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The Role of Organic Matter in the Fate and Transport of Antibiotic Resistance, Metals, and Nutrients in the Karst of Northwest Arkansas

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The Role of Organic Matter in the Fate and Transport of Antibiotic Resistance, Metals, and Nutrients in the Karst of Northwest Arkansas

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by

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ABSTRACT

Organic matter (OM) in the environment acts as a nutrient, but may also act as a transport vector for harmful chemical compounds and bacteria. Acetate is a labile form of OM produced during fermentation in anaerobic lagoons used to store animal fecal-waste from concentrated animal feeding operations (CAFOs). Dry and liquid fertilizers from CAFOs pose a threat to groundwater by introducing excessive amounts of nutrients (e.g. OM, nitrate and ammonia), metals, and antibiotic compounds. In the epikarst of Northern Arkansas in the Buffalo River watershed additional input of labile dissolved organic carbon (DOC) from liquid CAFO waste-fertilizers was hypothesized to increase microbial activity along groundwater flowpaths. In addition, high metal and antibiotic concentrations associated with increasing concentrations of DOC were hypothesized to be detrimental to microbial processes, with exception given to resistant bacteria species. Laboratory microcosm experiments were conducted to characterize microbial DIC production and denitrification. The microcosms were treated with acetate, nitrate, phosphate, and/or various metal species to characterize concentration effects on microbial activity. Field studies were used to calibrate laboratory conditions, and to compare biomass production and composition. Isotopes of dissolved inorganic carbon (δ^{13}C-DIC), nitrate (δ^{15}N-NO_3), and dissolved oxygen (DO) were used to assess microbial responses to increasing DOC concentrations. Fatty acid methyl ester (FAME) analysis was used to characterize biomass produced during the experiments.

Conversion of DOC-DIC and decreasing NO_3 concentrations were observed in the microcosms. Microbial productivity was greatest when DOC concentrations were 10 times greater than NO_3 concentrations. When metals were added to the microcosms, microbial activity was

inhibited with exception being microcosms containing metal concentrations below 10 µg/L. FAME biomarkers indicated gram-negative bacteria were present in biomass samples from the spring orifice and metal-treated microcosms, but microcosms amended with nutrients and DOC displayed indicators of predominantly gram-positive bacteria. Critical findings of this study were: bacteria species transported in spring discharge were resistant to antibiotics and metals, high concentrations of DOC and nitrate increase biological productivity in epikarst and metal exposure inhibits nitrate removal and causes ecological shifts in biofilms selecting for resistant bacteria strains.

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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

Pharmaceutical, heavy metal, nutrient, and biological constituents from Concentrated Animal-Feeding Operations (CAFOs) pose a potential threat to soils, groundwater and surface-water quality. Elevated nutrients may lead to eutrophic conditions, fish kills, and increased mortality among macroinvertebrates inhabiting or foraging in surface-water and groundwater ecosystems. Nitrate 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 concern (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 and Brown, 2003). High concentrations of dissolved organic matter (DOM), 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 focuses the role of carbon and nitrate cycling in antibiotic resistance (AR) in karst groundwater. The study applied multiple parameter approach targeting biofilms in karst flowpaths as environments harboring AR and as active mechanisms in the cycling of carbon and other essential nutrients. The objective of this research is to document the control of water-quality on microbial biofilm development in a karst environment. Biofilms 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 (OC) loading on microbial biofilm growth, nutrient assimilation, and metabolic response to environmental stressors in karst environments using a series of indicators.

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 (2012b), 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. 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 have 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 are not limited to organic matter (OM), other nutrients (e.g. nitrate, ammonia, and 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 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 conduits 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). 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, processing, and transport of other solutes such as endocrine-disrupting compounds (Yamamoto et al., 2003) and metals (Seiler and Berendonk et al., 2012).This dissertation focuses particularly on determining the impact of DOM concentration and species on the cycling of carbon and nutrients in biofilms and characterizing the role of DOM in transport of metals and antibiotic compounds. Formation of organo-metallic complexes and adsorption of metals by particulate OM occur commonly in aquatic and soil environments. The species of OM 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 CAFOs (Watanbe 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, such as synthetic antibiotics, 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).

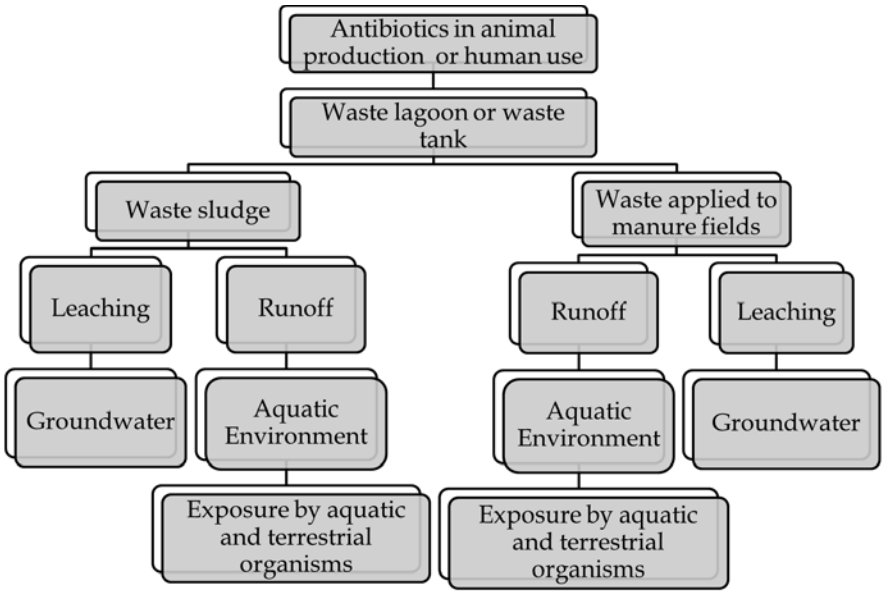

Figure 1 Fate and transport pathways of anthropogenic antibiotics in the environment

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 antibiotic resistant bacteria (ARB) and antibiotic-resistance genes (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 organic and 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 to be 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 OM 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 subjected to selective 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.

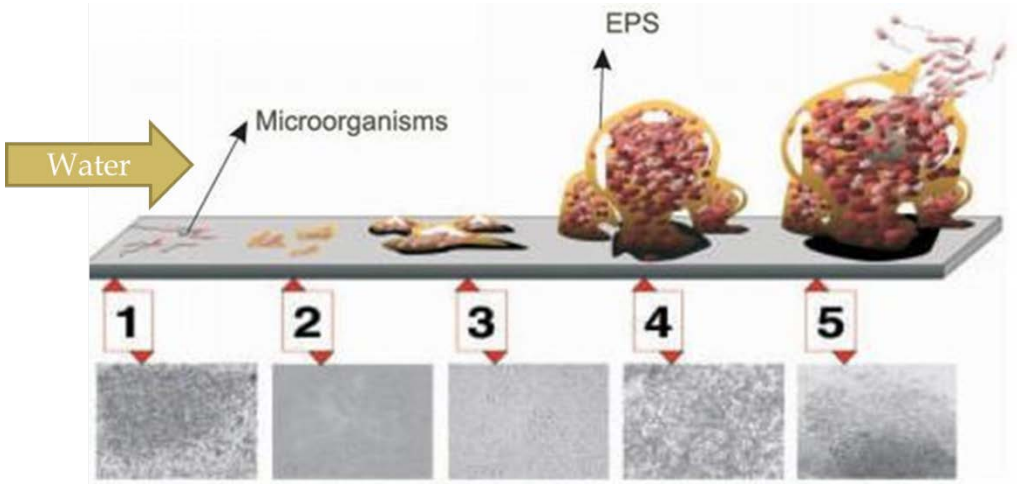

Figure 2 Five-stage microbial biofilm morphology diagram, describing the development of microbial biofilms (adapted from Monroe, 2007).

