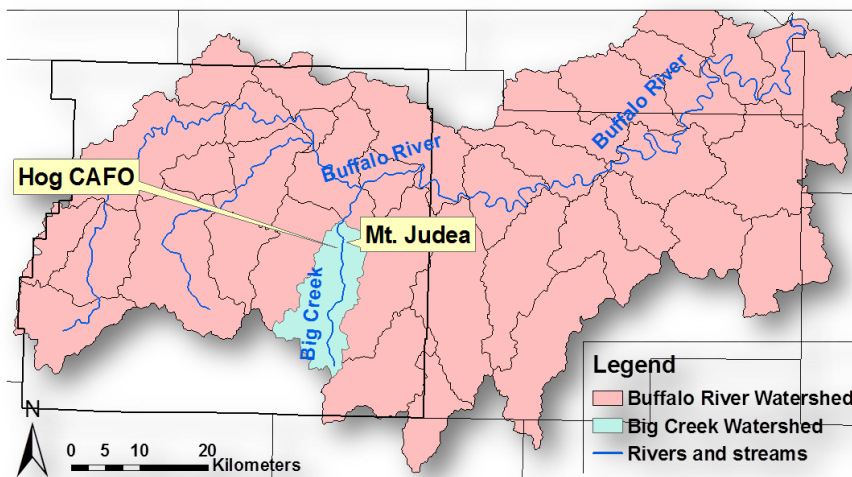


Figure 1 Buffalo River Watershed (pink) Big Creek Watershed (aqua)

The spring sampled in this study is an epikarst spring discharging groundwater from a perched limestone aquifer approximately 2 miles south of a swine CAFO. The spring is found in the Mississippian Boone Formation which consists largely of fossiliferous limestone with interbedded chert, as much as 70% in some areas. The spring is located within the Big Creek watershed, which is a major sub-watershed within the larger Buffalo River watershed (Figure 1). During baseflow spring discharge is approximately 1 cfs, but can increase more than 10 cfs post storm event.

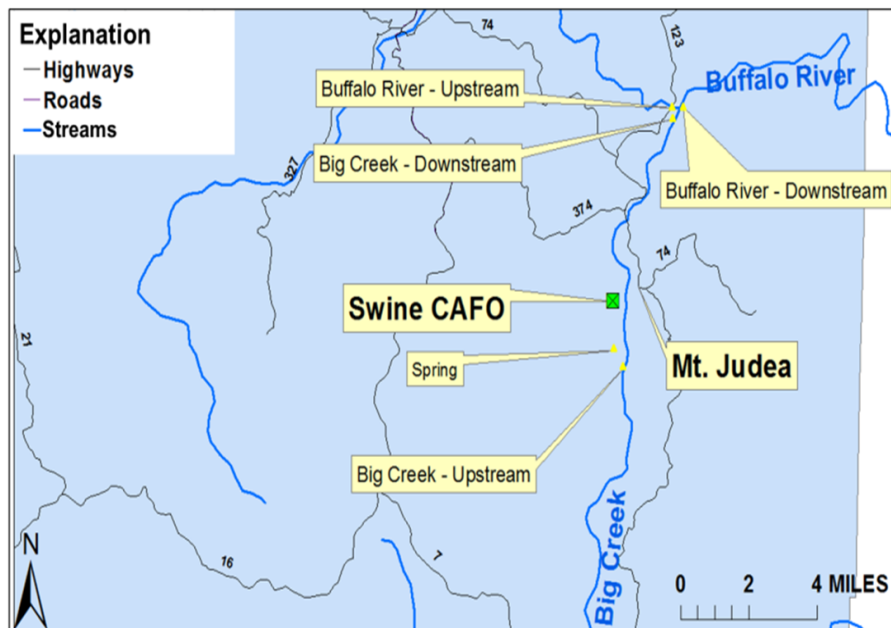


Figure 2 Sampling locations and location of CAFO

Land cover in the recharge area of the spring consists of agricultural pastures and forested areas. There are three large fields in the recharge area of the spring, two of which are sprayed with manure slurry from the CAFO and a third that receives no fertilizers from the CAFO. Temperature, pH, specific conductivity, and dissolved oxygen measurements were taken at the time of sampling. Water quality samples were collected in Nalgene or Teflon sample bottles at the locations denoted in Figure 2. Samples analyzed for total nitrogen and total phosphorus were

filtered and acidified using 0.2 % sulfuric acid. All samples were stored on ice during transit to the laboratory. Samples were stored at 4 °C before analysis. Total Phosphorus and total nitrogen were simultaneously analyzed using alkaline persulfate digestion (APHA, 4500-Pj). Sulfate analysis was conducted using barium sulfate turbidimetric method (USEPA 375.4). The method for the analysis of ammonia was conducted using the salicylate-hypochlorite method adapted from Reardon and others (1966). Biological water quality samples were collected in Teflon sample bottles and transported to the laboratory. The heterotrophic plate count method was modified to determine the concentration of live heterotrophic bacteria cells in water samples (APHA, 9215). Biological water samples were shaken before 10 µL aliquots were used to inoculate a 10% strength Trypticase Soy Agar media. Samples were allowed to incubate at 35°C for 48 hours.

Laboratory microcosm

Laboratory microcosms were conducted in a dark environment at 12 °C for 13 weeks to simulate and conditions in epikarst. Gravel was muffled to sterilized and eliminate in-situ organic carbon. Approximately 185 grams of gravel was added to 1.0 L mason jars and the jars were filled with water collected from the spring. Microcosms were run with three concentrations of sodium acetate ($C_2H_3NaO_2$); 1.0 mg/L, 10.0 mg/L, and 100 mg/L. Acetate was chosen as an organic carbon source because it is easily metabolized by bacteria, and is a major constituent in manure slurries commonly applied to pastures as fertilizer.. The microcosms also received three different nutrient treatments; potassium nitrate (KNO_3), sodium phosphate ($NaPO_4$), and nitrogen and phosphate together at 0.1 mg/L, 1.0 mg/L and 10 mg/L. Nutrient concentration ranges were determined based on historical phosphorus and nitrogen observations at the spring. Labeled ^{15}N -nitrate ($K^{15}NO_3$) and labeled- ^{13}C -acetate ($^{13}C_2H_3NaO_2$) were used to enrich the isotopic compositions of nitrate and dissolved organic carbon in the microcosms to 1000‰, respectively.

The microcosms were sampled weeks 1 – 3 and at week 11. Phosphoric acid conversion of DIC to CO₂ was used to measure $\delta^{13}\text{C}$ –DIC. Conversion of available nitrate to N₂O gas by the bacteria *Pseudomonad aereofaciens* was used to measure $\delta^{15}\text{N}$ -nitrate. Nitrate isotope values were measured relative to ambient air, and $\delta^{13}\text{C}$ –DIC were reported versus the Vienna Pee Dee Belemnite (VPDB) standard. Dissolved oxygen was also measured using the Winkler titration method.

Statistical Methods

Descriptive statistics were calculated for the data collected from field samples and laboratory microcosm studies using R Statistical package release 3.1.3. Two-way ANOVA was used to determine the statistical significance of variance between treatments of nitrate, phosphorus, and DOC. TUKEY HSD was used to quantify significant differences in mean values between treatment groups.

RESULTS

Field pH ranged from 6.38 to 9.64 standard pH units at all sites, Table 1. The mean surface-water pH was 8.23 ± 0.92 and had a range from 7.32 to 9.64. The mean groundwater pH of spring discharge was 7.07 ± 1.14 and had pH measurements ranging from 6.38 to 8.38. Water temperature at the spring during summer sampling was on average $17.73 \pm 0.25^\circ\text{C}$, and $13 \pm 2.45^\circ\text{C}$ in the winter. Mean specific conductance of the spring discharge was $413 \pm 19.7^\circ\text{C}$. Dissolved oxygen at the spring remained above anaerobic levels year round. The concentration of dissolved oxygen was lowest at the spring; however, water at all sites was aerobic.

Sampling Location	Sampling Date	Temperature (°C)	pH	Specific Conductance (µS/cm)	DO (mg/L)	Total Nitrogen (mg/L)	Total Phosphorus (mg/L)	NH ₃ -N (mg/L)	SO ₄ (mg/L)
Buffalo River (Upstream)	7/17/2014	25.9	7.32	217.3	7.00	0.18	<0.02	0.01	12.1
	1/30/2015	8.5	7.92	213	NA	0.21	0.02	NA	NA
Buffalo River (Downstream)	7/14/2014	26.6	7.44	225	8.01	0.22	1.77	<0.002	12
	1/30/2015	7.1	8.59	216.4	NA	0.22	<0.02	NA	NA
Big Creek (Upstream)	7/17/2014	19	7.51	149.7	9.89	0.16	0.14	<0.002	11.9
	1/30/2015	10.2	9.64	131.5	NA	0.07	0.02	NA	NA
Big Creek (Downstream)	7/14/2014	24.02	7.9	273.3	8.43	0.23	<0.02	0.01	12.8
	1/30/2015	7.7	9.49	236.8	NA	0.23	0.02	NA	NA
Spring	7/17/2014	17.55	6.45	407.1	6.65	2.52	0.04	0.05	11.7
	8/12/2014	17.9	6.38	435	6.38	NA	<0.02	NA	NA
	1/30/2015	13	8.38	397	NA	3.24	0.02	NA	NA

Table 1 Field parameters (measured at time of sampling), and measured water quality parameters. NA – constituent not measured, Total Phosphorus and total nitrogen MDL<0.02, Ammonia-nitrogen MDL<0.002 mg/L

Biological water-quality is presented in Figure 3. Mean heterotrophic bacteria counts were greatest upstream on Big Creek ($p<0.001$). Samples collected from the spring had the second greatest concentration of bacteria, 334 ± 73 cfu/10µL. There was no statistically significant difference in bacteria concentrations at upstream and downstream sites on the Buffalo River.

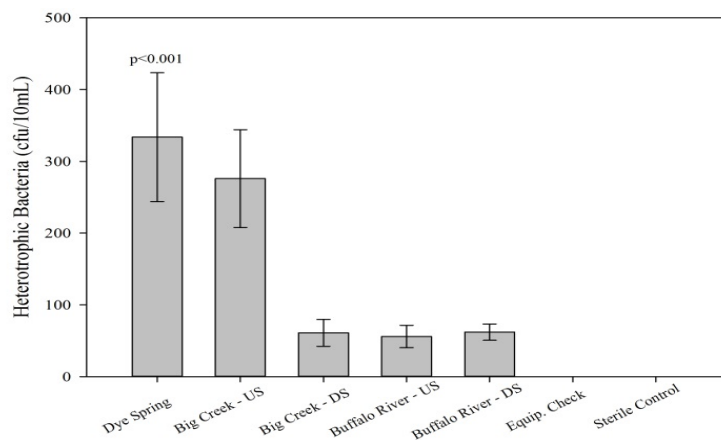


Figure 3 Heterotrophic Bacteria Concentration in biological water quality samples, shaded bar represents mean concentration, and error bars represent the \pm standard deviation

Laboratory microcosms study

In laboratory microcosms the concentration of DO was significantly impacted by the concentration of DOC in microcosms ($p=1.72 \times 10^{-8}$) (Figure 6). Individual treatments of nitrate, phosphate, or a combination of nitrate and phosphate had no significant effect on DO concentration ($p=0.946$) (Figure 5). Because DO concentrations were not found to be significantly impacted by the concentration of nitrate and/or phosphate, the mean DO concentration for each DOC level was used to create Figure 4. Microcosms with 1.0 mg/L DOC mean DO concentration was 0.78 mg/L greater than microcosms with 10 mg/L ($p=0.21$) and 2.9 mg/L greater than microcosms with 100 mg/L DOC ($p<0.05$) (Figure 6). Concentrations of DO were significantly greater in microcosms with 10 mg/L DOC than in microcosms with 100 mg/L by 2.1 mg/L ($p<0.05$). At time zero in the beginning of the experiment the average DO concentration in all microcosms was 8 mg/L (Figure 4). After week one DO concentrations were 5.4 mg/L, 2.8 mg/L, and 1.7 mg/L in 1.0 mg/L DOC, 10 mg/L DOC, and 100 mg/L DOC microcosms, respectively. At week 2, DO concentrations in all microcosms increased. Microcosms containing 1.0 mg/L DOC experienced a DO concentration increase to 5.7 mg/L. Microcosms with 10 mg/L DOC experienced the most drastic increase in DO concentration to 5.3 mg/L, and 100 mg/L DOC microcosms increased to 2.0 mg/L DO. Dissolved oxygen concentration increased throughout the remainder of the experiment in microcosms containing 1.0 mg/L and 10.0 mg/L; however, microcosms containing 100 mg/L decreased in week 3 to 1.7 mg/L and increased in the final sample to 4.0 mg/L DO. Week 11 DO concentrations in 1.0 mg/L DOC and 10.0 mg/L DOC were nearly identical with 6.4 mg/L DO and 6.5 mg/L DO, respectively.

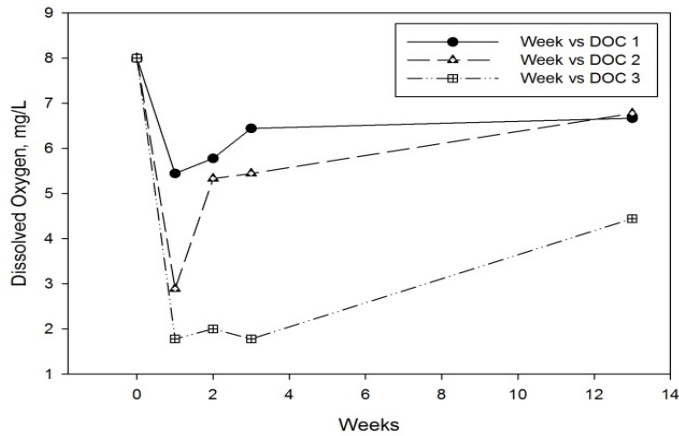


Figure 4 Results of dissolved Oxygen Analysis,

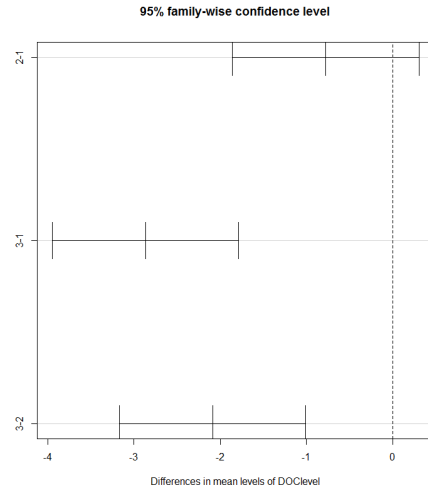


Figure 5 Difference in mean dissolved oxygen concentrations by DOC treatment

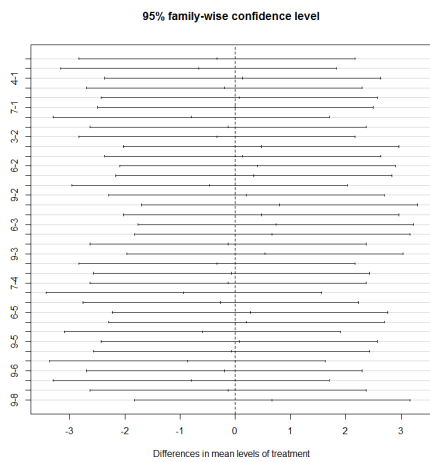


Figure 6 Difference in mean dissolved oxygen concentrations by nitrate and phosphate treatment

Results of isotopic analysis of ^{13}C -DIC indicated that the isotopic composition of DIC became significantly more enriched in the heavier isotopes in microcosms containing 100 mg/L DOC than in microcosms containing 10.0 mg/L DOC ($p < 0.05$) or 1.0 mg/L DOC ($p < 0.05$) (Figure 7). Microcosms containing 10.0 mg/L DOC was also significantly ($p = 7 \times 10^{-7}$) more enriched in the heavier isotope than microcosms containing 1.0 mg/L DOC by an average value of 79.6 ‰. Mean isotopic composition of microcosms with 100 mg/L DOC was 346.82 ‰ greater than microcosms with only 1.0 mg/L and 267.3 ‰ greater than microcosms with 10 mg/L DOC.

Analysis of interactions between DOC concentration and the nine treatment levels, there was no significant interaction ($p=0.98$) impacting the resulting $\delta^{13}\text{C-DIC}$. The DOC concentration was also found to be the only significant factor displaying a major effect on the resulting $\delta^{13}\text{C-DIC}$ values ($p=2 \times 10^{-16}$). Looking at the mean of all treatments in Figure 6, $\delta^{13}\text{C-DIC}$ increased by 190 ‰ to 350 ‰ in the first week of the experiment. In the following weeks, $\delta^{13}\text{C-DIC}$ continued to be enriched by 20 ‰ and by 80 ‰ in weeks 2 and 3, respectively. After week 3, $\delta^{13}\text{C-DIC}$ in the microcosms decreased slightly to 435 ‰. A different trend was observed in microcosms with 10 mg/L DOC and 1.0 mg/L DOC. In these microcosms $\delta^{13}\text{C-DIC}$ decreased with time in the first three weeks, and did not change at 11 weeks. The $\delta^{13}\text{C-DIC}$ of microcosms containing 10 mg/L DOC decreased from 100 ‰ to 90 ‰ within the first seven days, then to 85 ‰ and final to 83 ‰ in weeks two and three, respectively. In week 11, $\delta^{13}\text{C-DIC}$ decreased slightly to 80 ‰. In microcosms with 1.0 mg/L DOC very small change in $\delta^{13}\text{C-DIC}$ was observed over the entire experiment. Initially $\delta^{13}\text{C-DIC}$ was at 10 ‰ and decreased to 8 ‰ for the remainder of the experiment.

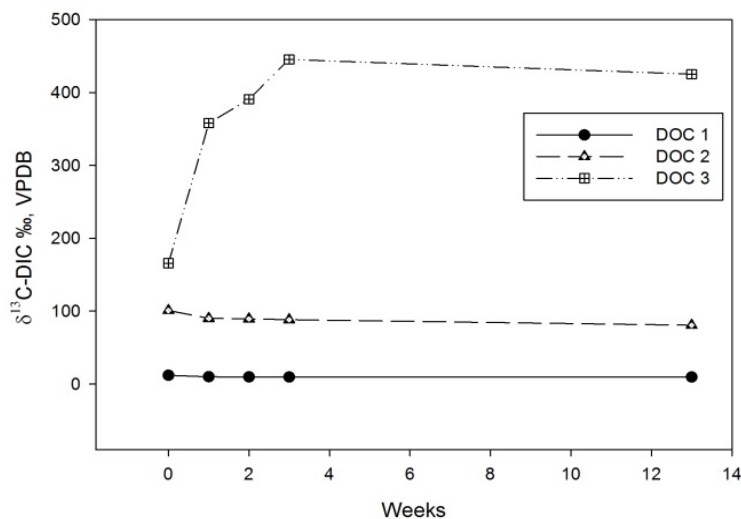


Figure 7 Isotopic composition of $\delta^{13}\text{C-DIC}$ of biologically derived $^{13}\text{C-DIC}$ in microcosm water samples

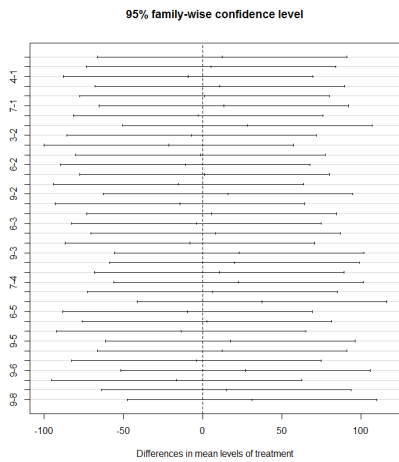


Figure 8 Difference in mean $\delta^{13}\text{C}$ -DIC isotopic composition by nitrate and phosphate treatment

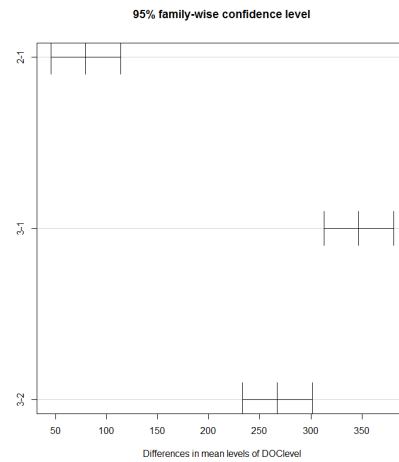


Figure 9 Difference in mean $\delta^{13}\text{C}$ -DIC isotopic composition by DOC treatment

Biologically derived DIC displayed many of the same trends observed in DO measurements and isotopic composition. The factor displaying a significant main effect was DOC concentration ($p=2 \times 10^{-16}$), the impact of the nine treatments on DIC concentration as not significant ($p=0.91$). With respect to DOC level, the concentration of ^{13}C -DIC produced by the bacteria is increasing with time before going unchanged for the remaining 8 weeks of the experiment. When DOC was most prevalent, biologically derived DIC continued to increase for the duration of the experiment, beginning just below 0.6 mg/L and trending towards concentrations that exceeded 1.5 mg/L Figure 11. Microcosms with less than 100 mg/L appeared to have a lag phase that lasted approximately one week, before DIC concentrations began to increase. The increase in DIC concentrations observed was approximately 0.2 mg/L in microcosms containing 10 mg/L DOC and less than 0.1 mg/L for microcosms containing 1.0 mg/L DOC.

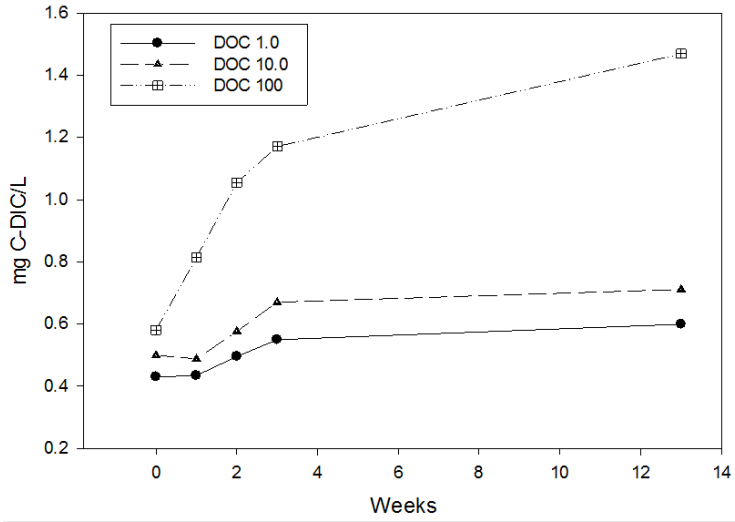


Figure 10 Biologically derived ¹³C-DIC concentration measured in microcosm water samples.

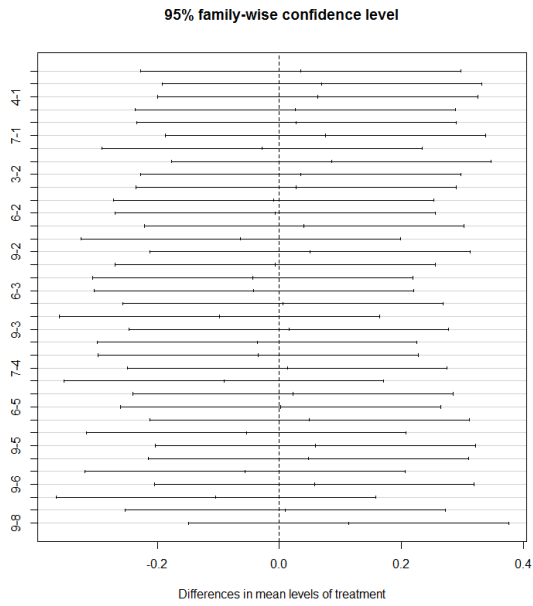


Figure 11 Difference in mean ¹³C-DIC concentration by nitrate and phosphate treatment.

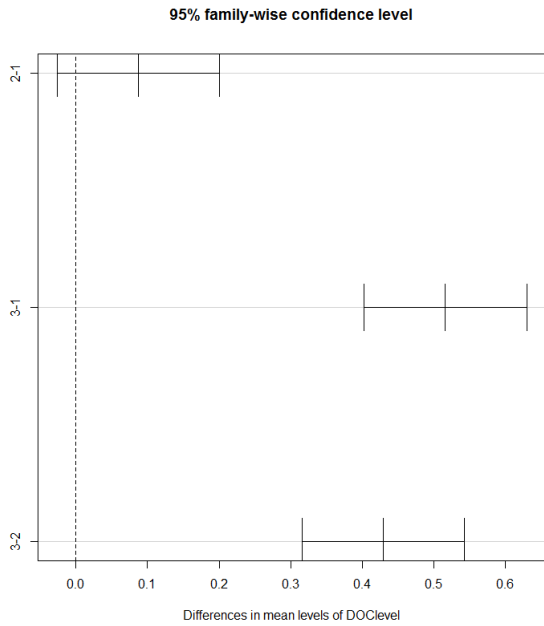


Figure 12 Difference in mean ^{13}C -DIC concentration by DOC treatment.

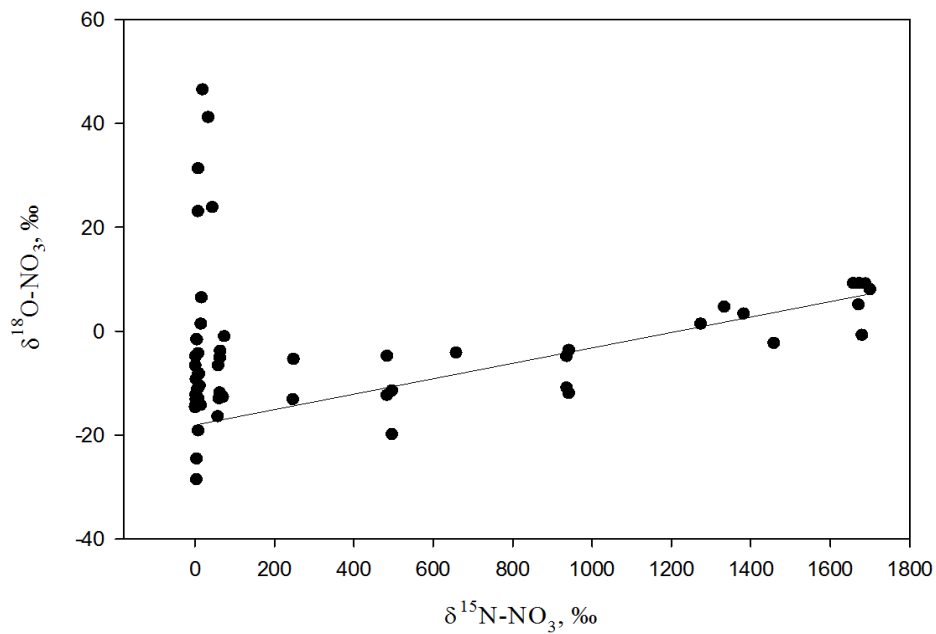


Figure 13 Isotopic composition of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in NO_3 measured in microcosm water samples.

Results of the isotopic analysis of $^{15}\text{N}-\text{NO}_3$ provided good indication of denitrification activity among other processes affecting the fractionation of nitrate Figure 13. Generally, $\delta^{15}\text{N}-\text{NO}_3$

values became more enriched temporally in microcosms containing greater concentrations of DOC. The rate of enrichment with respect to time was greatest in microcosms treated with 10 mg/L DOC at 106.6/week, followed by the microcosms treated with 100 mg/L DOC at 33.5/week. Microcosms treated with only 1.0 mg/L DOC displayed trends contrary to the other DOC levels, trending towards depletion of $\delta^{15}\text{N-NO}_3$ with a negative enrichment rate of -26.4/week. In figure 13, $\delta^{15}\text{N-NO}_3$ values became increasingly enriched in the heavier ^{15}N isotope at approximately half the rate of enrichment for the ^{18}O component of nitrate indicating denitrification.

DISCUSSION

The results of this experiment indicate DOC plays a significant role in microbial activity and the processing of nutrients in karst springs. Dissolved Organic Carbon is essential because it serves source of energy, thereby actively changing water chemistry. More labile forms of DOC such as acetate provide distinct boost in microbial activities even at concentrations as low as 1 mg/L. At low concentrations; however changes in DO, biological DIC production, and nitrate removal process are effected. Lower levels of DOC resulted in less oxygen consumption, DIC production, and less fractionation of residual DIC, and no isotopic indication of denitrification activity. The concentration of DOC increases microbial productivity and the resulting production of biomass significantly increases. Statistically, DOC concentration was a more significant factor in the results of the microcosm experiment than nutrient additions. In the experiment nitrate was the nutrient to be assimilated and denitrification was only observed in microcosms with more than 10 mg/L DOC. Denitrification is a terminal electron acceptor process or (TEAP), and requires adequate DOC to facilitate this metabolic process. Metabolic process are generally energy intensive, and the results indicate that in karst bacteria communities are capable of removing

nitrate, but the extent of removal is contingent on DOC availability and magnitude. The implications are that, nutrient rich waters, not accompanied by DOC will receive very little nutrient processing and thereby shifting processing of nutrients further down the flowpath; rendering epikarst as active transport conduits. However, seasonal variability in DOC species and flux related to hydrologic regimes also limits the efficacy of nutrient removal in the epikarst to periods when labile DOC is most prevalent. Fertilizing activity in the spring and fall accompanied with the typical winter groundwater recharge period in Northwest Arkansas, provide combined inputs of both nutrients and DOC.

Dissolved organic carbon species has a greater impact on the rate of nutrient removal and microbial metabolisms, and DOC concentration has more effect on the duration of removal and activity. In the results of the experiment, DO concentrations decreased early in the experiment, but increased over time as the available DOC was exhausted. The ^{13}C -DIC composition showed continued enrichment with time and DIC production increased with time in all of the microcosms as long as there was adequate DOC to sustain microbial activity. Biological oxygen demand in the microcosms was sustained longer in microcosms with greater quantities of DOC. At 11 weeks, the total change in DO was greatest in microcosms with more DOC, which is a likely indicator of complete exhaustion of the available DOC and potential endogenous decay of the microbial communities in the microcosms. Similarly, $\delta^{13}\text{C}$ -DIC, and the evolution of DIC within the microcosms continued to show indications of microbial respiration until the DOC was exhausted. Because acetate is a very easily degradable form of DOC bacteria respond rapidly, using DOC to fuel denitrification in aerobic and microaerobic. These conditions were largely present in microcosms with greater DOC and nitrate concentrations. At this point it is important to mention that denitrification can occur in aerobic conditions, where pH is ~ 7.0 , which is

consistent with the results and observations of this experiment. The spring-water sample used in the microcosms was of type CaCO₃ therefore increasing its ability to buffer the DIC evolution during microbial respiration in the individual microcosms. Without this capacity to buffer the pH of the water sample, CO₂ production would gradually decrease the pH of the water-sample potentially halting denitrification activity.

Nutrient utilization in the microcosms appeared to be largely limited by the concentration of nitrate in microcosms. Statistically, microcosms containing both nitrate and phosphate were identically in their use of DO, $\delta^{13}\text{C}$ -DIC composition, and DIC production. The only exception was found in microcosms that contained equal concentrations of nitrate and phosphate above 10 mg/L. It is likely that this is a threshold at which phosphate concentration becomes the limiting factor or potentially a source of interference in the process of denitrification. Broadly this balance of nitrate to phosphate should be noted because it represents a threshold when denitrification efficacy decreases or stops altogether and alternative nitrate processes for removing nitrate must be considered. In the recharge area surrounding the spring where the sample was collected for microcosms, concentrations of either nitrate or phosphate rarely reach this level, however, due to the high mobility of nitrate concentration could reach this concentration, but based on historical observations of phosphate in the watershed it would be unprecedented to observe concentrations in this range under normal conditions (e.g. barring direct input of phosphate to groundwater).

CONCLUSION

The removal of nitrate from infiltrating groundwater is dependent upon the availability of DOC. The findings of this study show that DOC concentration is a significant driving factor in the process of nitrate removal and the growth of biomass in subsurface flowpaths. The results of this

study have major implications for the fate nutrients, trace metals, and veterinarian pharmaceuticals that are known to be transported in tandem with DOC. Other solutes transported by DOC could impede enzymatic mechanisms that drive denitrification, as well as facilitate uptake and incorporation of these solutes and their components into the resulting biomass. The connection between biomass and DOC availability shows that more biomass could be expected when DOC is labile and readily available.

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CHAPTER 3: TOXIC EFFECTS OF METALS IN MICROBIAL ORGANIC CARBON TRANSFORMATION AND NITRATE REMOVAL

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Abstract

The epikarst is located at the interface of limestone bedrock and soil overburden, and is known to be important in many biogeochemical processes in karst landscapes. Literature on the coevolution of labile organic matter, dissolved nutrients, and metal fluxes in relation to the epikarst microbiota is limited in northwestern Arkansas. Two experiments were conducted to assess microbial response to dissolved organic carbon (DOC), nutrients, and dissolved metals at an epikarst spring. A gravel and spring-water sample was collected and portioned into 1-L mason jar microcosms to assess potential microbial nutrient utilization in the epikarst environment. Sodium acetate (CH_3COONa), potassium nitrate (KNO_3), and a suit of metals were added to the microcosms, and they were allowed to incubate in a dark- temperature-controlled environment for 11 and 4 weeks, respectively. Isotopically labeled-acetate ($\text{CH}_3^{13}\text{COONa}$) and nitrate (K^{15}NO_3) were added to the microcosms as stable isotopic tracers of microbial biological processes occurring throughout the experiment. Water samples were collected weekly from the microcosms without metal additions, and daily from microcosms with metal additions. Samples were analyzed for dissolved oxygen (DO) concentration, dissolved inorganic carbon (DIC) concentration, metal concentration, ^{13}C -DIC and ^{15}N - NO_3 isotopic compositions. Microcosms with metal additions showed no clear indication of denitrification activity, and DO concentrations remained aerobic throughout the experiment. Microcosms with dissolved metal concentrations above $10\ \mu\text{g/L}$ displayed inhibitory effects on microbial processes. These inhibited processes included DOC assimilation and denitrification. DIC production increased and $\delta^{13}\text{C}$ -DIC became more enriched after 2 days in microcosms with $<1\ \mu\text{g/L}$ dissolved metals. The results of this study indicate epikarst biogeochemistry changed dissolved nutrients and organic carbon were removed less efficiently when dissolved metal concentrations exceed $10\ \mu\text{g/L}$. Increased metal fluxes into groundwater-surface water systems appear to have major implications for biogeochemical cycles normally active in epikarst environments.