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 not only gives biofilms their slimy consistency, but provides protection and nutritional advantages for the bacterial communities living within the biofilm. Within biofilm communities, individual bacteria cells are protected from predatory organism such as protozoa, they are able to exchange genetic material with nearby bacterial cells allowing selection and propagation of desirable traits relative to growth environment conditions, and the exchange nutrients with neighboring bacteria cells. Water chemistry is a control on the microbial composition of biofilms, and of particular interest to the researchers are the effects of antibiotic

and metal species and concentration on the compositions of biofilms and activity of biofilm bacteria populations. Resistance mechanisms include limited diffusion of antibiotics across and 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). ARGs 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 into the extracellular environment (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 AR response in the absence of antibiotics (Alonso et al., 2001). Aquatic environments have been referred to as reservoirs of AR because of the diverse occurrence of antibiotics, ARGs, 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 OM, fewer nutrients, and toxic levels of antibiotics or metals) should cause changes in the communities of biofilms as observed in biofilms. This dissertation contributes data relating to changes in nitrate removal under varying metal exposures in karst environments. 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 ARGs and substrate for downstream bacteria. The

contribution to the fate and transport of AR is outside of the scope of this research and 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 (Liu et al., 2006; Wagner, 2007). Similarly detailed studies phosphorus and OC 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 NO_3 (Bengtsson and Annadotter, 1989). Bengtsson and Annadotter (1989) 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. 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, general chemistry, 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 in a marshy wetland to identify $^{15}NO_3$ that originated from groundwater, and concluded that denitrification accounted for the largest loss of $^{15}NO_3$ 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 $^{15}NH_4$ and $^{15}NO_3$ as both contaminants were rapidly degraded along the subsurface flowpath (Clark et al., 2008).

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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, $\delta^{15}N$ labeled-nitrate, and $\delta^{13}C$ 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), $\delta^{15}N$-NO_3, and $\delta^{13}C$-DIC. Microbial activity decreased rapidly after 3 weeks in all microcosm treatments as observed in $\delta^{13}C$ –DIC and dissolved oxygen data. The longest sustained microbial activity occurred in microcosms amended with 100 mg/L $\delta^{13}C$-acetate. The rate of biological inorganic carbon production in microcosms amended with less than 10 mg/L of acetate was statistically indistinguishable from one another. Treatments of phosphate and nitrate had no statistically significant ($p<0.05$) effect on biological inorganic carbon production with the only exception imparted to microcosms with 10 mg/L of both nitrate and phosphate and 100 mg/L acetate. Denitrification along with other processes was indicated in the results of $\delta^{15}N$ 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. Increasing labile organic carbon concentration increases the dissolved oxygen demand, and production of inorganic carbon, namely CO_2 increases. Substantial quantities of microbial biomass resulted from increasing acetate and nutrient concentrations. Increasing biomass is a response and factor in the evolution of surface-water and groundwater chemistry, and 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 nutrients, particularly OM. This practice achieves three major benefits for CAFOs; providing a receptor for waste, providing an economically benefit from waste, and supporting forage and hay-crop productivity for grazing livestock, and these benefits have been shown to be attainable with minimal/acceptable environmental effects in many environmental and hydrologic systems with application of effective waste management plans. However, nutrient migration occurring with retention-structure leaks and over fertilization, and spread of pathogens, veterinary pharmaceuticals, and trace metals are contamination risks for surface and groundwater. CAFO waste effluent in ponds and field application pose a threat to groundwater and surface-water in Northern Arkansas. The geologic setting of the region may be characterized as highly fractured, weathered, and karsted mixed-carbonate terrain which leads to widespread groundwater and surface water interaction. Biological treatment is often preferred over chemical and physical treatment processes when treating CAFO waste effluent because it is as a more cost effective method of managing waste, biological waste water treatment processes occur naturally under appropriate conditions, and because of the complex nutrient rich composition of CAFO waste effluent.

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 (VFAs). VFAs are 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). VFAs 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 of surface waters, gastrointestinal cancers, and methaemoglobinaemia (“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; hence, it is used in 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 (CO_2) and in many cases OM oxidation is coupled with other microbial terminal electron acceptor processes (TEAPs) 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 (SRB) 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 (BOD) during biological respiration. Increasing BOD and microbial respiration provide metabolic energy and contribute to creating anaerobic conditions necessary for denitrification. The input of residual OM from the soil zone into shallow groundwater in epikarst zones is advantageous because OM provides necessary substrate to an environment that is seasonally energy deprived; however, the presence of OM 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, veterinary pharmaceuticals, and other compounds mobilized in the soil, and the potential eutrophication of surface-water interacting with groundwater.

This study focuses on characterizing the control of DOC as an example of residual OM on denitrification and resultant microbial nitrate removal and biomass production in an epikarst-spring water sample. This study is part of a larger effort aiming to assess the role of OM in the transport and fate of antibiotics and the occurrence of antibiotic resistance (AR) 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 objective of this project is to develop a conceptual model describing the controls of nutrients, namely DOC as a surrogate of OM and nitrate, on microbial metabolism and productivity by: (1) to characterizing changes in microbial metabolic activity based on DOC concentration using laboratory microcosm studies, (2) to characterizing the effect of DOC concentration on nitrate removal, (3) to quantifying changes in biomass production

under different DOC and nutrient conditions.

METHODOLOGY

Site Description

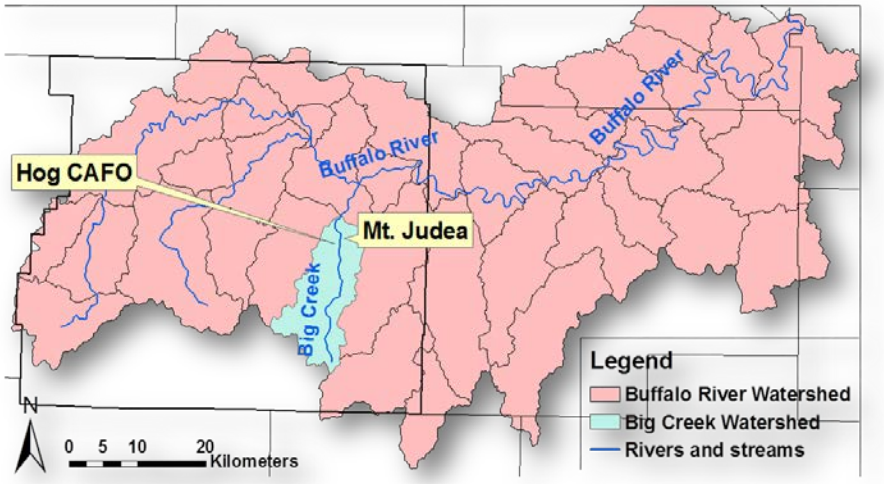

Figure 3Buffalo River Watershed (pink) Big Creek Watershed (aqua)

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.

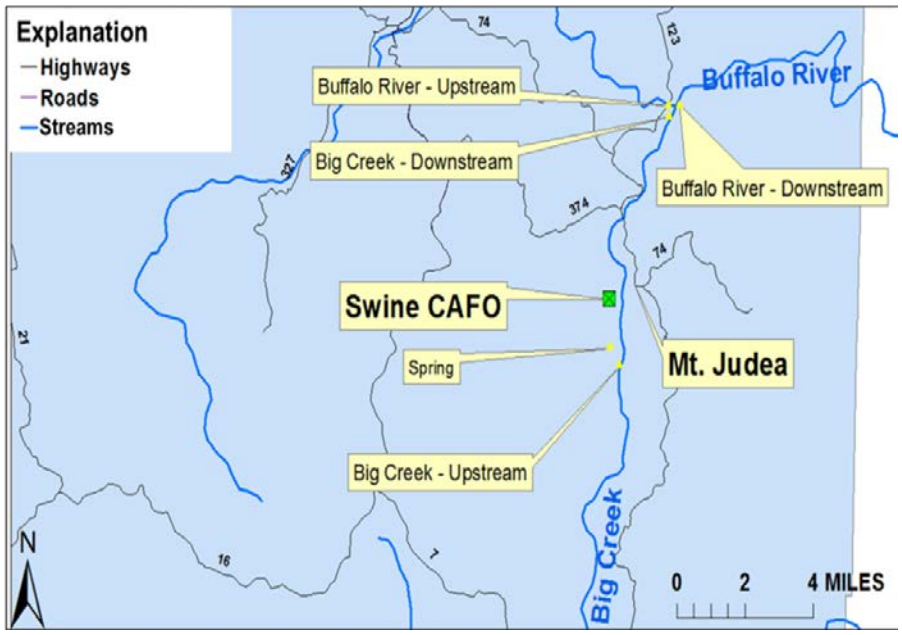

Figure 4 Sampling locations and location of CAFO

Land cover in the recharge area of the spring consists of agricultural pastures and forested areas. Three large fields lie in the recharge area of the spring, two of which are sprayed with manure slurry from the CAFO.