INTRODUCTION

The transport and fate of heavy metals in surface-water and groundwater has been studied extensively; however, far less attention has been given to the impact of heavy metals on the

geochemical evolution of water in the epikarst zone. The epikarst zone is the regolith zone underlying the soil overburden and above the more dense and lower porosity zone at the base of the vadose zone above the main karst body. Epikarst is characterized as having highly variable hydrology (Liu et al., 2007). Hydrological variation in the epikarst is largely attributed to extensive weathering of surficial limestone, in the case of carbonate rocks, which diminishes with depth in the vadose zone. Weathered epikarst limestone has a secondary porosity approximately 10%-30%, which is an order of magnitude greater than the thicker unweathered regions at the base of the vadose zone where secondary porosity is often <2% (Williams, 2008). Moreover, during storm-events when epikarst flow is high, the epikarst becomes saturated and is hydrologically connected to the main drainage system, and depending on the thickness of the epikarst this process may occur rapidly (Mangin, 1974; Aquilina et al., 2006). During these high flow storm-events, water chemistry should most closely reflect the chemistry of the overlying soil water and/or indicative of rock-water interactions occurring in connection to chemical and physical weathering. Epikarst water chemistry, which reflects surficial sources changes dynamically relative to precipitation events, in which the epikarst acts as a piston flushing a critical volume of epikarst water into the saturated zone and in the discharge of epikarst springs during storm flow conditions. In the context of epikarst hydrology, the evolution of epikarst water chemistry can be very broad spatially and temporally. Variable epikarst hydrology and water chemistry also creates a complicated ecological framework.

The epikarst zone provides habitat for various size organisms ranging from single celled microbes to multicellular invertebrates. Infiltrating water carries with it numerous dissolved and particulate chemical and biological constituents that are vital to downstream water chemistry. The evolution of water chemistry in epikarst is not only a result of physical and chemical

processes, but also biological processes. Anthropogenic and natural inputs of nutrients such as nitrate, ammonia, heavy metals, antibiotics, and organic matter create a unique chemical environment for epikarst inhabitants. The organisms living in epikarst must be robust and capable of adapting to challenges presented by dynamic hydrology and water chemistry present in epikarst. Because the epikarst is located so close to the surface, many of the aforementioned pollutants, often receive little attenuation outside of their retention time within the flow system, which may vary with precipitation conditions. As much as 74% of NO_3 applied to agricultural fields as fertilizer is discharged during background conditions via springs emanating from the epikarst of northwest Arkansas, while 26% of the nitrate was discharged during high-flow conditions (Peterson et al., 2002). Pollutant attenuation in epikarst occurs largely during base-flow conditions when residence time is greatest, and is carried out by physical, chemical, and biochemical processes. Biological processes were the focus of this study.

Various studies have shown several physical and biological processes occur in the epikarst such as denitrification (Panno et al., 2001), sulfate reduction, but a major question remains to be answered regarding the effect of heavy metal concentrations on biological geochemical processes and the conversion of DOC to dissolved inorganic carbon (DIC). Noted in the work of (Winston et al., 2005) 69% of nitrate (NO_3) and DIC concentration change quantified from the interflow zone that lies just above the surface of the epikarst zone, could be attributed to dilution from storm-event water and approximately 20% attributed to biological processing. Panno et al., (2001) using ^{15}N and ^{18}O isotopes of NO_3 , found the isotopic composition of NO_3 discharging from epikarst springs to be consistent with nitrification and reduced nitrogen fertilizers. Denitrification and several other biogeochemical processes are reliant on adequate available dissolved organic carbon, and as such are impacted by DOC concentration and species. Between

20-60% of the DOC pool in aquatic environments are humic acids or fulvic acids (Frimmel et al., 2008). Moreover, humic substances have a long residence time, taking decades for complete turnover in aquatic environments. Additional aquatic DOC species include Background concentrations of DOC in groundwater range from 1-40 mg/L (Thurman, 1985; Abbt-Braun et al., 2004). This places unique emphasis on the role of DOC in the fate and transport of potential pollutants, and additionally treating DOC as a pollutant in cases where DOC concentration far exceeds background concentrations for groundwater. These conditions are potentially met and exceeded in epikarst underlying regions with intensive spray manure application fertilization, concentrated animal feeding operations (CAFOs), and areas experiencing dramatic land-cover and land-use changes.

Metals are a focal point of this study because of their potential to form ligand complexes with DOC species and potentially negatively impact the ability of bacteria in an epikarst spring water sample to remove dissolved nitrate and to transform DOC to DIC. Metals may impede microbial activity in two main ways: acute toxicity or by way of competitive inhibition of enzymatic activity driving microbial processes. The study used a several dissolved metals; however, focuses on metals known to be associated with animal feed, road dust, and those that are naturally abundant in the geology of the study area. Water samples were collected of precipitation, a well, and a spring at the study site in addition to microcosm experiments run in a controlled laboratory environment using spring water samples. The results of the study will help to further elucidate the nature of water chemistry changes from the perspective of microbial activity and the contributions of microbial communities to the evolution of water quality in epikarst. Moreover, the results of this study could also make significant contributions to what is known about the

frequency and nature of co-selection for metal and antibiotic resistant bacteria populations in groundwater.

Buffalo River National Park is a major source of tourism revenue for the area, and is one of the last remaining undammed major rivers in the country. Nearly 1 million people visit the park annually. In 2012 a swine concentrated animal feeding operation (CAFO) was opened in close proximity to the Buffalo River and one of its major tributaries, Big Creek. The CAFO has a capacity to house 6,500 head of swine (2,000 sows and 4,000 piglets). Annually it is estimated that the CAFO could produce as much as 2 million gallons of waste effluent annually. The primary concern in the area is that waste effluent from the farm might have negative impacts on the groundwater and surface water in the area. The operation of the CAFO in such close proximity to a protected waterway has been the topic of much heated debate, and ongoing legal battles. The state of Arkansas has invested in a multi-year project to monitor water quality changes associated with the operation of the CAFO. Additionally several volunteers from the community and other concerned groups have also conducted extensive projects to monitor positive and negative ramifications of the CAFOs operation on groundwater and ecological resources in the area.

METHODS

Site Description

Field studies were conducted in Newton County, Arkansas. Newton County is located in the Ozark Highlands physiographic region of Arkansas. The topology of the area is rolling hills to mountainous with elevations ranging from 800 to 1500 feet. Annual mean temperature in the area ranges between 45 and 64 degrees F, and mean annual precipitation ranges from 47 to 59 inches. Soils in the study area are Noark very cherty silt loam, which allows soils to be well

drained although the clay fraction increases with depth. Because of the gravelly nature of the soils, they are not ideal for farming and therefore much of the land is used as pastures for grazing cattle. Mississippian Boone Formation limestone is the predominant outcropping geology in the area. The Boone Formation is highly weathered and fractured limestone with interbedded chert as much as 70% in some areas. The highly weathered limestone acts as the parent material contributing to the increasing clay fraction at the interface between the epikarst and soil zones. The Boone Formation has mature karst features, which allow for rapid interaction between surface and groundwater interaction via various dissolution features across the landscape.

Field Study

Precipitation and grab water samples were collected over a one month period June - July.

Precipitation samples were collected using Palmex Rain Collector as shown below, and transferred into a 250 mL Nalgene sample bottle. Grab samples were collected at the sites shown in Figure 14. All samples were collected and acidified using 2% nitric acid and stored on ice during transportation to the lab and stored in a cold environment until analysis. Trace metal analysis was conducted using inductively coupled mass spectrometry (ICP-MS). At each sampling time, dissolved oxygen (DO), pH, specific conductance (SC), and temperature were measured.

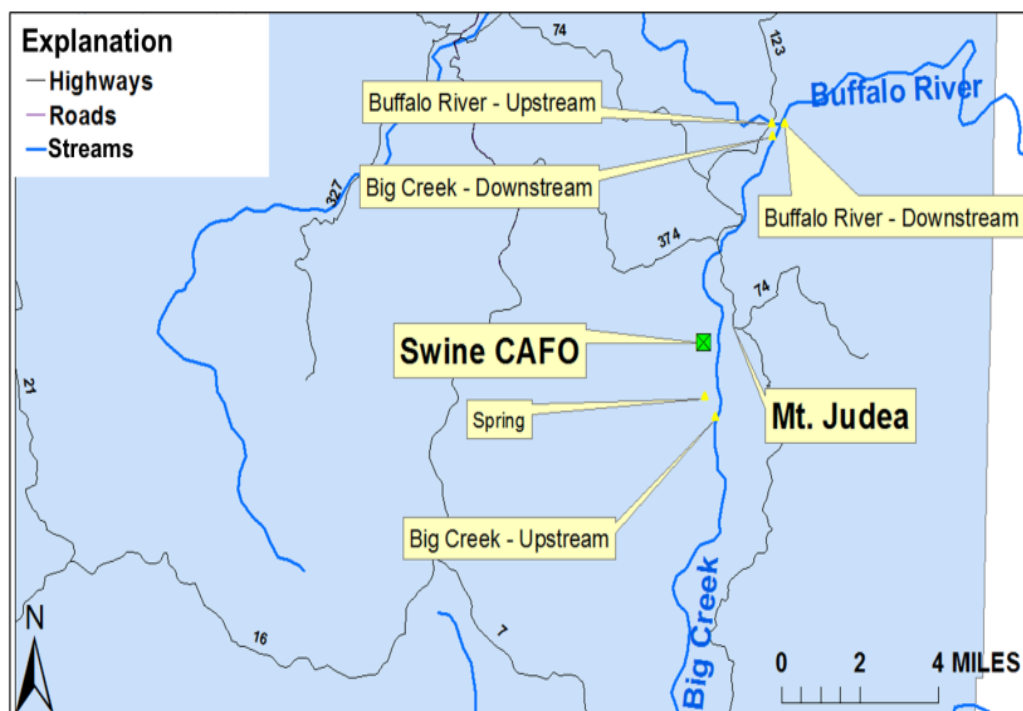


Figure 14 Study sampling locations indicated by yellow triangles.

Laboratory Study

The laboratory microcosm experiment was conducted using a sample of water from the spring and gravel collected from the spring mouth. The gravel was cleaned and baked in the muffle furnace for 4 hours at 550°C. Autoclaved water from the spring water sample was used for an abiotic control in the experiment. Four microcosms were spiked with 100 mg/L of sodium acetate (CH_3COONa) and 1 mg/L of potassium nitrate (KNO_3). Three of the microcosms were amended with increasing concentrations: 1.0 $\mu\text{g/L}$, 10.0 $\mu\text{g/L}$, and 100.0 $\mu\text{g/L}$ - of a trace-metal solution containing equal concentrations of As, Cr, Zn, Pb, and Cu. Additionally, isotopic enrichment was used to assess fractionation of dissolved inorganic carbon and dissolved nitrate. Four microcosms were enriched with ^{13}C -labeled sodium acetate ($\text{CH}_3^{13}\text{COONa}$) and ^{15}N -labeled potassium nitrate (K^{15}NO_3) to 1000 ‰. Abiotic spring-water, raw spring-water, and non-metal control microcosms were run alongside microcosms spiked with trace-metals. The microcosms were incubated in a dark environmental chamber at a temperature of 12°C. Water samples were collected from the microcosms at 0, 24, 48, and 72 hours and again at 4 weeks. Samples taken

from the microcosms were analyzed for their ^{13}C -DIC and ^{15}N - NO_3 composition, DO concentration using the Winkler Titration method, and DIC concentration. DIC concentration was calculated from the isotopic data using the steps outlined below. Isotopic data collect for ^{13}C -DIC were measured against the Vienna Pee Dee Belemnite (VPDB) standard, and ^{15}N - NO_3 measurements were made relative to the AIR standard. Isotopic data was presented using delta-notation. The isotopic delta value is a ratio the heavier to the light isotope with respect to a standard using the relationship described below for values of $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$.

$$\delta, \text{‰} = \frac{\left(\frac{R_H}{R_L}\right)_{\text{sample}} - \left(\frac{R_H}{R_L}\right)_{\text{standard}}}{\left(\frac{R_H}{R_L}\right)_{\text{standard}}} \times 1000$$

$$\text{mol CaCO}_3 = \text{mass}_{\text{calcite standard}} \times \left(\frac{1 \text{ mol}_{\text{calcite}}}{F.W._{\text{calcite}}}\right)$$

$$[\text{CO}_2]_{\text{standard}} = \frac{\text{Mol, CaCO}_3 \times F.W._{\text{CO}_2}}{\text{Volume}_{\text{standard}}}$$

Step 1. Calculate the concentration of CO_2 in standards

$$A = \frac{[\text{CO}_2]_{\text{standard}}}{\text{Peak Area}}$$

Step 2. Determine response coefficient, A

Statistical methods

Descriptive statistics were calculated for all for all data collected. Non-parametric one-way ANOVA was used to analyze differences between treatment groups in laboratory experiments. Linear regression and Pearson correlation coefficient was used to determine the significance of correlation and relationships between individual variables in laboratory experiments.

RESULTS & DISCUSSION

Mean metal concentrations from the three sampling events are presented below in Figure 14. Zinc was the most abundant metal observed in the water samples. Zinc was most abundant in precipitation samples, followed by the well, and then the spring. This trend was also observed in Cu concentrations. No As was detected in precipitation samples, and precipitation Cr concentrations were lowest. Cadmium was only detected in precipitation and in very low quantities. Lead concentrations were detected above 1 µg/L. In well water Cr and Cu concentrations were above 1 µg/L. All As detections were below 1 µg/L, but the greatest concentration was observed in well samples. In spring samples, Cr and Zn were the only metals detected above 1 µg/L. Moreover, the spring had the greatest detected amount of Cr. Spring-water Cu concentrations were second lowest, and As concentrations were the second lowest.

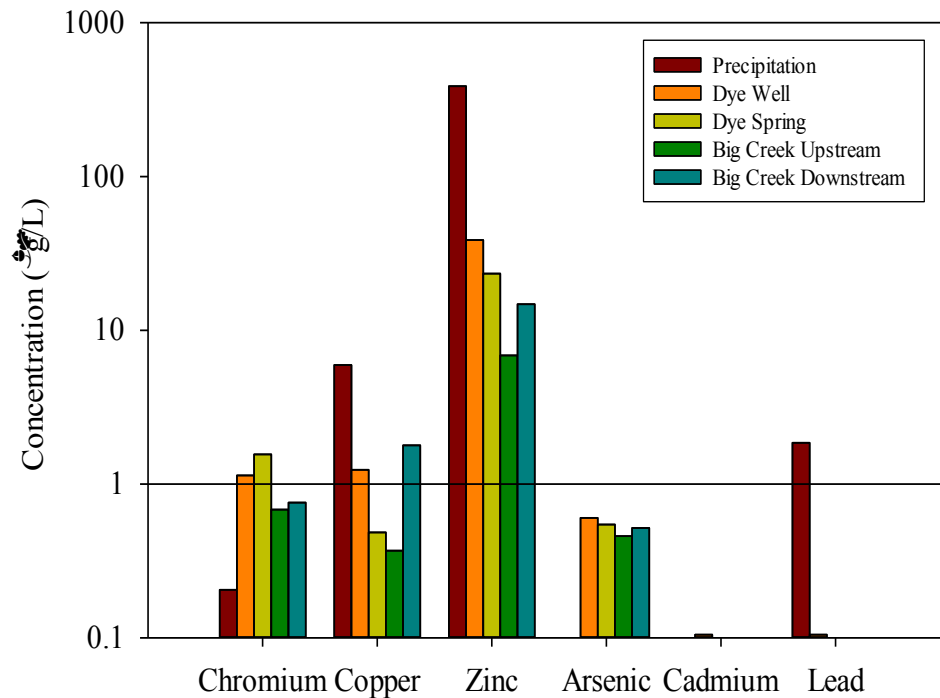


Figure 15 Dissolved metal concentrations measured in precipitation, wells, springs, and surface-water in the study area.

In the laboratory studies metal treatments had no statistically significant effects distinguishing microbial activity in microcosms from one another. The mean DIC concentration was statistically identical in all microcosms ($p=0.932$). The mean DIC concentration in abiotic control samples and raw control samples were 0.48 mg/L and 0.45 mg/L, respectively. In the non-metal control the average DIC concentration was 0.46 mg/L, and for microcosms with metal additions mean DIC concentrations were 0.45 mg/L, 0.41 mg/L, and 0.41 mg/L for levels 1, 2, and 3 respectively Figure 18. The trend over the duration of the experiment; however, did indicate that DIC concentration began to increase toward the end of the experiment between 48 and 72 hours. This trend was observed in the microcosms without metal addition and in the lowest level metal addition microcosm. A similar trend was also observed in the $\delta^{13}\text{C}$ -DIC observations.

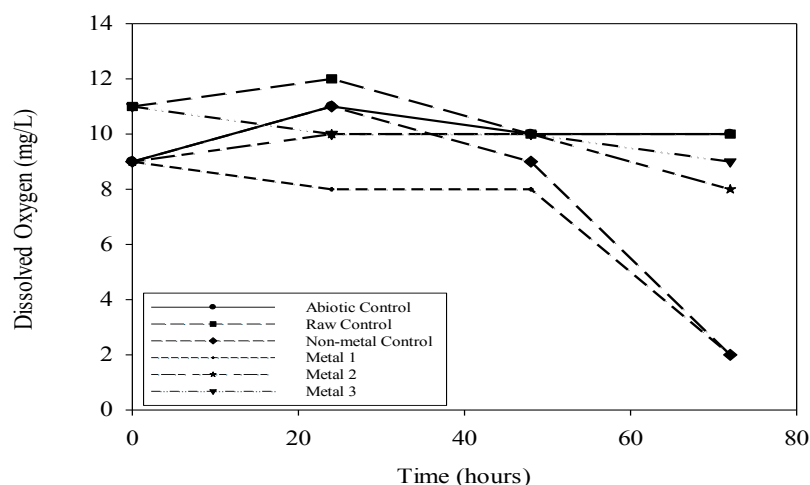


Figure 17 Dissolved oxygen concentration measured in microcosm water samples.

The mean isotopic composition of the microcosm was statistically identical ($p=0.389$). All microcosms had a mean isotopic value of -12.4‰ , VPDB Figure 17. After 24 hours, the isotopic composition of DIC in microcosm with the lowest level of metal addition and the microcosms with only DOC and nutrient additions became isotopically enriched. These two microcosms also showed a slight uptick in DIC concentration Figure 17. In the microcosm containing the largest addition of metals, activity appeared unchanged as seen in both DIC concentration and isotopic composition Figure 17 & 18. To compliment DIC concentration and DIC isotopic composition, DO concentration was also measured rendering aerobic DO concentrations throughout the experiment in all microcosms with exception given to the non-metal microcosm and the lowest level metal addition microcosm. The DO concentration in these microcosms decreased to 2 mg/L at 72 hours Figure 16.

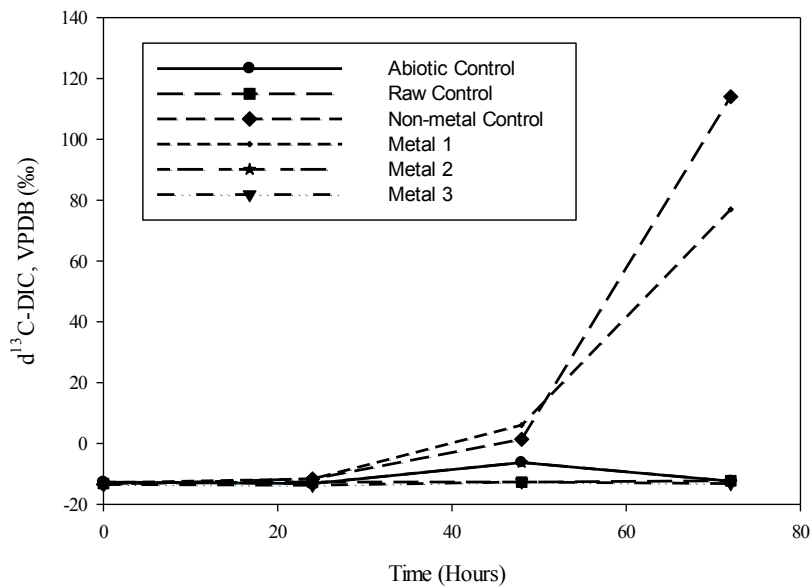


Figure 18 $\delta^{13}\text{C-DIC}$ isotopic composition measured in microcosm water samples.

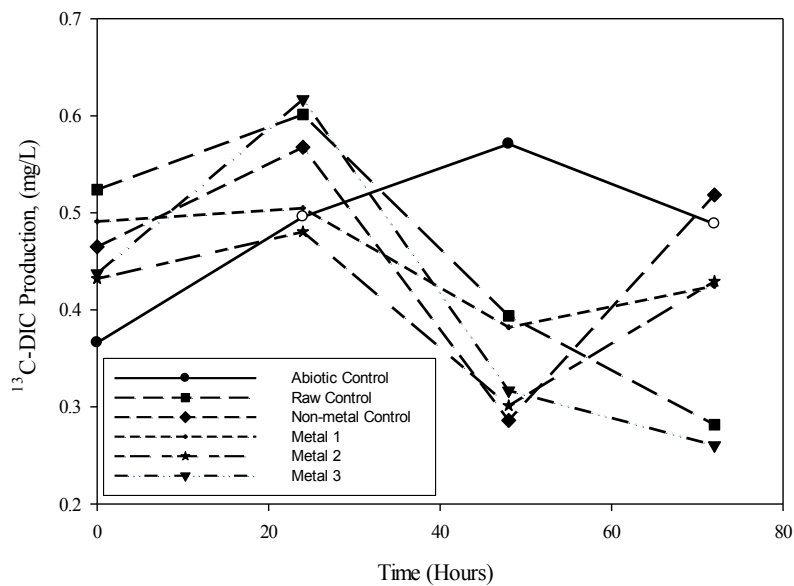


Figure 19 $^{13}\text{C-DIC}$ concentration measured in microcosm water samples.

Isotopic data collected from the microcosms showed no significant enriching of $\delta^{15}\text{N-NO}_3$ (Figure 19). In all microcosms the amount $\delta^{15}\text{N-NO}_3$ values fluctuated, but not to a significant degree indicating processing of amended NO_3 . Nitrate concentration data confirmed that denitrification was not occurring at the time samples were collected because there was no significant reduction in nitrate concentrations in any of the microcosms (Figure 20). Lacking

evidence in support of denitrification activity is in agreement with $\delta^{13}\text{C-DIC}$, DIC concentration, and DO concentration data, which all indicate that there is a lag or potential inhibition or toxic response effecting nitrate and DOC processing in the microcosms.

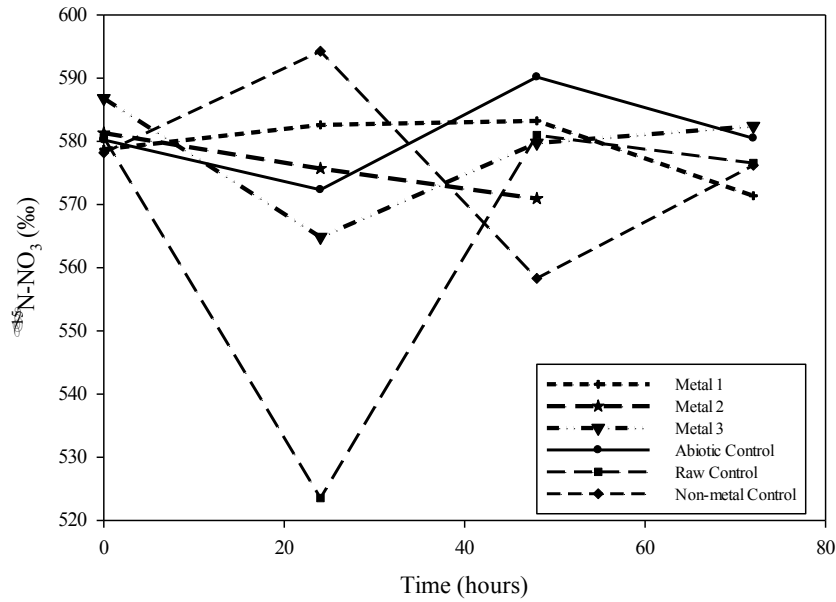


Figure 20 $\delta^{15}\text{N-NO}_3$ isotopic composition measured in microcosm water samples.

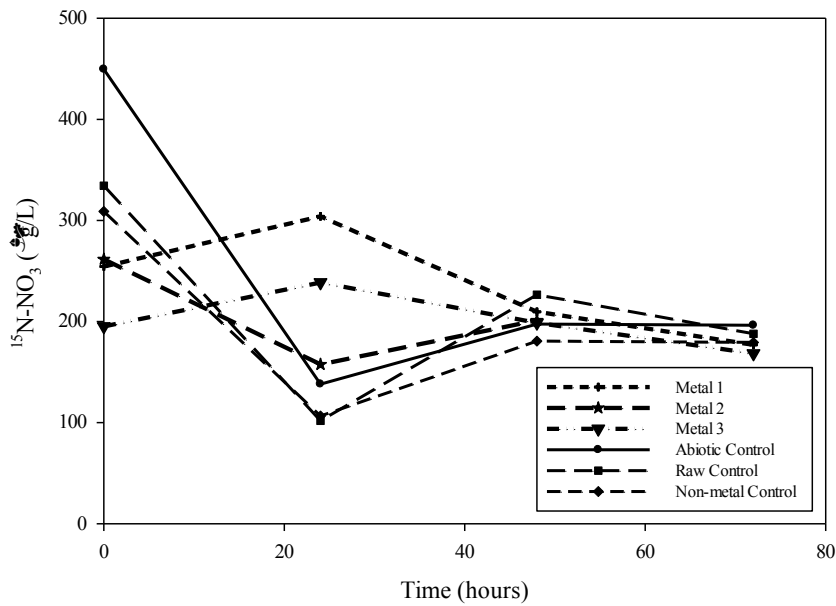


Figure 21 $^{15}\text{N-NO}_3$ concentrations measured in microcosm water samples.

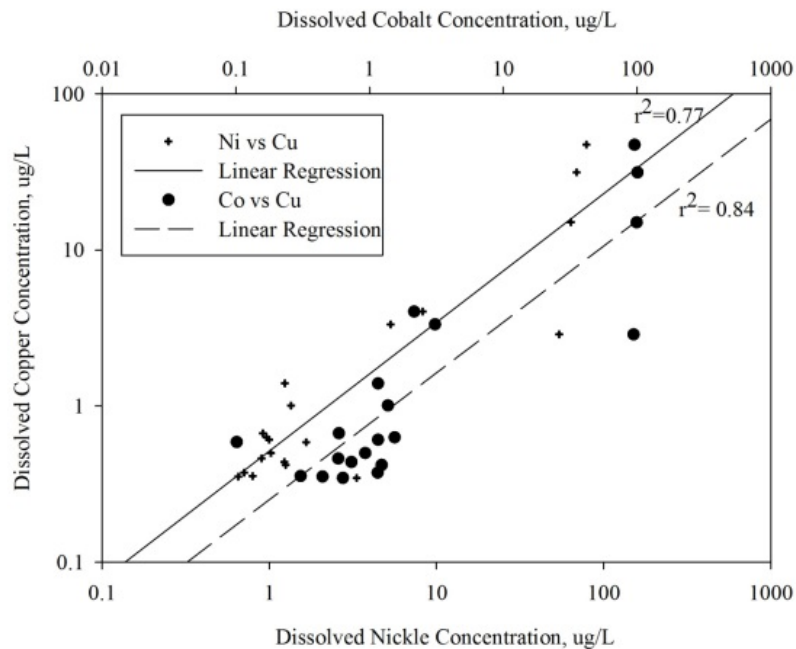


Figure 22 Dissolved copper, nickel, and cobalt concentrations from microcosm water samples.

Trace metal interaction and biogeochemical activity is important because it has major implications for exposure pathways of microbial communities to potentially toxic metals derived from the overlying soil and in recharge. Of interest in this study is the role of DOC in the fate of trace metals. Looking to metal data collected from microcosm samples, clear indications of the affinity of some trace elements to bind to DOC was observed by looking at the relationships between dissolved Cu, Ni, and Co concentrations fig(). Competition for binding sites on organic molecules is $Cu > Ni > Co$, Cu and Ni are able to bind to a wider range of functional groups in organic molecules resulting in more bonding and thereby greater transport efficacy in relation to DOC entering groundwater flowpaths (Fairchild and Hartland, 2010; Hartland et al., 2011; Hartland et al., 2012). Labile DOC species, such as those used in the experiment, tend to be most rapidly degraded and most abundant as the epikarst receives pulses of recharge during storm events increasing the potential for metal-microbiota interaction by way of DOC. Cu(II) specifically is a micro-nutrient and has an active role in aerobic metabolic processes in microbial communities, but at toxic concentrations increases microbial iron and sulfur acquisition

(Macomber and Imlay, 2009; Dupont et al., 2011). However, it should be noted that the indirect effects of Cu(II) toxicity in biofilms may be overcome, as the morphology of microbial communities in the biofilm effectively create a buffer composed of dead microbial cells, which allows the community to adapt to stress and grow (). The results of the microcosm experiment perhaps display a combination of these responses by the microbial communities most clearly in microcosms treated with the greatest quantity of metals. Inactivity in the microcosm was observed as microbes adapted to the Cu induced stress within the first 24-48 hours, concurrent with increasing Fe(II) concentrations beyond 48 hours. What distinguishes this microcosm with relatively large amounts of metals to the other microcosms is microbial inactivity, which is not seen in microcosms with less than 1 $\mu\text{g/L}$ of metals added. In these microcosms, microbial activity resumes when Cu(II) concentrations fall below 0.4 $\mu\text{g/L}$.

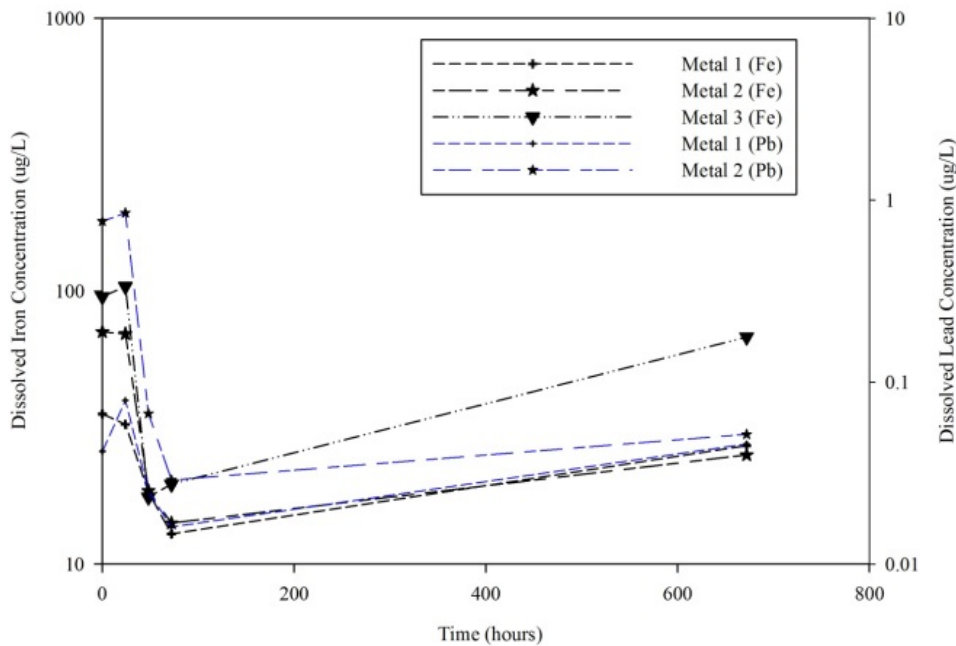


Figure 23 Time-series dissolved iron and lead concentrations in microcosm water samples.