Temperature, pH, specific conductance (SC), and dissolved oxygen (DO) measurements were taken at the time of sampling. Water quality samples were collected in HDPE or PTFE sample bottles at the locations denoted in Figure 2. Samples analyzed for total nitrogen (TN) and total phosphorus (TP) 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. TP and TN were simultaneously analyzed using alkaline persulfate digestion (APHA, 4500-Pj). Sulfate analysis was conducted using barium sulfate turbidimetric method (USEPA 375.4). Ammonia analysis was conducted using the salicylate-hypochlorite method adapted from Reardon and others (1966). Biological water-quality samples were collected in PTFE 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 epikarst-conditions. Gravel was baked at 550°C for 5 hours to sterilize 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‰ $\delta^{15}N$ and $\delta^{13}C$, respectively. The microcosms were sampled weeks 1 – 3 and at week 11. Phosphoric acid conversion of DIC to CO_2 was used to measure $\delta^{13}C$ –DIC. Conversion of available nitrate to N_2O gas by the bacteria *Pseudomonas aereofaciens* was used to measure $\delta^{15}N$-nitrate. Nitrate isotope values were measured relative to ambient air, and $\delta^{13}C$ –DIC were reported versus the Vienna Pee Dee Belemnite (VPDB) standard. Dissolved oxygen was also measured using the Winkler titration method.

	DOC 1 (1.0 mg/L); D1	DOC 2 (10.0 mg/L); D2	DOC 3 (100.0 mg/L); D3
NO_3 1 (0.1 mg/L); N1	D1N1	D2N1	D3N1
NO_3 2 (1.0 mg/L); N2	D1N2	D2N2	D3N2
NO_3 3 (10.0 mg/L); N3	D1N3	D2N3	D3N3
NO_3 + PO_4 1 (0.1 mg/L); NP1	D1NP1	D2NP1	D3NP1
NO_3 + PO_4 1 (1.0 mg/L); NP2	D1NP2	D2NP2	D3NP2
NO_3 + PO_4 1 (10.0 mg/L); NP3	D1NP3	D2NP3	D3NP3
PO_4 1 (0.1 mg/L); P1	D1P1	D2P1	D3P1
PO_4 2 (1.0 mg/L); P2	D1P2	D2P2	D2P2
PO_4 3 (10.0 mg/L); P3	D1P3	D2P3	D3P3

Table 1 Laboratory microcosm study treatment plan.

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 spring groundwater discharge pH was 7.07 ±1.14 ranging from 6.38 to 8.38. Water temperature at the spring during

summer sampling was on average 17.73 ±0.25°C, and 13 ± 2.45°C in the winter. Mean specific conductance of the spring discharge was 413 ± 19.7°C. Dissolved oxygen at the spring remained above anaerobic levels year round. The concentration of dissolved oxygen was lowest at the spring when compared to measurements taken at surface water sites; however, water at all sites was aerobic.

Biological water-quality data are presented in Figure 5. 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. No statistically significant difference was determined for bacteria concentrations at upstream and downstream sites on the Buffalo River.

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 2 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

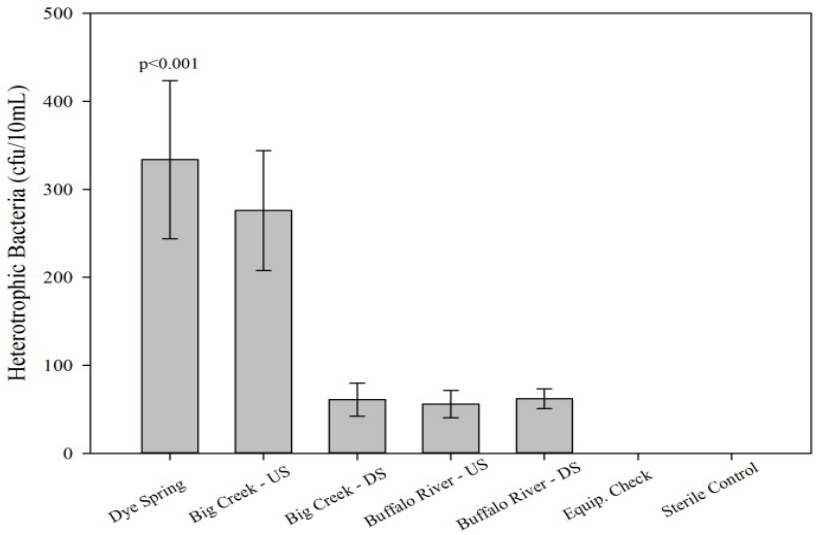

Figure 5 Heterotrophic Bacteria Concentration in biological water quality samples, shaded bar represents mean concentration, and error bars represent the ± standard deviation

Laboratory microcosms study

At time zero in the beginning of the experiment the average DO concentration in all microcosms was 8 mg/L (Figure 6). 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.

In the microcosms the concentration of DO was significantly impacted by the concentration of DOC in microcosms ($p=1.72x10^{-8}$) (Figure 7). Individual treatments of nitrate, phosphate, or a

combination of nitrate and phosphate had no significant effect on DO concentration (p=0.946) (Figure 8); however, DOC appears to show some control because DO concentrations were significantly lower throughout the experiment for microcosms amended with greater concentrations of DOC. In the microcosms with the lowest amended DOC, 1.0 mg/L DOC mean DO concentration was 0.78 mg/L and 2.9 mg/L greater, respectively, than microcosms with 10 mg/L (p=0.21) and100mg/L amended DOC (p<0.05) (Figure 7). Concentrations of DO were significantly greater by 2.1 mg/L (p<0.05) in microcosms with 10 mg/L DOC than in microcosms with 100 mg/L.

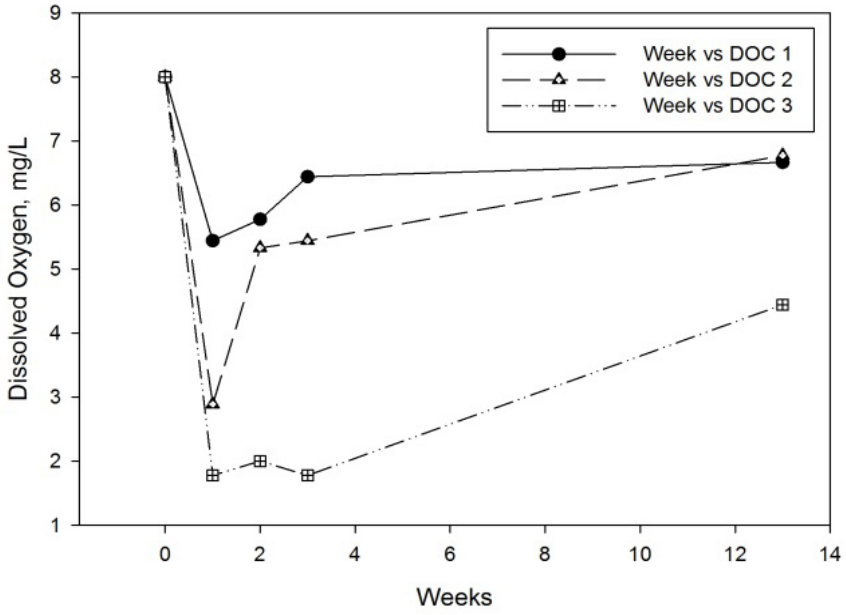

Figure 6 Results of dissolved Oxygen Analysis; DOC 1: 1.0 mg/L DOC, DOC 2: 10.0 mg/L DOC, and DOC 3: 100.0 mg/L DOC