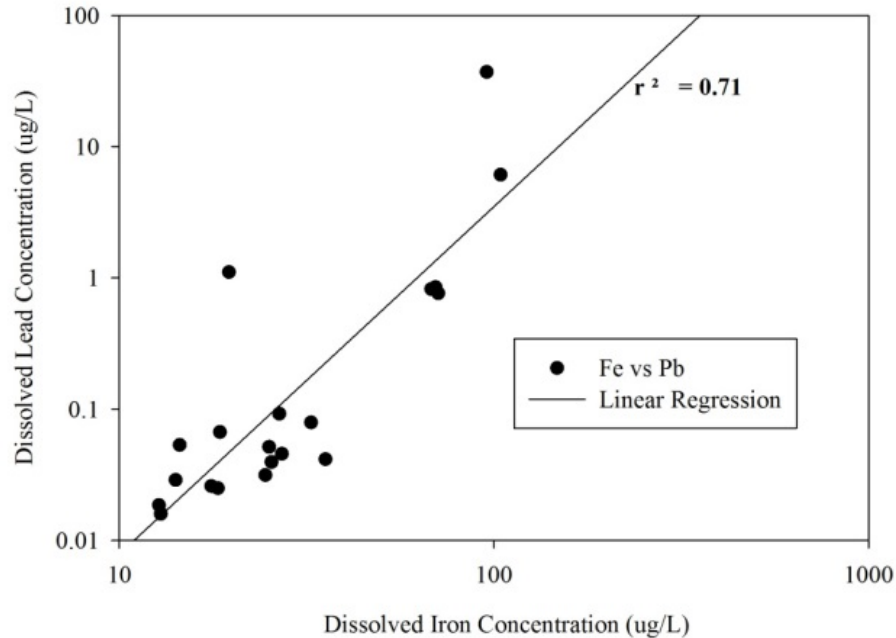


Figure 24 Dissolved iron concentration versus dissolved lead concentration measured in water samples from microcosms. Lead mobility is closely linked to the prevalence of oxidized iron and magnesium compounds. Lead is able to adsorb to these particulates resulting in removal of aqueous Pb. Fe concentrations in the microcosms began with greater concentrations of Fe(II) and upon oxidation of ferrous iron Fe(II) into ferric iron Fe(III), Pb and Fe(II) concentrations decreased. A fraction ferric iron in the microcosms was reduced to ferrous iron, which provides an explanation for increasing Pb concentrations over time; however, the concentration was only a fraction of what was added in each microcosm. Moreover, Pb concentrations overall were reduced to observed background concentrations in the spring sample $<0.1 \mu\text{g/L}$. The only exception to this trend was the microcosm with the greatest amount of Pb added where the concentration of Pb decreases as the concentration of Fe increased. Cadmium (Cd) concentration trends were similar to those observed in the case of Pb over the length of the experiment; however, what must be considered in the dynamics of Cd and each of the other metals used in the experiment to varying extents is the occurrence of biosorption of metals to cells and biofilms that grew over the course of the experiment. Biofilm samples were harvested at the conclusion of the experiment, but they were

not analyzed for their metal content. However, the work of other research have confirmed the adsorption of heavy metals to mineral surfaces as well as to biological surfaces in the sub-surface and for this reason a better understanding of biosorption is necessary. In the work of (Boyanov et al., 2003), they found pH as a major control on the adsorption of Cd to *Bacillus subtilis* cell walls, in particular they reported increasing adsorption of Cd to the cell wall of this gram positive strain of bacteria as pH increased to 7.8. The results from the microcosm studies do not clearly corroborate the findings of Boyanov et al., 2003, because specific taxonomic description of the microbial communities in the microcosms was not completed, but generally Cd concentrations rapidly decrease within the initial 72 hours of the experiment, and after four weeks.

CONCLUSION

This study successfully demonstrated the negative effects of heavy metals on the activity of bacteria living in the epikarst. In a 72 hour period, microbial activity within the epikarst spring water sample was drastically reduce as demonstrated by reduced oxygen use, $\delta^{13}\text{C}$ -DIC fractionation, and DIC production. The study also successfully demonstrated the ability of bacteria to adapt to acute exposure to toxic concentrations of heavy metals, as seen in microcosm treatments containing background heavy metal concentrations and low-level heavy metal concentrations $\leq 1.0 \mu\text{g/L}$. These demonstrations provide evidence as to the nature of geochemical evolution of waters in epikarst springs, and provide further evidence for the complex biogeochemical processes which occur in the epikarst zone. Water chemistry in the epikarst evolves due to several environmental interactions: rock-water, soil-water, and bacteria-water interactions. As a driving factor of denitrification, DOC must be available in sufficient quantities. Moreover, complexing of DOC with heavy and transition metals, pose a series of

problems for microbial communities in the epikarst. The problems include competitive inhibition for enzymatic activity necessary to drive denitrification, toxicity due to adsorption of heavy metals and transition metals to biofilms, as well as the incorporation of these metals into individual bacteria cells.

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CHAPTER 4: RESPONSE OF BACTERIA IN EPIKARST SPRING DISCHARGE TO ERYTHROMYCIN, TETRACYCLINE, AND METALS AS INTERPRETTED BY FATTY ACID METHYL ESTER COMPOSITION OF BIOMASS

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ABSTRACT

The determination of changes in microbial communities as a result of increasing organic carbon, nutrient, and/or metal concentrations is important in understanding the evolution of epikarst water chemistry. The objective of this study was to identify microbial communities in biofilms exposed to a range of organic carbon concentrations, nutrients, trace and heavy metals, and antibiotics. Biofilm samples were collected from laboratory microcosms and field microcosms. Laboratory microcosms conducted using acetate, nitrate, and phosphate additions were allowed to incubate in a light- and temperature controlled environment for 11 weeks, then harvested using abrasion and by filtering the suspended biofilms. Laboratory experiments using metal additions were allowed to incubate in a light and temperature controlled environment for 4 weeks. The field component of the study included sampling biofilms grown in-situ on ceramic plates over the course of 3 weeks. Dose response studies were conducted on bacteria harvested from a spring site used in the biofilms study. Antibiotics (tetracycline and erythromycin) were added to Tryptic Soy Agar growth media and the colonies were counted daily for three days. Results of the study indicate that bacteria cultured in the laboratory were resistant to erythromycin up to 10 mg/L, but were less resistant to tetracycline. Analysis of fatty acid methyl esters from the field and laboratory microcosms displayed biomarkers of both gram-positive and gram-negative eubacteria. Under metal stress, gram-negative indicators became more prevalent relative to the total fatty acid composition, and gram-positive biomarkers became more prevalent carbon to nutrient rations.

INTRODUCTION

The response of epikarst bacteria to increasing labile carbon has not received much attention in karst research. Specifically, little is known about microbial community dynamics within epikarst biofilms in response to labile carbon. Due to well drained soils and the high porosity of weathered limestone in northern Arkansas, there is a great deal of interaction between surface water and groundwater, with the epikarst representing a critical zone in the evolution of water

chemistry. Due to its physical position above the karst and below the soil, epikarst represents a zone of much transition physically, chemically and biologically. Much of the microbial life in the epikarst is derived from overlying soils; additionally the soil environment is also responsible for the initial chemical and biological alteration of infiltrating water. A prime example, which is pertinent to this study, is the application of manure slurries to fields as fertilizer. Manure slurries carry copious amounts of fecal bacteria, nutrients (NH_3^+ , NO_3^- , PO_4^-), metals (Cr, As, Zn, Cu), and antibiotics (roxarsone, tetracycline, sulfadiazine). These constituents are capable of passing through the soil and into the groundwater where they can be disseminated on a much larger scale creating a potential for eutrophication of water ways, human health problems, decreasing biodiversity, and economic turmoil particularly when natural resources are economic staples (e.g. national parks).

As is the case with many ecosystems, it may be assumed that increasing labile carbon would increase productivity within biofilm microbial communities resulting in increased microbial activity. In the soil environment, organically rich water infiltrating provides key nutrition for a broad range of organisms. Additionally the soil environment also provides a matrix for the adsorption of organic compounds and metals, effectively removing them from the water as it moves through the soil profile; however, adsorption and biological removal of organic matter, nutrients, and metals is not without limits in the soil environment. Once the ability of the soil to act as a primary buffer for the groundwater environment has been surpassed, these constituents make their way toward the groundwater table, but not without encountering the epikarst where it is present. Within epikarst, variable hydrology and the range of water chemistry conditions of the water entering creates a unique environment with variable water chemistry. Identifying one characteristic response of microorganism communities to this chemical variability is the

objective of this study. Bacteria communities form biofilms as a method of adapting to environmental stressors. Typically, biofilms contain several bacteria communities, all living in symbiosis with one goal, survival. The general functions of biofilms are to protect from toxins and predatory organisms (e.g. protozoa), self-preservation (e.g. exchange of genetic information), nutrition (e.g. terminal electron acceptor processes nitrification to denitrifying bacteria), as well as providing a stable environment.

The popular understanding of biofilm morphology can be described in a five step process shown in the diagram below. A single bacteria cell attaches to a surface, and replicates, before more cells are present representing several types of bacteria, and the production of extracellular polymeric substance (EPS) begins. The composition of EPS varies across community structures, but contains polysaccharides, genetic information, proteins. The function of EPS, outside of serving as a protective agent, is the topic of much study. The EPS is, believed by many, to be a “highway” for quantum signaling between communities of bacteria within the biofilm. However, as the biofilm continues to mature shear force increases in turn causing the biofilm to slough and break away, carrying the genetic information and viable cells down the flowpath. In the context of this study, we hope to identify changing activity in biofilms by identifying changes in their community structure by looking at their fatty acid compositions under different chemical conditions. Fatty acids are phospholipid molecules used to store energy within the cells of organisms. These molecules are present in all living organisms; however their abundance and complexity makes it possible to distinguish different organisms (e.g. bacteria, algae, plants, and mammals). In this study we harvested the biofilm from two laboratory studies and one field study. The laboratory studies represented biofilm growth under varying dissolved organic carbon (DOC) and nutrient concentrations as well as trace metals concentrations. The field study was

conducted as an environmental control, to compare the results of the laboratory experiment to in-situ conditions.

METHODS

Site Description

Field studies were conducted in Newton County, located in the Ozark Highlands physiographic region of northern Arkansas. The topology of the area is rolling hills to mountainous with elevations ranging from 800 to 1500 feet. Annual mean temperature in the area ranges between 45 and 64 degrees F, and mean annual precipitation ranges from 47 to 59 inches. Soils in the study area are Noark very cherty silt loam, which allows soils to be well drained although the clay fraction increases with depth. Because of the gravelly nature of the soils, they are not ideal for farming and therefore much of the land is used as pastures for grazing cattle. Mississippian Boone Formation limestone is the predominant outcropping geology in the area. The Boone Formation is highly weathered and fractured limestone with interbedded chert as much as 70% in some areas. The highly weathered limestone acts as the parent material contributing to the increasing clay fraction at the interface between the epikarst and soil zones. The Boone Formation has mature karst features, which allow for rapid interaction between surface and groundwater interaction via various dissolution features across the landscape.

Antibiotic Sampling and Analysis

Three replicate grab samples were collected for the analysis of antibiotics. Samples were processed in the field in a clean chamber created using virgin plastic bags attached to a frame constructed from pvc pipes. Clean hands-dirty hands protocol was followed to reduce the risk of sample contamination. A new clean chamber was constructed for each sample collected. Samples were filtered using a pre-combusted glass fiber filter (45 μ m) on a stainless steel plate filter.

Sample water was collected in a Teflon carboy, from which three sample aliquots of 250 mL were collected in amber glass bottles. The bottles were then wrapped in foam sleeves, placed into two ziplock plastic bags and stored on ice before being mailed to the USGS Kansas Organic Geochemistry Laboratory for analysis. If samples were not shipped same day, they were stored up to 48 hours at 4°C before being mailed overnight for analysis. Before and after sampling from each site, all equipment was washed with soap, rinsed with tap water, distilled water, methanol, distilled water, and organic free water. The equipment was wrapped in foil and double bagged in plastic bags after cleaning.

Antibiotic Inhibition Dose Response Assay

An antibiotic inhibition dose-response study was conducted by using media made of 2% agar (w/v) and 10% Tryptic Soy Agar (TSA). This concentration of TSA was selected based on findings of previous studies conducted by Byl and others (2014), which concluded karst groundwater bacteria grew better on 10% strength media than on full strength TSA. Appropriate quantities of sterile-filtered stock solutions of tetracycline, and erythromycin (Sigma Chemical, St. Louis, MO) were mixed into the 10% TSA just prior to pouring into 9 x 50 mm Petri plates. Antibiotic additions to the 10% TSA resulted in antibiotic concentrations of 0.00, 0.01, 0.10, 1.00, and 10.0 mg/L agar media. Each plate had three replicate treatments. The plates were inoculated with 10 µL of raw water from sampling sites after the water samples were shaken to re-suspend bacteria cells. The plates were inoculated by spreading bacteria cells evenly over the media using a sterile bent glass rod. The plates were labeled, inverted, and placed in an incubator at 25°C. Bacteria colonies were counted at 1, 2, and 3 days. The results are reported as colony forming units per 10 µL (cfu/10 µL).

Laboratory Microcosms

Two laboratory microcosm experiments were conducted; one to compare microbial activity under varying concentrations of DOC, NO_3 , and PO_4 , and a second microcosm experiment to compare increasing trace and heavy metal concentration on denitrification and DOC conversion to dissolved inorganic carbon (DIC). Gravel and 30-L epikarst spring-water sample were used to construct the microcosms in 1-L Mason Jars. Gravel (185 g) and a spring-water sample (0.8 L) was added to 30 mason jars. The experiments used sodium acetate as a DOC amendment and potassium nitrate as a nitrate amendment. The nutrient and DOC microcosm experiment was allowed to incubate in a light- and temperature- controlled environment to simulate conditions in an epikarst environment. The microcosms were allowed to incubate at 12°C in the dark for a period of 11 weeks. Weekly water samples were collected from the microcosms. Biomass samples were collected at the end of the eleven week experiment and prepared for FAME analysis. The results of the nutrient and DOC experiment indicated that the most microbial activity was observed in microcosms with DOC concentration of 100 mg/l and NO_3 concentration of 1 mg/L. Additionally, it was determined that DOC was rapidly converted to DIC with the first week of the experiment, therefore an experiment looking at the first week of activity would be beneficial to further understand the impact of metals on DOC-DIC conversion and denitrification. The second laboratory experiment used a single DOC (100 mg/L) and NO_3 (1.0 mg/L) concentration. The microcosms were spiked with a suite of metals at three concentrations levels: 1.0 $\mu\text{g/l}$, 10 $\mu\text{g/L}$, and 100 $\mu\text{g/L}$. Water samples were collected from the microcosms each day for 3 days and one additional sample was taken at 4 weeks. Biomass was harvested from the microcosms at the conclusion of the experiment and prepared for FAME analysis.

Field Study

In-situ biofilm samples were collected on ceramic plates with a surface-area of 20.3 cm². The ceramic plates were placed at an upstream well and a spring that discharges downstream of the well. The ceramic plates were placed in wire mesh pouches and tied and weighed down below the water table in the well and below the water's surface in the spring housing. Three packets were placed at each location and collected at the same time with water samples.

Field samples and microcosm samples of biomass were analyzed for their fatty acid composition using the modified method of (Findlay & Dobbs, 1993). Biomass samples were collected from the microcosms at the end of the 4 week period and dried. The samples were weighed and allowed to sit overnight in a 50µM phosphate buffer solution. The phosphate solution was collected and fractions of dimethyl chloride (DCM), methanol, and UltraPure water were added in a 2:2:1 ratio. The samples were shaken then allowed to sit overnight. The organic fraction was removed, and dried down under a stream of nitrogen gas, before being eluted with DCM. The solution was heated at 100°C for 1 hour then derivitized using 1 mL of boron trifluoride (BF₃) and heated for an additional 20 minutes. The reaction was quenched with 1 mL of hexane and 1mL of ultrapure water, and rinsed with an additional 2mL of hexane. Hexane used to rinse the sample was collected in 1.8 mL GC vials. Samples were analyzed using the Agilent 6890 Gas Chromatography – Mass Spectrometer (GC-MS).

Statistical analysis

Descriptive statistics were calculated for each of the FAs detected in field and laboratory microcosms. Non-parametric statistical methods and statistical methods robust to non-normally distributed data were used. Statistical analysis for the antibiotic dose-response study included the Wilcoxon Rank Sum test was used to determine statistical significance in differences between treatments and controls. One-way ANOVA was used to determine the significance of the

variation of total FA compositions of laboratory microcosms augmented with metals and microcosms augmented with nutrients and DOC. Additionally, the statistical significance of correlations in microcosm experiments was determined by calculating the Spearman Correlation Coefficient. All statistical analysis was conducted using the Sigma Plot software package release 12.

RESULTS

Antibiotic Sampling and Dose-Response Study

Grab samples from sampling locations all returned negative for any measurable concentrations of the six classes of antibiotics and antibiotic degradation products analyzed. Results of the dose response assay provided some indication of resistance to both erythromycin and tetracycline. The greatest quantity of bacteria were cultured from samples taken at the upstream Big Creek sampling site, followed by the sampling site on the Buffalo River, and lastly Dye Spring. Samples taken downstream on Big Creek and upstream on the Buffalo River had less culturable bacteria overall. Excluding samples taken upstream on the Buffalo River, bacteria grew at all concentrations of erythromycin; however, as concentrations of erythromycin increased the concentration of bacteria decreased Figure 25. At all study sites, the bacteria seemed capable of growing with erythromycin, but tetracycline appeared to be more effective halting bacteria growth at concentrations above 10 mg/L. The exceptions were downstream on the Buffalo River beneath the confluence with Big Creek.