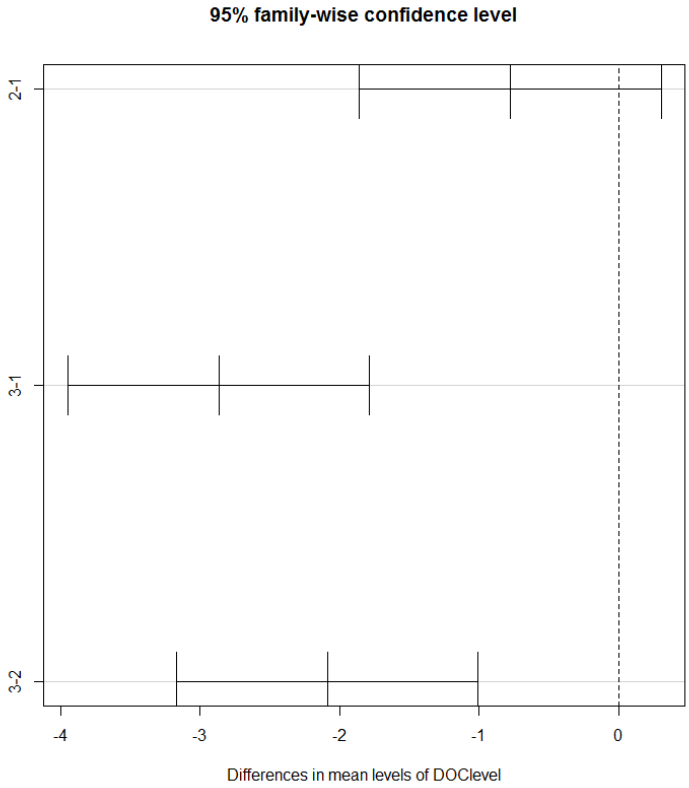

Figure 7 Difference in mean dissolved oxygen concentrations by DOC treatment; DOC 1: 1.0 mg/L DOC, DOC 2: 10.0 mg/L DOC, and DOC 3: 100.0 mg/L DOC

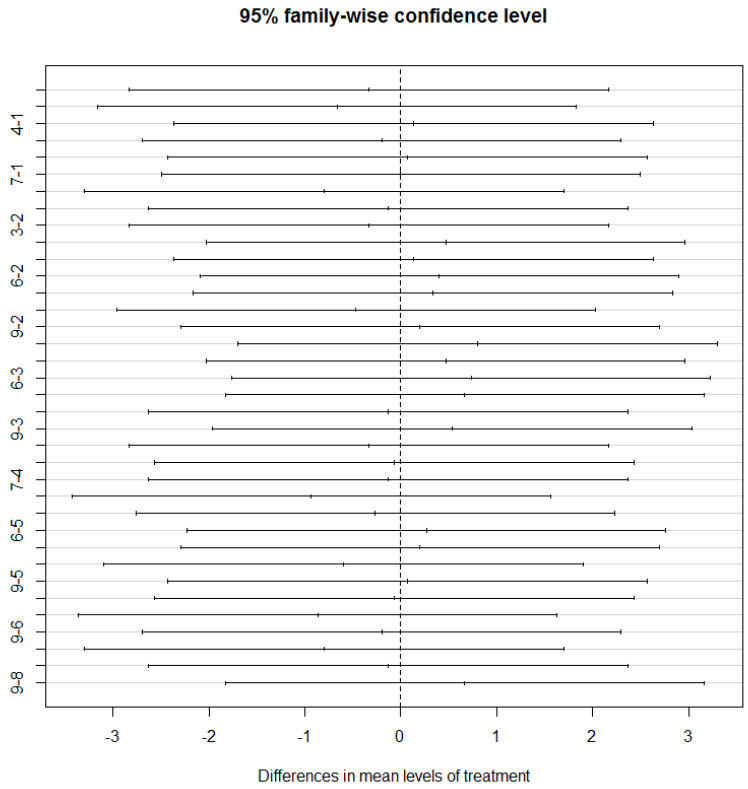

Figure 8 Difference in mean dissolved oxygen concentrations by nitrate and phosphate treatment

Results of isotopic analysis of ^{13}C-DIC indicated that the isotopic composition of DIC became significantly more enriched in the heaver isotopes in microcosms amended with 100 mg/L isotopically enriched DOC than in microcosms receiving 10.0 mg/L DOC (p=<0.05) or 1.0 mg/L DOC (p<0.05) (Figure 9). Microcosms amended with 10.0 mg/L DOC were also significantly (p=7x10^-7) more enriched in the heavier isotope than microcosms amended with 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 chemical interactions between DOC concentration and the combined nine NO_3, NO_3 and PO_4, and PO_4 treatment levels, indicated was no significant chemical interaction (p=0.98) impacting the resulting δ^{13}C-DIC. The DOC concentration was also found to be the only significant factor displaying a major effect on the resulting δ^{13}C-DIC values ($p=2x10^{-16}$). Looking at the mean of all treatments in Figure 11, δ^{13}C-

DIC increased by 190 ‰ to 350 ‰ in the first week of the experiment. In the following weeks, δ^{13}C-DIC continued to be enriched by 20 ‰ and by 80 ‰ in weeks 2 and 3, respectively. After week 3, δ^{13}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 δ^{13}C-DIC decreased with time in the first three weeks, and did not change at 11 weeks. The δ^{13}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, δ^{13}C-DIC decreased slightly to 80 ‰. In microcosms with 1.0 mg/L DOC very small change in δ^{13}C-DIC was observed over the entire experiment. Initially δ^{13}C-DIC was at 10 ‰ and decreased to 8 ‰ for the remainder of the experiment.

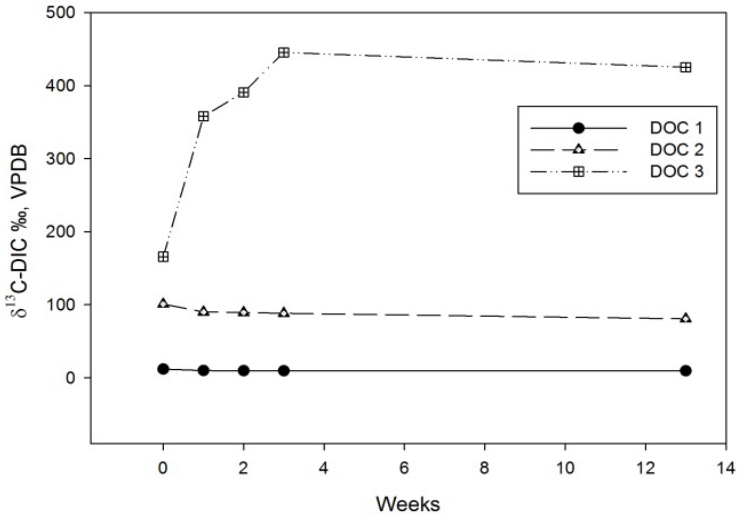

Figure 9 Isotopic composition of δ^{13}C-DIC of biologically derived ^{13}C-DIC in microcosm water samples; DOC 1: 1.0 mg/L DOC, DOC 2: 10.0 mg/L DOC, DOC 3: 100 mg/L DOC

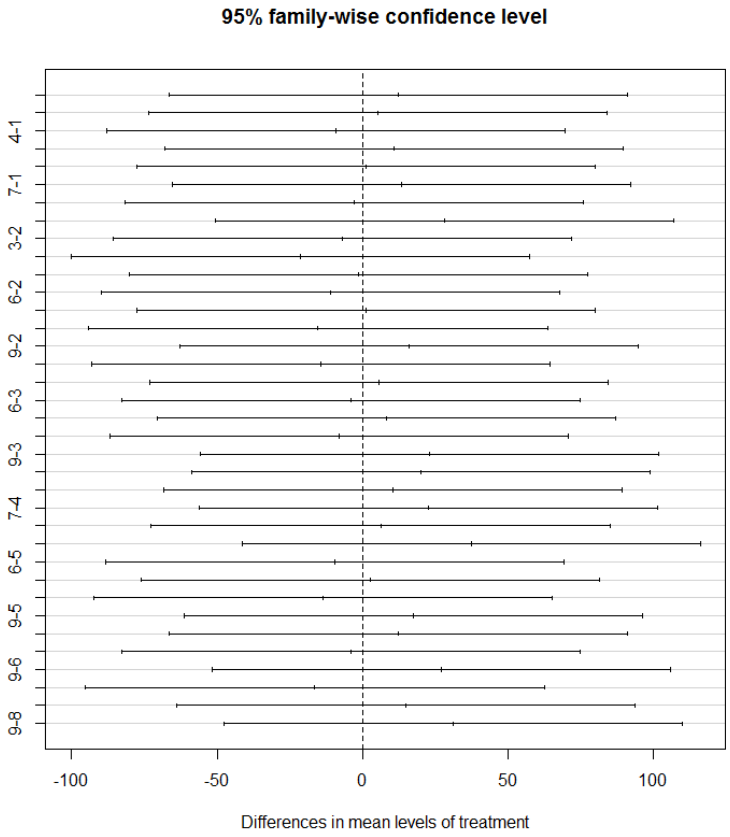

Figure 10 Difference in mean δ^{13}C-DIC isotopic composition by nitrate and phosphate treatment

Biologically derived DIC displayed many of the same trends observed in DO measurements and isotopic composition. With respect to DOC concentration 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 present at higher concentrations, 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 12). 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.