Fluoroquinolones (0.005 µg/L)	Macrolides	Sulfonamides	Tetracyclines (0.010 µg/L)	Pharmaceuticals	Others
Ciprofloxacin	Azithromycin	Sulfachloropyridazine (0.005 µg/L)	Chlorotetracycline	Carbamazepine (0.005 µg/L)	Chloramphenicol (0.100 µg/L)
Enrofloxacin	Erythromycin (0.008 µg/L)	Sulfadiazine (0.1 µg/L)	Oxytetracycline	Ibuprofen (0.050 µg/L)	Lincomycin (0.005 µg/L)

Lomefloxacin	*Erythromycin-H2O (0.008 µg/L)	Sulfadimethoxine (0.005 µg/L)	Tetracycline	Ormetoprim (0.005 µg/L)
Norfloxacin	Roxithromycin (0.005 µg/L)	Sulfamethoxazole (0.005 µg/L)	Doxycycline	Trimethoprim (0.005 µg/L)
Ofloxacin	Tylosin (0.010 µg/L)	Sulfamethazine (0.005 µg/L)	*Epi- chlorotetracycline	
Sarafloxacin	Virginiamycin (0.005 µg/L)	Sulfathiazole (0.05 µg/L)	*Epi-iso- chlorotetracycline *Epi-tetracycline *Iso- chlorotetracycline	

Table 2 Measured antibiotic and antibiotic degradation compounds measured in water samples.

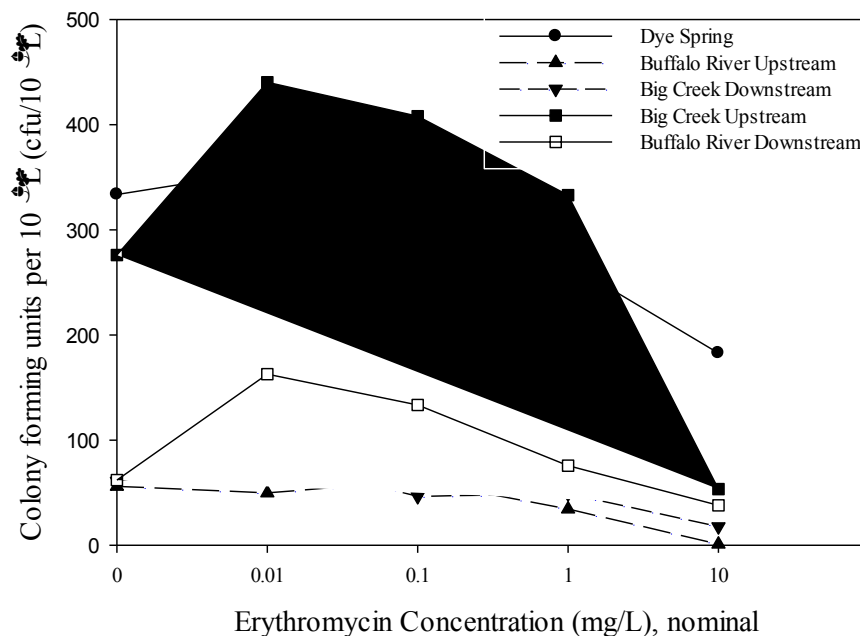


Figure 25 3 Colony counts of bacteria grown on erythromycin dosed growth media.

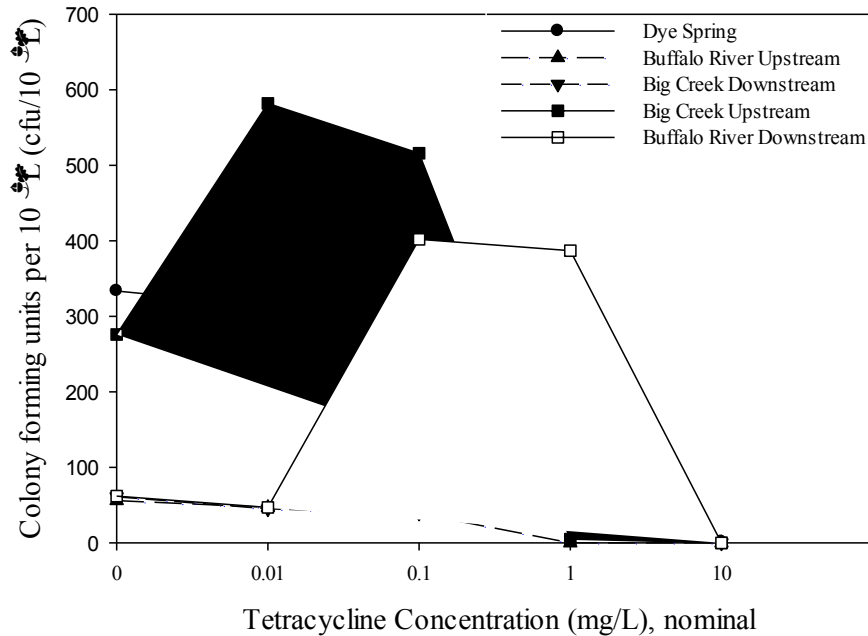


Figure 26 Colony counts of bacteria grown on tetracycline dosed growth media.

DOC, Nitrate, Phosphate Addition Microcosm Study

Samples collected from the microcosm studies using varying concentrations of DOC, NO₃, and PO₄ show that the FA compositions of all of the microcosms were similar. Palmitic (16:0) and stearic acid (18:0) made up the largest fraction of FAs in each of the microcosms observed. Stearic and palmitic acid are also the most abundant FAs in the natural environment and accounted for the largest fraction of FAs in each microcosm as a whole. In the microcosm experiment, the percentage of saturated FAs was significantly greater than the percentage of unsaturated FAs ($p < 0.001$). The median percentage of total FAs from the biofilms harvested from the microcosms that were saturated FAs was 75.5%, and the median percentage of monounsaturated FAs was 13%. The mean percentage of palmitic acid in the microcosms was $42.1\% \pm 3.78$. The range of palmitic acid fractions in the microcosms was broad, from 10.45% to 100%. The Abiotic control microcosm and the D3P3 microcosms were the only microcosms to have 100% of their FA compositions attributable to palmitic acid. Because of the abundance of palmitic acid in the environment, palmitic acid in the abiotic microcosm is likely residual DOC

in the samples after microcosm preparation. Across all microcosms, considering DOC, NO₃, and PO₄ concentrations samples containing only PO₄ had significantly greater amounts of palmitic acid than microcosms containing only NO₃ ($p=0.05$). The fraction of palmitic acid in microcosms containing equal parts of NO₃ and PO₄ was negatively correlated with the amended DOC concentration ($\rho=-0.69$, $p=0.04$); however, a similar relationship with concentrations of NO₃ and PO₄ was not statistically significant ($\rho=-0.11$, $p=0.78$). A positive trend was observed between palmitic acid fraction and DOC concentration; however, this trend did not meet the criteria of statistical significance ($\rho=0.58$, $p=0.09$).

Stearic acid comprised of the next largest fraction of saturated FAs in the biofilms harvested from the microcosms. On average stearic acid comprised of approximately $27.8\% \pm 2.23$ of the total FA composition of the biofilm samples. The range of stearic acid fractions in the biofilms was 51.75%, with the greatest fraction present in the Raw Control (51.75%) microcosm and the smallest fraction present in the Abiotic Control and D3P3 microcosms, which both had no stearic acid present. Although not statistically significant, a positive trend was observed between the percentage of stearic acid and DOC concentration in microcosms containing only NO₃ and equal parts of NO₃ and PO₄. The stearic acid percentage was, however, negatively correlated with DOC concentration in microcosms containing only PO₄ ($\rho=-0.69$, $p=0.04$). There was no statistical difference in the fraction of stearic acid contained within the biofilms of microcosms augmented with NO₃ only and PO₄ only at all DOC concentrations.

Nonadecylic acid (19:0) and lauric acid (12:0) were the only other saturated FAs observed in biofilms samples from the microcosms experiment. Nonadecylic acid was detected in nearly all of the samples, while lauric acid was detected in a sample from only the D2P2 microcosm (51.5%). Because lauric acid is relatively rare in the environment, and was not detected in other

samples, it is plausible that its detection may be an outlier. Nonadecylic acid, however, was detected in samples on average constituting $3.63\% \pm 0.69$ of the total FAs in the samples. The maximum detected fraction of nonadecylic acid was detected in the D1NP3 microcosm at 11.21%. Nonadecylic acid fractions were statistically identical in all microcosm treatments. Generally, nonadecylic acid fractions in samples trended negatively with increasing DOC concentration, but this was not a statistically significant relationship in microcosms with only NO_3 , only PO_4 , or equal parts of NO_3 and PO_4 .

Monounsaturated FAs are key in the identification of gram-negative bacteria. Two monounsaturated fatty acids were observed in biofilm samples from the microcosm experiments palmitoleic acid (16:1) and oleic acid (18:1c9). Palmitoleic acid comprised greater fractions of FAs in all microcosm treatments with a mean percentage of $7.83\% \pm 0.96$, and the mean oleic acid fraction was $3.17\% \pm 0.59$. The maximum fractions of palmitoleic acid and oleic acid were observed in D2NP3 and D1N1 microcosms at 16.61% and 10.43%, respectively. The percentage of total FAs that were monounsaturated fatty acids (MUFAs) decreased with increasing DOC concentration slightly, but this was not a statistically significant finding. No significant changes were detected in the percentage of total FAs that were MUFAs with increasing concentrations of NO_3 .

Metal Addition Microcosm Study

In the metals microcosm experiment palmitic acid comprised of more than 25% of all samples. The average fraction of palmitic acid in each of the metal microcosms was $33.98\% \pm 6.512$. Fractions of palmitic acid in the samples ranged from 26.89% as observed in the microcosms with the greatest metal addition to 44.23%, which was observed in the microcosm augmented with only DOC and NO_3 . The palmitic acid percentage in metal microcosms followed a

decreasing trend with increasing concentrations of metal amendments. There was not a statistically significant negative correlation between the palmitic acid fraction of the total FA of biofilms collected from the metal microcosms and increasing metals concentrations ($\rho=-0.96$, $p=0.182$). Palmitoleic acid fractions in the metals microcosms ranged from 6.19% to 42.39%, with the greatest fraction present in the Metal 2 microcosm and the smallest fraction in the Raw Control microcosm. Microcosms with additions of DOC and NO_3 each had greater fractions of palmitoleic acid than the raw control sample; however, the Metal 3 microcosms had the lowest fraction 11.13%. The palmitoleic acid percentage in the microcosms followed an increasing trend in microcosms treated with DOC, NO_3 , and metals with the exception given to the Metal 3 microcosm. The increasing palmitoleic fraction in the Non-metal control, Metal 1, and Metal 2 microcosms had a Pearson coefficient of $\rho=0.96$, but was not significant ($p=0.192$). Stearic acid was most abundant in the Raw control making up 45.1% of the total FA composition of the biofilm in that microcosm. The stearic acid percentage, similar to the palmitic acid percentage, decreased from the Non-metal control microcosm (19.66%) to the Metal 3 microcosm (9.46%). The smallest fraction of all the FAs observed in any cumulatively was oleic acid. Oleic acid was present most in microcosms Metal 3 (9.79%), but was not observed in the Metal 2 and Non-metal control microcosms. Raw control and Metal 1 oleic acid fractions were 7.25% and 3.96%, respectively. Nonadecylic acid was observed in all of the microcosms, comprising a varying percentage of the total FAs. Nonadecylic acid fractions ranged from 3.9% to 11.45% with a mean value of $7.28\% \pm 2.8$. Microcosms Metal 2 had the greatest percentage of nonadecylic acid (11.45%), and Metal 1, the Non-metal control, and the Raw control each had decreasing fractions with 8.27%, 6.64%, and 6.15% respectively.

Field Microcosm Study

The results of the field microcosms varied, but in most instances the greatest contribution to the FA distribution of the collected samples reflected the ubiquitous nature of palmitic acid and stearic acid. Results of analysis of data collected from each site, palmitic acid was on average $27.1\% \pm 4.94$ of the total fatty acid composition, and the mean fraction of stearic acid was $17.39\% \pm 4.7$. Palmitic and stearic acid were most abundant in the July biofilm spring sample, but it should be noted palmitic and stearic acid were the only FAs observed in this sample at 53.7% and 46.3% respectively. The June spring sample showed a much broader range of FAs, and this was the general trend with spring biofilm samples.. Palmitic acid and stearic acid were the majority of the FA composition of the sample, but tridecylic acid (13:0), arachidic acid (20:0), and behenic acid (22:0) were also observed in the sample. Well samples had a simpler FA composition, with a more discrete range of saturated and unsaturated FAs. Biofilm samples from the well were composed of palmitic and stearic acids, but linolenic acid (18:3) was detected as well as oleic acid (18:1c9). Linolenic acid was observed in only one sample at 7.3% and oleic acid was observed in an earlier sample at 9.1%. Because biofilm samples were abraded from the growth surface in a known volume of UltraPure water, the supernatant was also analyzed for the FA composition. In most cases there were no significant differences between biofilm samples and their supernatant; however there was a significant difference overall FA composition. Palmitic and stearic acid were a larger fraction within the aqueous samples, and had a lower abundance relative to biofilm samples.

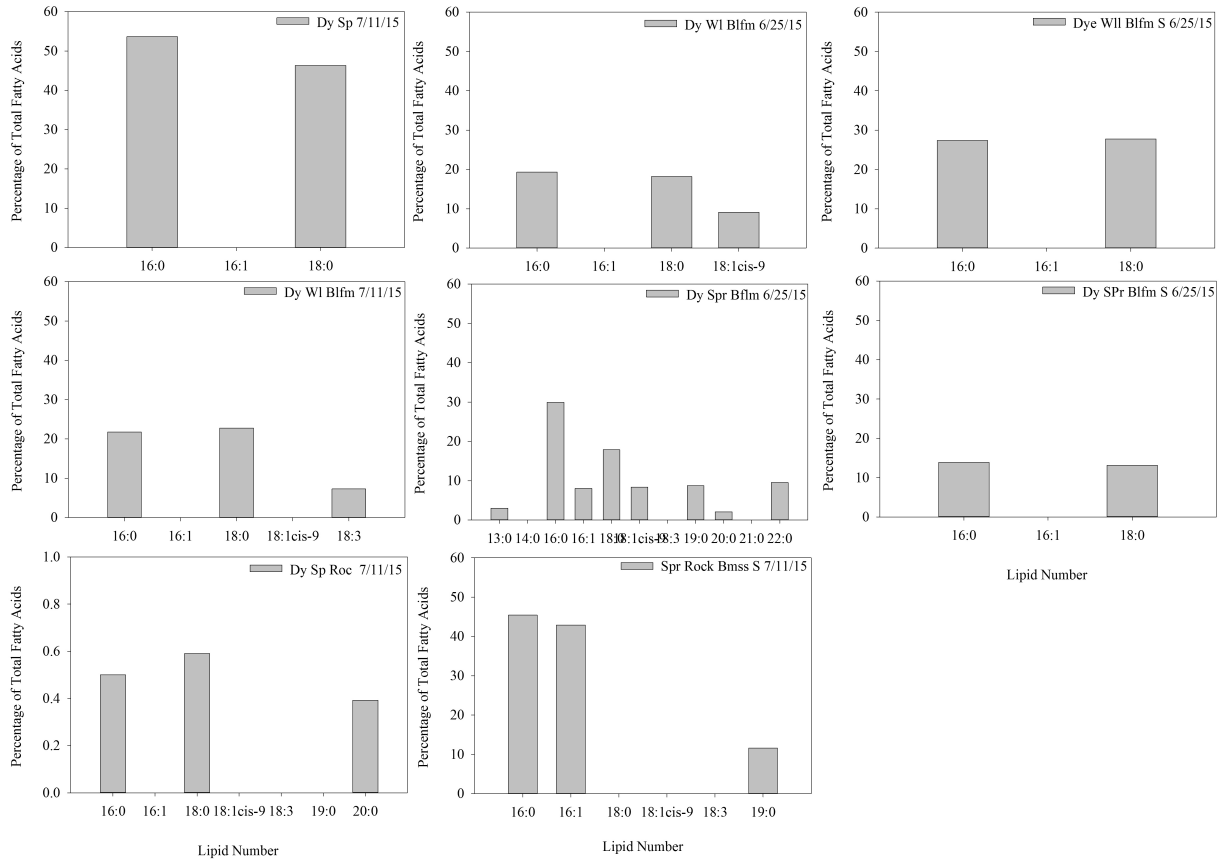


Figure 27 Fame distributions of biofilm samples collected from field biofilm study.