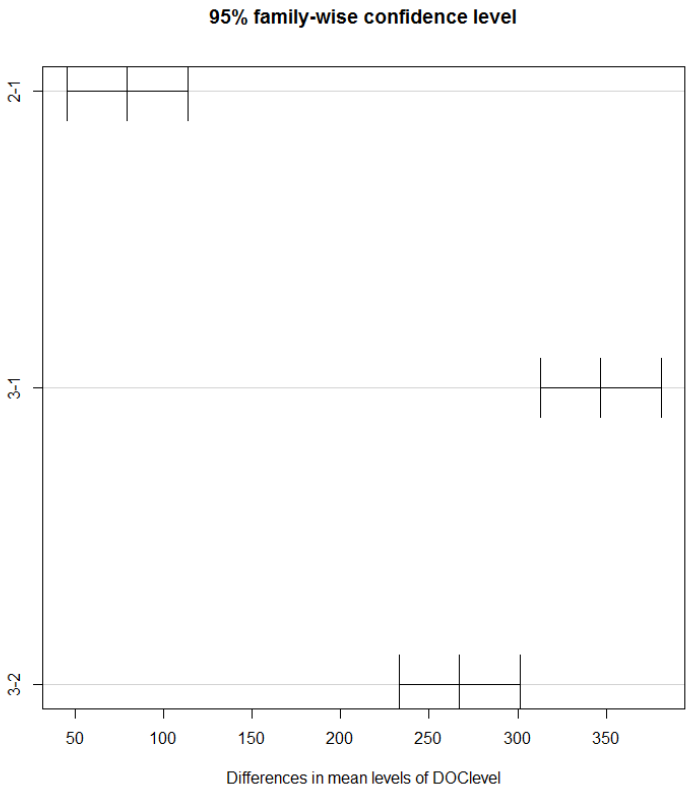

Figure 11 Difference in mean δ^{13}C-DIC isotopic composition by DOC treatment; DOC 1: 1.0 mg/L DOC, DOC 2: 10.0 mg/L DOC, DOC 3: 100 mg/L DOC

Factors displaying a statistically significant main effect was DOC concentration (p=2x10-16) (Figure 14). The impact of the nine NO_3, NO_3 and PO_4, and PO_4 treatments on DIC concentration was not significant (p=0.91) (Figure 13).

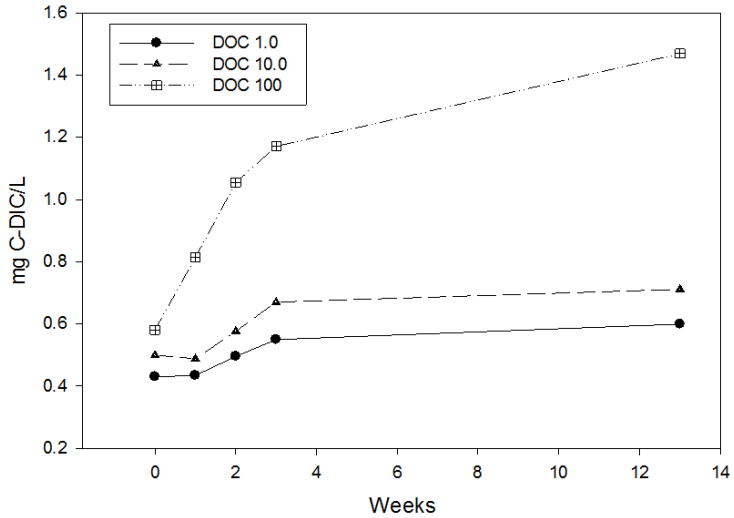

Figure 12 Biologically derived ^{13}C-DIC concentration measured in microcosm water samples

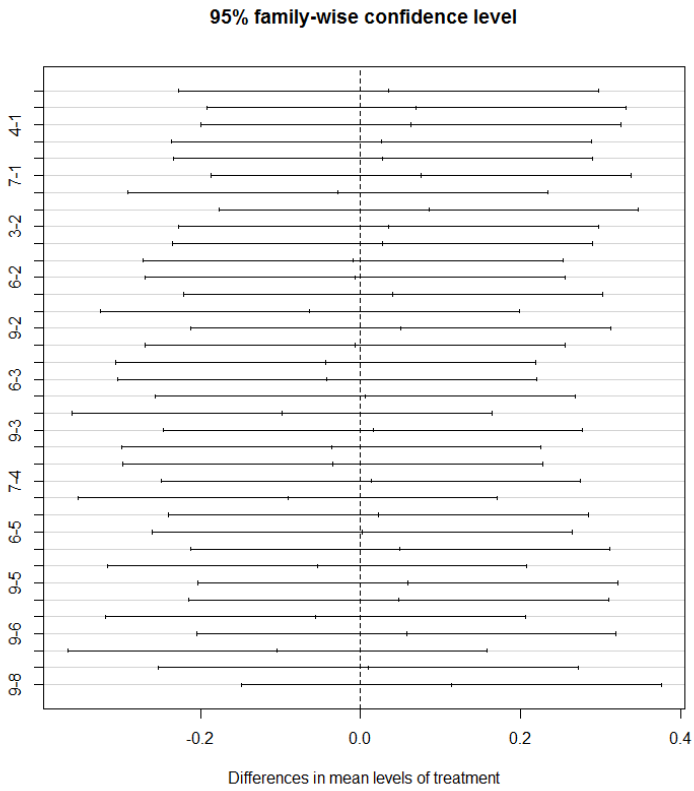

Figure 13 Difference in mean ^{13}C-DIC concentration by nitrate and phosphate treatment

Results of the isotopic analysis of $^{15}N\text{-}NO_3$ provided good indication of denitrification activity among other chemical, biological, and physical processes potentially affecting the fractionation of nitrate (Figure 15). Also of note is the broad range of $\delta^{18}O\text{-}NO_3$ values measured. At lower isotopic values of $\delta^{15}N\text{-}NO_3$ values of $\delta^{18}O\text{-}NO_3$ ranged from -30‰ to 50‰. Generally, $\delta^{15}N\text{-}NO_3$ 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 $\delta^{15}N\text{-}NO_3$ with a negative enrichment rate of -26.4/week. In figure 13, $\delta^{15}N\text{-}NO_3$ 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.