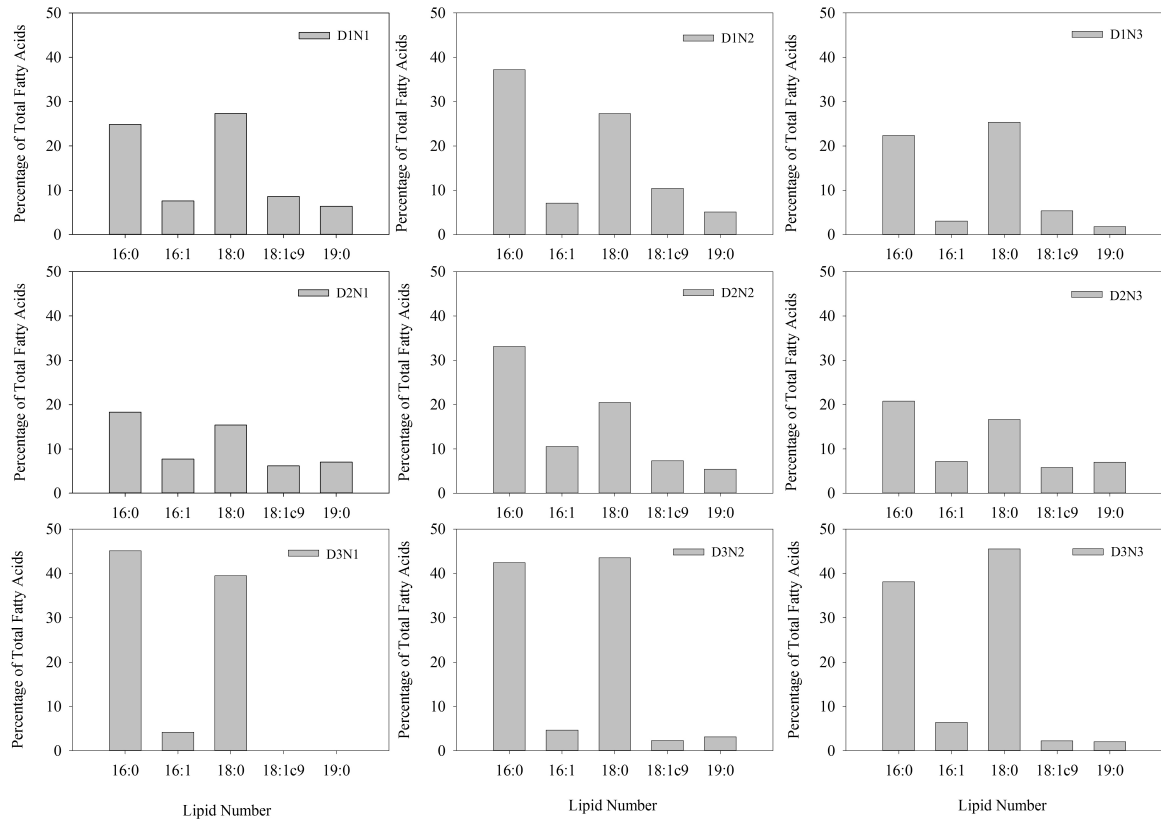


Figure 28 FAME distributions of biofilms harvested from nutrient study.

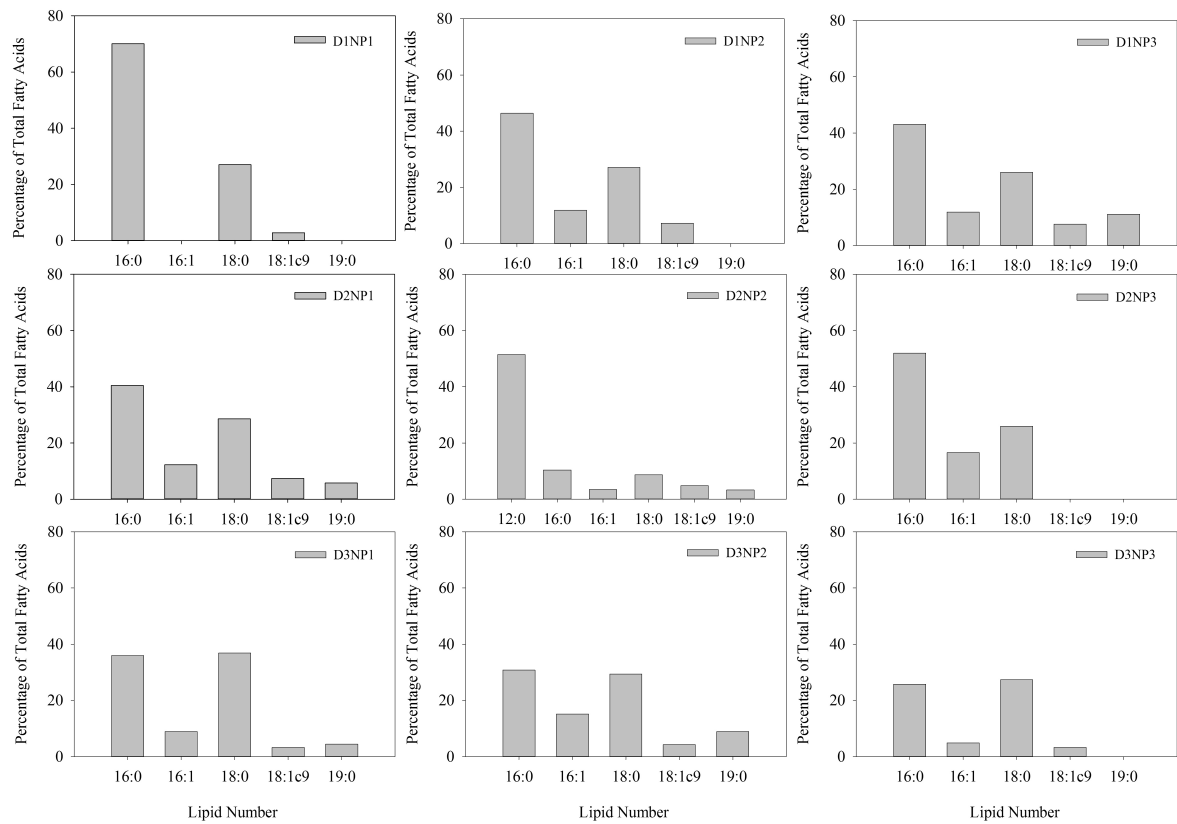


Figure 29 FAME distributions of biofilms harvested from nutrient study.

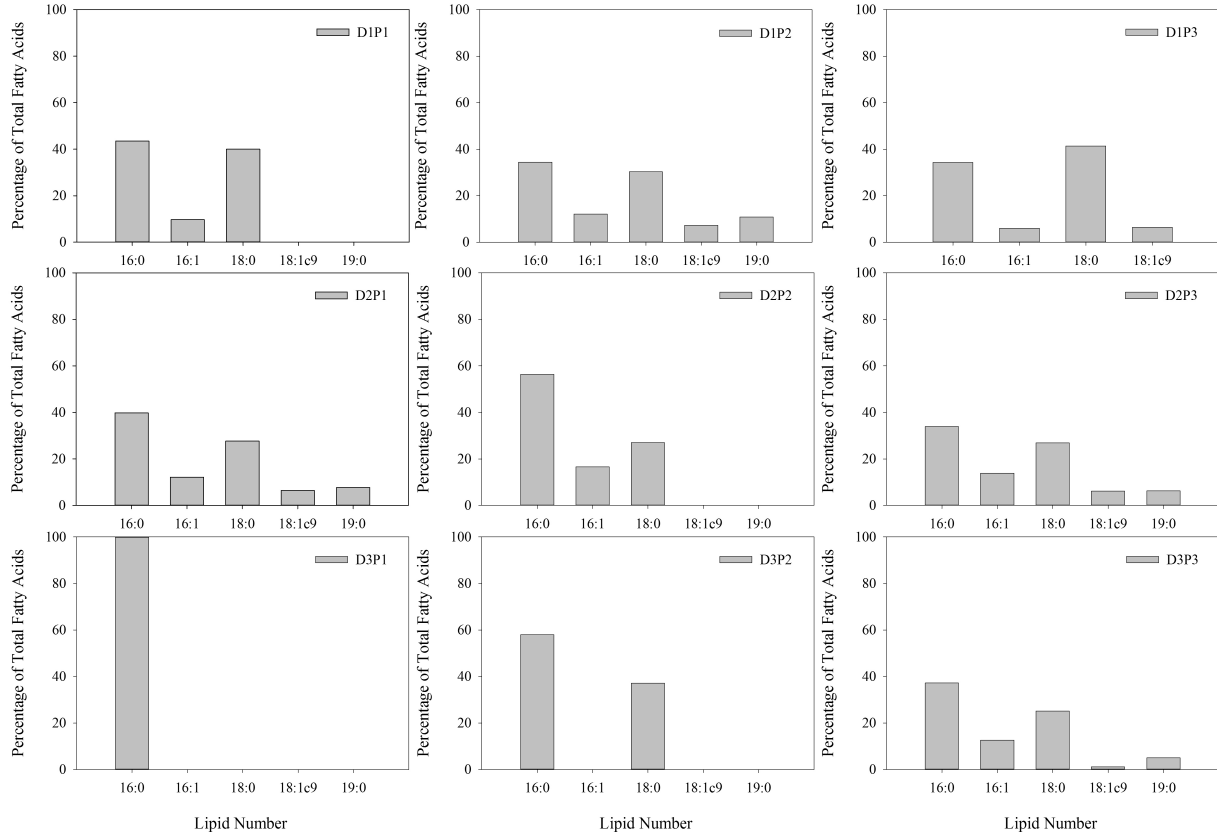


Figure 30 FAME distributions of biofilms harvested from nutrient microcosm study.

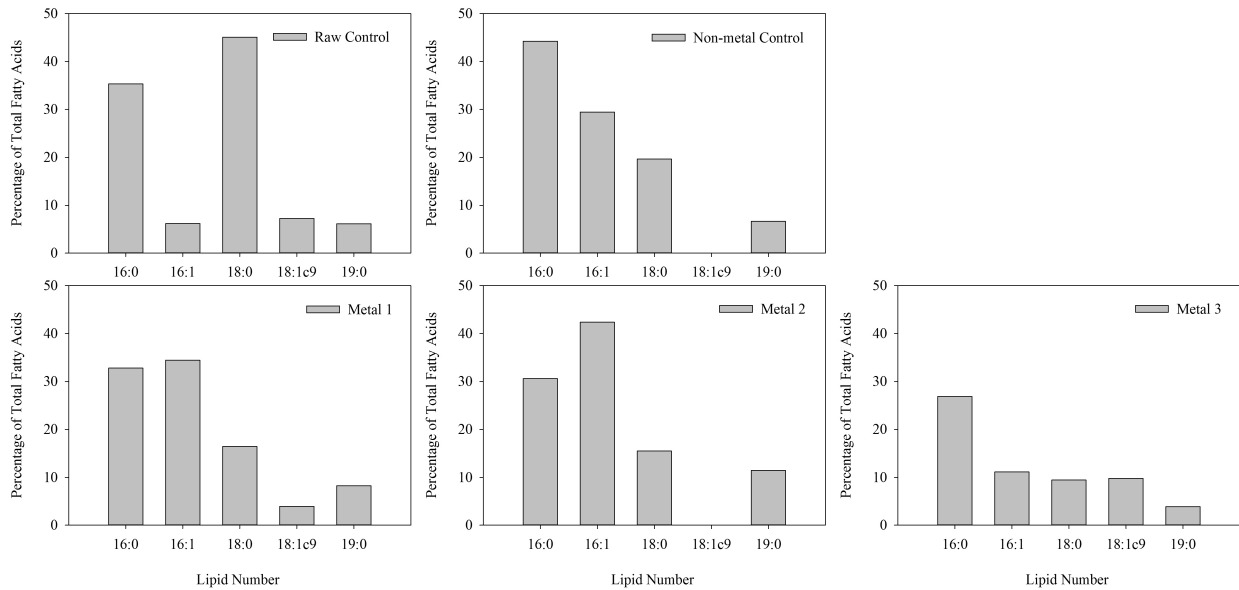


Figure 31 FAME distributions of biofilms harvested from metal microcosm study.

DISCUSSION

Antibiotic Sampling and Dose-Response Assay

The results of the dose response study provided data that showed that bacteria communities cultured from spring water samples were capable of being cultured in the lab on growth media augmented with tetracycline and erythromycin antibiotics. This finding is significant because it provides evidence that confirms the presence of antibiotic resistant bacteria in the discharge of this epikarst spring. Springs are great integrators of water chemistry originating from a number of environments within the recharge area of the spring. The epikarst spring recharge area is overlain mainly by pastures, but also contains small forested environments as well. The range of land uses and land cover types provides a broad range of organic matter, nutrients, metals, antibiotics and other chemical constituents. Antibiotics and metals receive particular attention in this study because of their agricultural use in the prevention of pathogens in concentrated animal feeding operations (CAFOs), and the resulting development of antibiotic resistance in microbial species.

In our study we observe tetracycline antibiotics to be more effective at reducing microbial populations in water samples collected from an epikarst spring and in surface-water samples. In unpublished studies, *E.coli* concentrations in the area of Big Creek and the spring are highly variable. Upstream of the CAFO and spring, concentrations of *E.coli* were typically greater in water samples than samples taken downstream near the confluence of Big Creek and the Buffalo River. A major factor in this trend is the increasing discharge in Big Creek near the Buffalo River, which increases the potential for dilution and overall decreasing *E.coli* concentration. Additionally, the movement of *E.coli* is also closely related to sediment movement and turbidity of streams, but the work of Pronk et al. (2007) disputes this idea in favor of particle size distribution as better indicators of sediment associated bacterial transport. Increasing sediment

loads and resulting turbidity of the water is positively correlated with *E.coli* concentrations. However, samples collected downstream of the confluence of Big Creek and the Buffalo River did show indication of resistance to 10 mg/L concentrations of tetracycline. Turbulent flow at the confluence of the two streams creates a mixing zone resulting in the resuspension of bacteria living in streambed sediments. Bacterial contributions from Big Creek and resuspension of bacteria from streambed sediments resulted in increasing bacteria concentrations downstream on the Buffalo River as well as increasing resistance to tetracycline. In a karst study looking at the transport of multi-antibiotic resistant *E.coli*, transport of the resistant bacteria was found to be closely related to rainfall events, demonstrating the role of overland runoff and leaching process in the transportation of antibiotic resistant bacteria (Laroche et al., 2010). The transport of this resistance was outside of the scope of this study, but the widespread use of tetracycline antibiotics and its natural occurrence among specific species of bacteria increases the probability that tetracycline resistance may also be observed further downstream.

Tetracycline antibiotics inhibit protein synthesis during the translation of mRNA (Schnappinger & Hillen, 1996). Tetracycline antibiotics were once widely used; however, due to the evolution of tetracycline resistant bacteria species their uses have become increasingly limited (Chopra & Roberts, 2001). There are three known resistance mechanisms associated with tetracycline antibiotic resistance: enzyme inactivation of tetracycline, efflux, and the generation of protective ribosomal proteins. Efflux and protective ribosomal proteins are the most prevalent resistance mechanism to tetracycline antibiotics. Efflux pumps are active transport mechanisms encoding for the synthesis of proteins that remove tetracycline and other toxins from the cell, thereby inhibiting the accumulation of toxins to toxic levels. Ribosomal protective proteins function by removing bound tetracycline from the ribosome allowing translation to continue. There are

bacteria species with inherent resistance to tetracycline antibiotics such as *Pseudomonas aeruginosa* (*P. aeruginosa*) via efflux and low permeability of the outer cell membrane (Nikaido, 1989; Ma et al., 1994). Additionally, observations of tetracycline and fluoroquinolone selected resistance has been observed in resulting increased efflux activity in the work of Cohen et al. (1989). However, tetracycline resistance is typically transferred horizontally, which increases the potential of transport of the resistance mechanism within biofilms.

Bacteria from all sites were resilient to erythromycin. Albeit bacteria counts decreased with increasing concentrations of erythromycin, bacteria cultures were able to grow at clinical dosages. Erythromycin is also a protein synthesis inhibitor, but it also affects the function and structure of critical proteins for life and the translation of tRNA and subsequent replication. Erythromycin is more effective at greater concentrations, as is confirmed in the results of the dose response study, but there is also a wide range of bacteria species with inherent resistance. Erythromycin belongs to a group of antibiotics known as macrolides. Macrolide antibiotics are typically used against gram-positive bacterial infections, but are effective against limited gram-negative bacterial infections (Halling-Sørensen, 2000). Macrolide antibiotics tend to be less effective against gram-negative bacteria species due to an inability of hydrophobic antibiotic molecules to diffuse across the outer cell membrane via porin channels (Nikaido, 1989). Sutcliffe et al. (1996) observed genetic indicators of not only erythromycin disabling resistance mechanisms, but also efflux resistance mechanisms in clinical isolates of three different strains of *E. coli*. This point is important to note because of the prevalence of gram-negative bacteria within the study area, most notably *E. coli*. The results of the study show significantly more erythromycin resistant bacteria grew at sampling sites that historically had greater concentrations of *E. coli* and fecal bacteria relative to the other sampling sites.

Fatty Acid Laboratory and Field Microcosms Analysis

The results of the FA study provide insight about the community structure of the biofilms when nutrient and energy sources are variable as well as under potential metal stress conditions. In our study we observed mainly long chain FAs with aliphatic tails ranging from 13-21 carbons long. Carbon chain length and the complexity of FAs present in samples provide a basis to determine whether biomass sampled contains prokaryote cells, or those belonging to higher organisms e.g. plants, mammals. Eukaryote cells are distinguished from eubacteria cells in that they contain polyunsaturated FAs, which are rare in prokaryote cells Madigan et al. (1997), and thus we observe contributions of bacterial cells with indications of both gram-positive and gram-negative communities. As a biomarker of gram-negative communities we look to the presence of MUFAs in the samples and their abundance relative to the total FA composition. The presence of MUFAs serve as an indicator of gram-negative bacteria since MUFAs typically compose less than 20% of FAs in gram-positive bacteria (Zelles, 1999). As observed in the results of the FA assay, gram-negative biomarkers are present, but their abundance relative to the total FA composition in samples appears to shift in reaction to DOC concentration, phosphate concentration, and metal concentration.