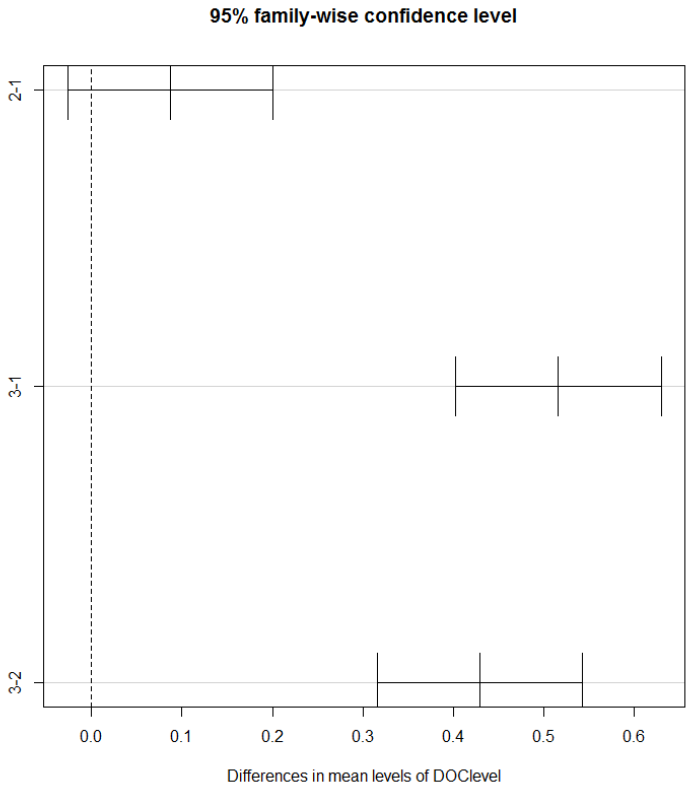

Figure 14 Difference in mean ^{13}C-DIC concentration by DOC treatment
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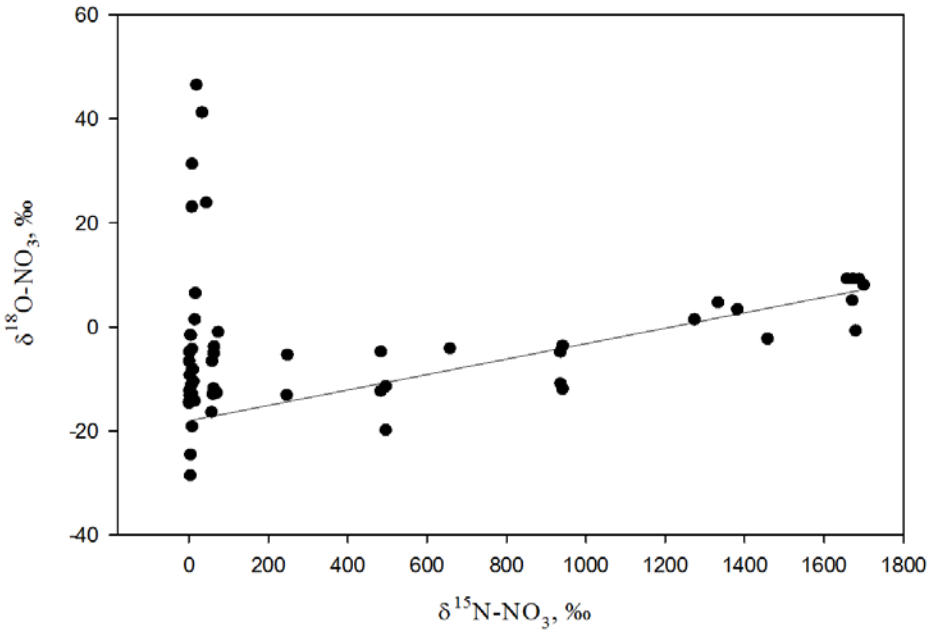

Figure 15 Isotopic composition of $\delta^{15}N$ and $\delta^{18}O$ in NO_3 measured in microcosm water samples

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 as a source of energy (electron donor), contributing to changes in water chemistry. More labile forms of DOC such as acetate provide a distinct boost in microbial activities even at concentrations as low as 1 mg/L. At low concentrations of DOC; however changes in DO, biological DIC production, and nitrate removal process are effected. Lower levels of DOC resulted in less oxygen consumption, less 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 significantly increased production of biomass. 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. Available DOC was a limiting factor in the complete use of all available DO, which was necessary for the

microcosms to shift from the more efficient electron acceptor O_2 to the less efficient electron acceptor NO_3, which results in denitrification. Denitrification is a terminal electron acceptor process or (TEAP) that will proceed in the general or local absence of oxygen, and requires adequate DOC as the electron donor to facilitate this metabolic process. Metabolic processes are generally energy intensive, and the results indicate that 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 undergo little nutrient processing, allowing nutrients to move 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 available. 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 have 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. During the course of the experiment, DO concentrations decreased early, but increased after 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 while DOC was available to sustain microbial activity. Biological oxygen demand in the microcosms remained elevated 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 that more of the amended DOC was transformed to DIC. Similarly, δ^{13}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 labile form of DOC bacteria respond rapidly, metabolizing the DOC in aerobic conditions via respiration and fueling denitrification in microaerobic conditions. 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 slightly 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 a $CaCO_3$-type water, therefore, the water was capable of buffering the evolving DIC during microbial respiration in the individual microcosms. Without this capacity to buffer the pH of the water sample, CO_2 production would have gradually decreased the pH of the water-sample potentially limiting denitrification activity.

Fractionation of $^{18}O\text{-}NO_3$ and $^{15}N\text{-}NO_3$ during denitrification warranted discussion, specifically related to observed greater variability in $\delta^{18}O\text{-}NO_3$ values at low enrichment levels of $\delta^{15}N\text{-}NO_3$. As values of $\delta^{15}N\text{-}NO_3$ became more enriched, the variability of $\delta^{18}O\text{-}NO_3$ values decreased. Although not conclusive, these results were contributed to two plausible causes; (1) noise generated during analysis, and (2) the signature of soil water $\delta^{18}O\text{-}CO_2$ and potentially atmospheric CO_2. Carbon dioxide (CO_2) has an atomic weight ranging from 44-49 depending on the isotopes of C and O present (e.g. ^{12}C, ^{13}C, ^{16}O, ^{18}O). The atomic weight of CO_2 overlaps the range of atomic weights of N_2O, which is generated during analysis resulting in variable values depending on the ability of specialized software to parse and integrate precursory CO_2 peaks. Additionally, these values were also consistent with a range of values for soil water $\delta^{18}O\text{-}CO_2$ values (29‰ – 39‰) (Amundson et al., 1998). The slight enrichment observed in $\delta\,^{15}N\text{-}NO_3$ associated with the $\delta^{18}O\text{-}NO_3$ in samples displayed very small isotopic effects, which is expected with respect to the diffusion of inorganic nutrients at low substrate concentrations, such as the

conditions in microcosms with DOC concentrations less than 10 mg/L and NO_3 concentrations below 1 mg/L. Further investigation is required to explore the dynamics of O_2 fractionation and speciation during nitrogen and carbon cycling in epikarst.

Productivity 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, δ^{13}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. This likely defines a threshold at which phosphate concentration potentially interferes in the process of denitrification. In aerobic conditions phosphate is removed from the microcosms, but nitrate remains because the O_2 is the present and more efficient electron acceptor. When conditions become anaerobic orthophosphate is released as phosphate accumulating organisms utilize the available acetate to restore intracellular energy reserves. Competitive interference arises between organisms removing nitrate and organisms removing phosphate as both sets of organisms attempt to oxidize as much of the available DOC as quickly as possible. Broadly this balance of nitrate to phosphate should be noted because it represents a threshold when denitrification efficacy decreases or stops altogether and alternative processes for removing nitrate must be considered. In the spring recharge area where the sample for the microcosms was collected, nitrate and phosphate concentrations rarely reach the levels used in the study. However, because of agricultural practices and nitrate's mobile nature, there exists a potential for high nitrate concentrations on the order of the concentrations used in this study. Historically phosphate concentrations in the watershed remain below 1 mg/L, and to observe measured concentrations at the intermediate to high range of concentrations used in this study would be highly unprecedented baring the outfall from human activity (e.g. leaking or overflowing waste storage

lagoons, unscrupulous disposal of phosphorus-rich waste products).

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. Denitrification activity was contingent on anaerobic and pH neutral conditions, both of which required active oxidation of DOC and subsequently increased BOD. In aerobic conditions O_2 was the preferred electron acceptor and nitrate was not used, denitrification did not occur. After the O_2 pool was exhausted, denitrification began and nitrate was removed. With a range of DOC, NO_3, and PO_4 concentrations from low to high used in the study, DOC was the only distinguishing variable. DOC provided energy for denitrifying bacteria when conditions were anaerobic, and provided energy to phosphate accumulating organisms under the same conditions. However, when equal high concentrations of NO_3 and PO_4 were present productivity remained high, but nitrate was not removed. This observation means that in phosphate dominated systems aerobic conditions would facilitate the removal of phosphate, but in anaerobic conditions nitrate removal can only occur if the pool of DOC is adequate to support the denitrifying organisms and the phosphorus accumulating organisms. Productivity increased with DOC concentration, which also resulted in significant biomass growth. 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 should 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. 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 ($CH_3{}^{13}COONa$) 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 µg/L displayed inhibitory effects on microbial processes. These inhibited processes included DOC assimilation and denitrification. DIC production increased and δ^{13}C-DIC became more enriched after 2 days in microcosms with <1 µg/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 µg/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 have 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 denser, lower porosity zone at the base of the vadose zone above the main karst bedrock. 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 generally on the order of 10%-30%, which is an order of magnitude greater than the thicker unweathered intervals 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 hydraulically 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 be 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; precipitation-induced flow in epikarst acts as a piston flushing a critical volume of epikarst water into the saturated zone to discharge from epikarst springs. In the context of epikarst hydrology, the evolution of epikarst water chemistry can be highly variable. Variable epikarst hydrology and water chemistry also creates a complicated ecological framework.

The epikarst zone provides habitat for various organisms ranging from single celled microbes to multicellular invertebrates. Infiltrating water carries numerous dissolved and particulate chemical and biological constituents that affect 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 and ammonia, heavy metals, antibiotics, and organic matter dictate the 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 well connected to the surface, many of the aforementioned pollutants often are little attenuated within the flow system, although retention time and potential for attenuation will vary with hydrologic conditions. As much as 74% of NO_3 applied to agricultural fields as fertilizer is discharged during base-flow conditions via springs emanating from the epikarst of northwest Arkansas, while 26% of the nitrate is 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 attenuating nutrient concentrations and related metal-concentrations controls 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, and others, but a major question remains to be answered regarding the effect of heavy metal concentrations on biogeochemical processes, nutrient uptake, and the conversion of DOC to dissolved inorganic carbon (DIC). Winston et al. (2005) observed that 69% of the change in nitrate (NO_3) and DIC concentration observed during storm events could be attributed to dilution and approximately 20% attributed to biological processing. Panno et al., (2001) observed that the isotopic composition of NO_3 discharging from epikarst springs to be consistent with nitrification of reduced nitrogen fertilizers. Denitrification and several other biogeochemical processes depend on adequate available dissolved organic carbon (DOC), and as such are impacted by DOC concentration and speciation. Between 20-60% of the DOC pool in aquatic environments comprises humic acids and/or fulvic acids (Frimmel et al., 2008). Moreover, humic substances are relatively refractory (poor bioavailability) and have a long residence time, taking decades for complete processing or turnover in aquatic environments.

In groundwater background DOC concentrations 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. High DOC concentration potentially may occur in epikarst underlying regions with intensive liquid or dry 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 transform dissolved nitrate and DOC. Metals may impede microbial activity by two primary means: acute toxicity or by way of competitive inhibition of microbial enzymatic activity. The study used several dissolved metals; and 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. Microcosm experiments were 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 if its major tributaries, Big Creek. The CAFO has a capacity to house 6,500 head of swine (2,000 sows and 4,000 piglets). 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—leaking from the storage lagoon or leaching from fields on which it is applied. The operation of the CAFO in such close proximity to a protected waterway has been the topic of 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 conducted extensive projects to monitor positive and negative ramifications the CAFO operation's potential effect 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 topography of the area comprises moderate- to high-relief terrain typical of the dissect plateaus of the Ozarks with elevations ranging from 243 m to 457 m. Annual mean temperature in the area ranges from 3°C to 27°C throughout the year, and mean annual precipitation ranges from 49 mm to 172 mm (Weather.com, 2016). 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. The gravelly nature of the soils renders them less than ideal for farming; therefore, as the primary land use is pasture for grazing cattle. Mississippian Boone Formation limestone is predominant in outcrop in the area. The Boone Formation is highly weathered and fractured limestone with interbedded chert more

than 70% in some areas. Weathering of the limestone is thought to have contributed to the higher 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.

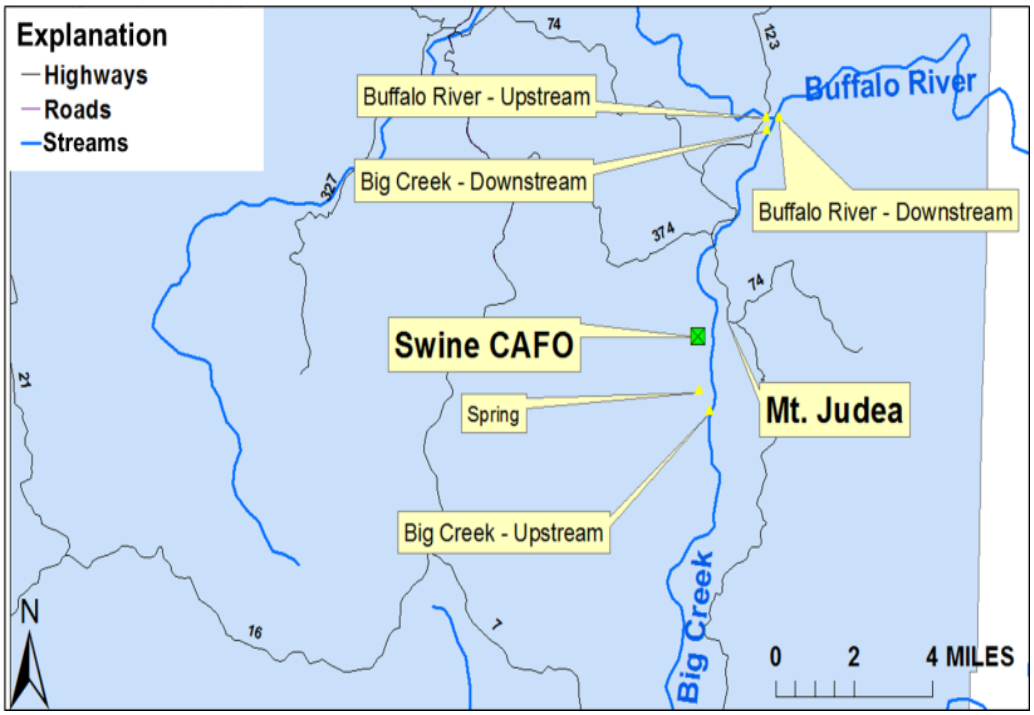

Figure 16 Study sampling locations indicated by yellow triangles.

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 differing concentrations: 1.0 μg/L, 10.0 μg/L, and 100.0 μg/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 ($CH_3{}^{13}COONa$) and ^{15}N-labled potassium nitrate ($K^{15}NO_3$) to 1000 ‰. Abiotic spring-water, raw spring-water, trace-metals spiked, and non-metal control microcosms were run together under identical conditions. 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 ^{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 relative to 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 of the heavy to the light isotope with respect to a standard using the relationship described below for values of $^{15}N/^{14}N$ and $^{13}C/^{12}C$.

$$\delta, ‰ = \frac{\left(\frac{R_H}{R_L}\right)_{Sample} - \left(\frac{R_H}{R_L}\right)_{Standard}}{\left(\frac{R_H}{R_L}\right)_{Standard}} \times 1000$$

$$mol\ CaCO_3 = mass_{Calcite\ standard} \times \left(\frac{1\ mol_{Calcite}}{F.W._{Calcite}}\right)$$

$$[CO_2]_{Standard} = \frac{Mol, CaCO_3 \times F.W._{CO_2}}{Volume_{Standard}}$$

Step 1. Calculate the concentration of CO_2 in standards

$$A = \frac{[CO_2]_{Standard}}{Peak\ Area}$$

Step 2. Determine response coefficient, A

$$[CO_2]_{sample} = A * Peak\ Area_{sample}$$

Step 3. Determine concentration of CO_2 in sample

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 were used to determine the significance of correlation and relation between individual variables in laboratory experiments.

RESULTS & DISCUSSION

Zinc was the most abundant metal observed in the water samples (Figure 17). Zinc concentrations were highest in precipitation samples, the well exhibited intermediate values, while the spring had the lowest zinc concentrations. Zinc concentrations in Big Creek increased between upstream and downstream locations. This trend was also observed in Cu concentrations. Arsenic was not detected in precipitation samples, but was present in the samples collected from the spring, well, and Big Creek. Chromium concentrations were lowest in precipitation samples and highest in well and spring samples. Chromium concentrations in samples from Big Creek upstream and downstream exhibited intermediate values, and increased from upstream to downstream. Cadmium was only detected in samples taken from the well in very low quantities. Lead was detected in precipitation and well samples. Lead concentrations were highest in precipitation samples.