The results of both the nutrient and metal microcosm studies, we see good indication of gram-positive bacteria in the fraction of palmitic acid present in samples. As noted earlier, palmitic acid is abundant in the environment and we do see it even in abiotic controls; however, analysis of fatty acid methyl esters (FAMES) captures the FA composition of viable and non-viable cells. In the microcosms containing metals, we did observe a trend indicating that the fraction of gram-positive bacteria cells in the microcosms were slightly decreasing as metal concentrations decreased. When DOC and NO₃ were provided, we also see increases in both gram-negative and gram-positive bacteria biomarkers, suggesting increased productivity within the culturable

bacteria communities or a shift in community structure. Haldeman et al. (1995) report a similar finding, where community FA structure and biomass was statistically indeterminate, but culturable counts of bacteria increased steadily. Green & Scow (2000) concluded that the study conducted by Haldeman experienced this discrepancy either due to limitations of the FA study to capture the phenomena, or community changes and culture counts were not connected via FA data. The Green & Scow critique of the Haldeman study provides important contextual information for interpreting the results of our study, because often we saw no strong statistical evidence of communal shifts with exception to gram-negative and gram-positive bacterial species. To further elucidate the responsible phenomena and mechanisms responsible for changes in microbial communities exposed to excessive nutrients, metals, or antibiotics it may very well be more important to look to the ratio of gram-positive to gram-negative microbial communities. When interpreted in this context, the results show that when metals are present gram-negative biomarkers have a tendency to become larger fractions of the total FA composition in samples, and conversely when there is an imbalance of nutrients and energy source gram-positive indicators become more abundant. The field microcosm data provides some supporting evidence of the presence of both gram-negative and gram-positive bacteria comprising the microbial communities captured in biofilms, and provides an additional consideration to interpreting laboratory results in that we are not able to culture all bacteria and that the epilimnetic microbial community is much more diverse than what is reflected in observations of biomarkers from laboratory experiments. However, the results of the experiments do provide some indication of physiochemical and community changes that potentially could occur if conditions in the epilimnetic were to undergo similar perturbations.

CONCLUSION

The results of this study indicate antibiotic resistance and co selection of metal resistance in the epikarst is very complex, and is subject to a number of environmental factors such as the sediment transport in subsurface flowpaths and mixing of distinct surface water end-members. We were able to confirm that there is inherent antibiotic resistance in the study area, and based on the results of FA analysis, this may be largely attributed to the distribution of gram-positive to gram-negative bacteria. In biofilm samples collected from the spring there were biomarkers of dominant gram-negative bacteria species, although in nutrient and DOC studies gram-negative and gram-positive biomarkers were observed. Given the inherent resistance of many gram-negative bacteria erythromycin, and to a lesser extent tetracycline we concluded *E.coli* and *P.aeruginosa* were likely organisms displaying resistance to the antibiotics used in the dose response assay. The use of metals as alternatives to antibiotics may be beneficial, however FA biomarkers of gram-negative bacterial species increased under perceived metal stress, until toxic concentrations of metals were used and we observed a marked reduction in gram-negative and gram-positive bacteria. The study has confirmed that in the absence of antibiotic use, there still exist antibiotic resistant bacteria that also display resistance to metals. In parallel studies, we will analyze the function of these communities related to the transformation of DOC to DIC and in nitrate removal processes; however, this study has provided a small, but useful contribution to the evolution of antibiotic resistance and co-resistance in bacterial biofilms in the epikarst of Northern Arkansas.

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CHAPTER 5: CONCLUSIONS

This study used isotopes as tracers of biological processes effecting the evolution of water chemistry in the epikarst. Using biofilms as a focus, the primary intension of the study was to determine the impact of influent water chemistry on epikarst microbial communities, with particular emphasis placed on trace elements commonly found in the environment and those whose use is associated with animal feed. Overall, the study was able to identify conditions that were favorable for the proliferation of epikarst microbial species, as well as identifying potential circumstances that would reduce microbial impacts on water-quality.

DOC to DIC Conversion, Nitrate Removal, and Biomass Production

Natural sources of dissolved organic carbon will vary seasonally, and because of this seasonal variation, the epikarst environment receives a range of DOC species and concentrations.

Depending on the complexity of DOC species these molecules may have rather long residence times in the subsurface environment, which implies a comparable exposure time for the microbial biofilms in subsurface flowpaths. More labile DOC species will promote biomass production in the epikarst. In laboratory microcosm studies conducted, more biologically derived DIC was produced as the concentration of DOC increased. Conversion of DOC-DIC is an indicator microbial productivity, and as observed in laboratory experiments, bacteria were more productive when adequate concentrations of DOC were available. A secondary indicator of microbial metabolic processes was the concentration of dissolved oxygen. Terminal electron acceptors are used by bacteria to metabolize DOC and the subsequent conversion of DOC to DIC. Oxygen is the most efficient electron acceptor and necessary for aerobic respiration to occur, and thereby is the first choice of obligate aerobic bacteria or facultative anaerobic bacteria. Temporal observations of DO concentration from the laboratory microcosms showed a trend of decreasing DO concentration over time, providing further indication of respiration.

However, the trends of DO concentration, DIC production, and the isotopic composition of DIC were statistically independent of nutrient species and concentrations. Microbial respiration in the epikarst was first limited by the concentration of DOC.

The second microbial process observed in this study, denitrification, was observed using isotopic tracers as well as nitrate concentrations measured in water samples from the microcosms.

Denitrification is a process that culminates in the removal of NO_3 from the water column, but requires anoxic conditions and neutral pH. Based on these two conditions for the onset of denitrification, denitrification was not observed equally across all DOC and nutrient microcosm treatments. Denitrification was most prevalent in microcosms treated with more than 100 mg/L of DOC and 1 mg/L NO_3 . This is a significant finding because it indicates that nitrate removal potential in the epikarst will occur when DOC concentrations are relatively high e.g. late fall and winter. Increasing DOC input into the epikarst drives the biological demand for oxygen and provides conditions necessary for denitrification removal to occur. Conceptually, the combination of available labile DOC and appropriate nutrient loading must occur in concert to achieve maximum impact on water-quality in the epikarst with regards to NO_3 removal. Biomass production will also follow a similar trend. Biomass collected from microcosms also suggests that DOC quantity and biomass production trended positively with one another, meaning more biomass was produced at higher DOC concentrations.

Synthesizing the findings of this study to provide a conceptual model to a larger picture, consideration to precipitation regime in the northern Arkansas must also be considered.

Precipitation in Arkansas occurs primarily in two seasons, spring and late fall. During spring and late-fall more precipitation results recharge to the epikarst and groundwater occurs; however, during the spring vegetative growth on the surface reduces the volume of water going to recharge

and therefore larger portion of groundwater recharge occurs in the winter when vegetative growth and losses associated with evapotranspiration is minimal. In the winter, when the bulk of recharge is occurring the epikarst will also receive an increased flux of DOC derived from leaf litter and other detritus. Adding to the winter influx of DOC to the epikarst is reduced soil bacteria activity, due to falling day and night time temperatures, which slows microbial processes. The flux of DOC, particularly labile DOC, will be metabolized and converted to DIC and utilized to produce more biomass. More microbial biomass increases the potential for DOC conversion to DIC and nitrate removal when conditions are favorable.

Metal toxicity and nitrate removal

Several heavy metals and trace elements have been documented to possess bacteriocidal, antiviral, and bacteriostatic characteristics which has led to their use in animal feed and veterinary pharmaceuticals. Additional to the use of metals to prevent the spread of pathogens, metals may also be used to encourage livestock to reach maturation more quickly and to increase dressed weight of livestock. Heavy metal and trace element exposure of metals in the subsurface occurs from a number of processes. Leaching of heavy metals from local geology and soils, road dust, wet and dry deposition from winds and precipitation events are all transport vectors of metals into subsurface flowpaths. In each case, these dissolved or particulate metal and trace element species will have some impact on the evolution of water chemistry and specifically the microbial ecology of biofilms within subsurface flowpaths. Many trace elements such as Zn, Se, and Cu are micro-nutrients for many bacteria and other organisms, however exposure time and concentration can move these trace constituents from nutritional for organisms to toxic for organisms. Secondary to the concentrations of trace constituents necessary for life is the transport vector of these constituents to bacteria communities biofilms and the role of organic matter. Based on laboratory studies using varying concentrations of labile DOC, NO₃, and PO₄,

concentrations of the ideal DOC and nutrient treatment to render the most biomass production, while reflecting epikarst conditions observed in the field were used to assess the effect of increasing concentrations of a suite of metals on microbial respiration and denitrification activity.

Isotopic tracers were again used to assess DOC-DIC conversion, and denitrification, as well as concentration observations of DIC, NO₃, DO, and (Cd, As, Pb, Zn, and Cu). The results of the experiment concluded that metals were effective at slowing bacterial processing of DOC and NO₃. The epikarst spring water sample used in this laboratory microcosm experiment contrasted the original nutrient study, in that the conversion of DOC to DIC was very gradual. Indications of microbial respiration began more than 24 hours after the initialization of the experiment in microcosms treated with only 100 mg/L DOC, 1 mg/L NO₃, and in microcosms with the same treatment of DOC and NO₃ with an additional 1 µg/L of trace and heavy metals. After approximately 48 hours these microcosms with DOC, NO₃, and metals began producing biologically derived DIC, isotopic fractionation of DIC showed enrichment of the δ¹³C-DIC in the heavier ¹³C isotope, as well as DO concentrations declining to micro-aerobic conditions. Because DO concentrations did not reach anaerobic conditions during the period of the experiment, denitrification was not observed in the experiment, even in microcosms that showed respiration activity.

Metal concentrations in the experimental data provided evidence suggesting many complex interactions occurring in the microcosms. In cases where microbial respiration was observed, Pb concentrations were closely correlated with dissolved iron concentrations. Dissolved lead concentrations increased as the concentration of dissolved iron in the microcosms increased. This result confirms the close relationship between Pb and Fe oxyhydrides that form as ferrous iron

(Fe²⁺) is oxidized to ferric iron (Fe³⁺). The transition between iron species produces particulate iron that has a high affinity for surface adsorption of Pb, subsequently removing lead from the water column. However, over time as geochemical conditions in the microcosms are altered by the gradual increase in microbial activity. Redox conditions in the microcosms shift from oxidizing to reducing, which explains observations of gradually increasing Pb concentrations later in the experiment. Competition between metals for active sites on enzymes and the formation of organometallic compounds with DOC was also indicated in the results of this experiment. The affinity of three metals Cu<Ni<Co to bind to organic molecules was indicated by looking at the concentration ranges of their concentrations over the course of the experiment. Copper displayed a greater affinity to bind either to DOC, biofilm, and intracellular structures with biofilms produced during the experiment. This finding is important because it is an indication of potential inhibition of denitrification reductases, several enzymes responsible for the regulation of denitrification. Lastly, a likely the most significant finding of this study, was the temporary nature of apparent metal inhibition. As described in literature, one response to toxic metal exposure would be a physical buffering between viable bacteria cells and dead bacteria cells. The slow reactivity in the microcosms in the initial hours of the experiment was due to shock from the metal exposure; however, bacteria appear to rebound and in time potentially fully recover. The response described might be the first indicator of metal tolerance of bacteria in the epikarst spring water samples; a model of what could be expected of microbial biofilms communities exposed to toxins, as well an indicator of shifts in community structure and activity.

Biomass FAME Analysis and Antibiotic Resistance

The objective of this study was to identify bacteria communities present in biofilms grown in the controlled environment of the laboratory and in in-situ field experiments. The study utilized data gathered from previous antibiotic resistance studies to demonstrate the resistance of bacteria

collected from water samples from an epikarst spring to doses of the antibiotics erythromycin and tetracycline. FAMES extracted from biomass collected from the field and those harvested from laboratory microcosms were compared and contrasted to identify biomarkers of predominant community structure within biomass samples. Biomass samples were taken from two laboratory experiments using a water sample collected from the same epikarst spring. The first laboratory microcosms was designed demonstrate concentration effects on microbial response to highly labile DOC, NO₃, and PO₄. The second microcosm study conducted in a laboratory setting demonstrated toxic effects of metals on microbial respiration and denitrification activity. A third study was designed to collect wild-type biofilms growing in the flow path of the spring and at the spring orifice.

The antibiotic dose-response study demonstrated that many bacteria in the study area had a tolerance, or in some cases a resistance to the antibiotics erythromycin and tetracycline. Water samples collected and analyzed for a suite of antibiotics returned negative results for the presence of the antibiotics used in the dose-response study as well as many other antibiotics and antibiotic degradation products. Therefore, antibiotic occurrence, at the time of sampling, could not be related to the presence of erythromycin and tetracycline tolerant bacteria communities. FAME analysis of biomass grown in the laboratory and in the field provided some insight as to what communities of bacteria were present in water samples from the epikarst, displaying resistance to antibiotics. Comparison of the results of the FAME analysis from the three groups of samples confirm that samples taken from biomass grown in the field had a greater range of short to long chain fatty acids comprising the total fatty acid distribution within the samples when compared to fatty acids extracted from biomass grown in laboratory microcosms. The larger more complex fatty acids observed in field samples are likely due to input from higher

species eukaryotic species and plants, however there were indications of the presence of sulfate reducing bacteria, as well as strong indication of gram-negative bacteria. FAME distributions from those microcosm not using metal exposure as a variable were very tightly constrained to just to primarily medium to long chain FAs, primarily saturated fatty acids such as stearic and palmitic acid. Both stearic and palmitic acid are among the most abundant observed in the environment; however, the closed nature of the microcosms experiment limited the presence of saturated fatty acids observed in the biomass to predominately gram-positive bacteria species.

In contrast, the biomass collected from microcosms using metal exposure as a variable showed a slight larger range of FAs, particularly monounsaturated FAs that may be used as indicators of the presence of gram-negative bacteria species. This is the most significant finding of this study because it provided a plausible explanation for erythromycin resistant bacteria in spring—water samples. Erythromycin, a macrolide antibiotic, is hydrophobic which makes it difficult to cross the cell membrane via porin channels, which renders this class of antibiotics minimally effective against gram-negative bacteria. In addition to the hydrophobic nature of macrolide compounds, the pore sizes of many gram-negative bacteria are too small for these hydrophobic antibiotic compounds to break the cell membrane and enter intracellular space. Tetracycline resistance was only observed in samples taken downstream on the Buffalo River, however, common gram-negative bacteria such as *P.aeruginosa*, have documented inherent resistance to tetracycline antibiotics due to the low permeability of the cell membrane as well as active efflux mechanisms, which export tetracycline and other toxins e.g. metals from inside bacteria cells.

Implications

In Northern Arkansas animal husbandry is a major contributor to the regional economy, but natural resources also contribute to the regional economy by way of tourism. Protecting these resources and enacting effective legislation, and promoting education on water-water quality and human interaction with such are essential to a sustainable economic future for the region. The work presented in this dissertation reflects conditions in groundwater flowpaths under current land management and land use circumstances. However, studies conducted in laboratory environments only provide a small glimpse into processes occurring in the wild environment. This dissertation demonstrated the response of bacteria to labile carbon and increasing nutrient concentrations, which was to increase biomass and have an increasing impact on the evolution of sub-surface water quality and subsequently surface water-quality. For the natural environment increasing availability of labile DOC will result in more biomass present in subsurface flowpaths and an increasing biological influence on water chemistry, and potentially leading to eutrophic subsurface conditions if not managed properly. The seasonality of DOC and recharge in any given watershed must be considered in addition to peak nutrient demand for pastures, and in this respect this dissertation provides additional considerations of resource managers and land managers in regions with karst.

Antibiotic resistance in karst regions is a critical issue because of the rapid and expansive scale upon which karst flowpaths disseminate solutes and other materials. Conceptually, recharge to epikarst is greatest during winter months, which happens to coincide with relatively large flux of DOC and nutrients to the epikarst. Organic antibiotic compounds, inorganic metal compounds, and organometallic compounds have the greatest potential to breakthrough to groundwater. Through the remainder of the year, bacteria are exposed to pockets of this influent water, leading to increasing biomass production and the proliferation of resistant organisms. Under the accepted

morphological model for the maturation of biofilms, genetic material and viable cells will disperse throughout the karst flow system transporting antibiotic throughout the subsurface and potentially the surface flow system as biofilm communities mature. Antibiotic resistance has the potential to be highly mobile in karst environments, scalable with other contaminant transport models in karst across varying temporal scales. But moreover, this study speaks to the relevance of managing OM as it is the primary vector of transport for antibiotics, metals, and bacterial cells.