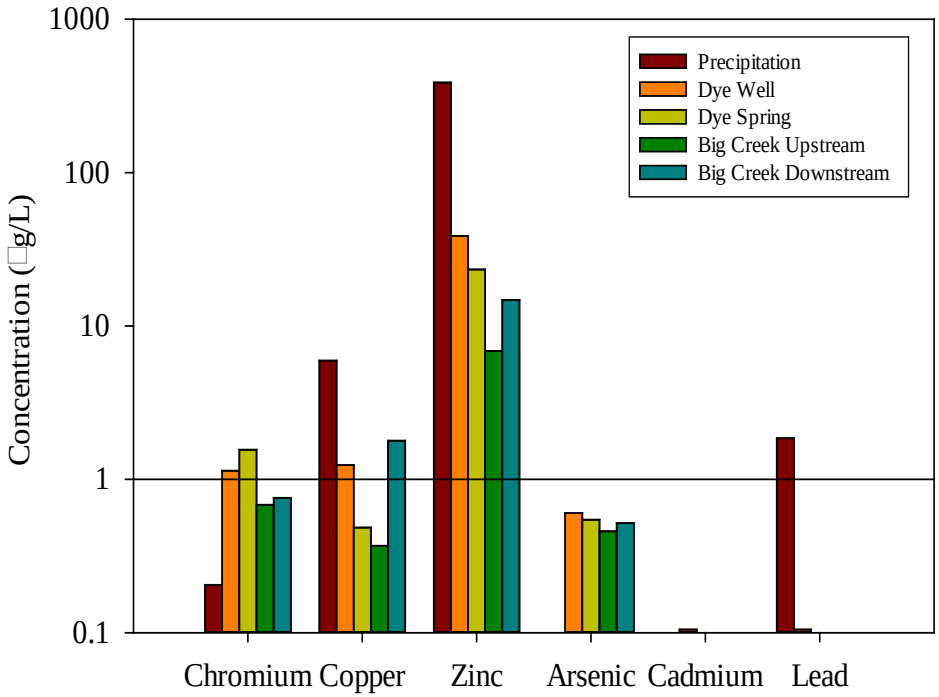

Figure 17 Dissolved metal concentrations measured in precipitation, wells, springs, and surface-water in the study area.

In the laboratory studies metal treatments had no statistically significant effects distinguishing on microcosms' microbial activity. 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 metal-concentration levels 1, 2, and 3 respectively Figure 21. 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. Mean DIC concentrations, however; were statistically identical in all microcosms ($p=0.932$). A similar trend was also observed in the δ^{13}C-DIC observations.

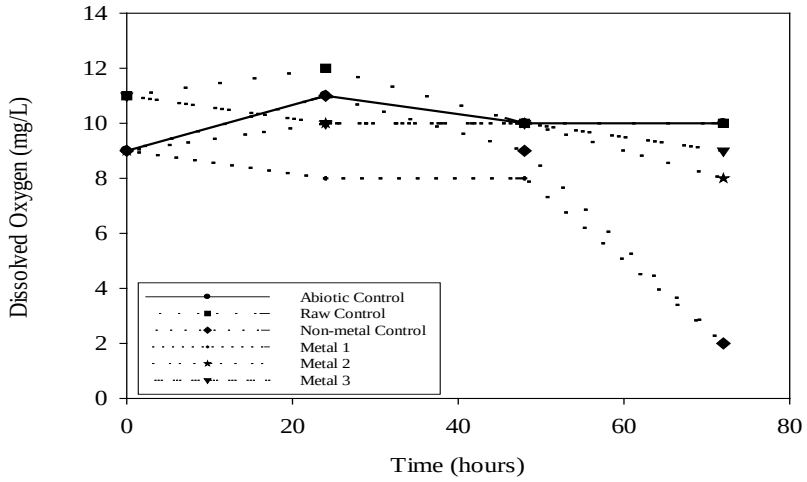

Figure 19 Dissolved oxygen concentration measured in microcosm water samples.

All microcosms had a mean δ^{13}C-DIC value of -12.4 ‰, VPDB (Figure 20). The mean isotopic composition of the microcosms was statistically identical (p=0.389).After 24 hours, the δ^{13}C-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 21). In the microcosm containing the largest addition of metals, activity appeared unchanged as seen in both DIC concentration and isotopic composition (Figure 20 & 21). Measured DO concentrations in the microcosms were aerobic 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 19.

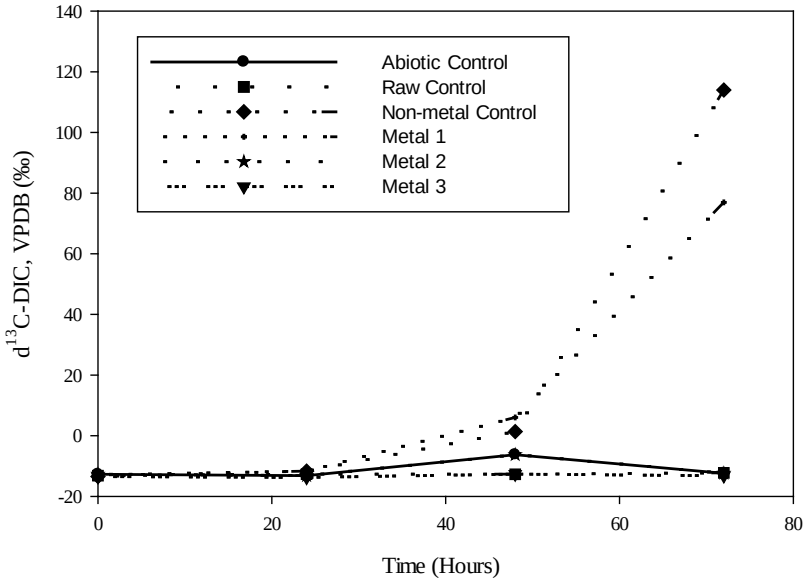

Figure 20 δ^{13}C-DIC isotopic composition measured in microcosm water samples.

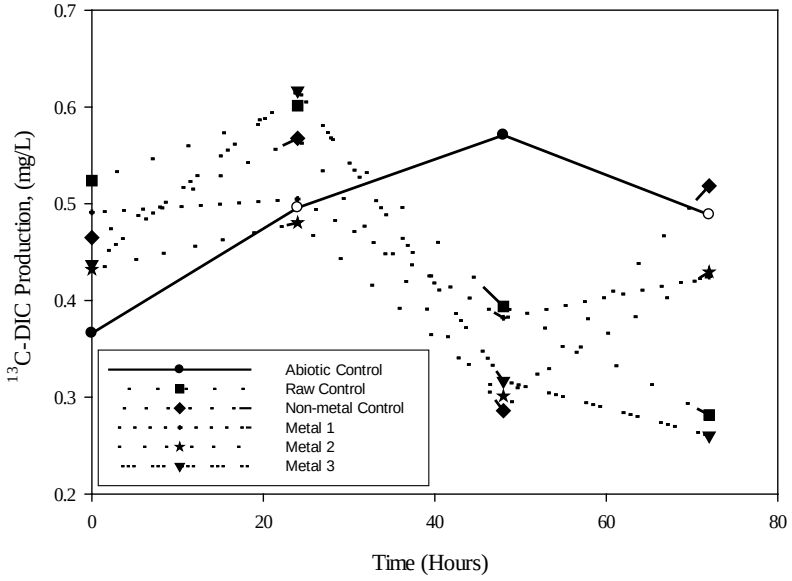

Figure 21 ^{13}C-DIC concentration measured in microcosm water samples.

There was no significant indication of enrichment in δ^{15}N-NO_3 data from the microcosms (Figure 22). In all microcosms values of δ^{15}N-NO_3 fluctuated, but not to a significant degree that would indicate processing of amended NO_3. Nitrate concentrations showed no decline, corroborating that denitrification was not occurring (Figure 23).Nitrate concentration and isotope data are consistent with δ^{13}C-DIC, DIC concentration, and DO concentration data, which all

indicate a lag, potential inhibition, or toxic response affecting nitrate and DOC processing in the microcosms.

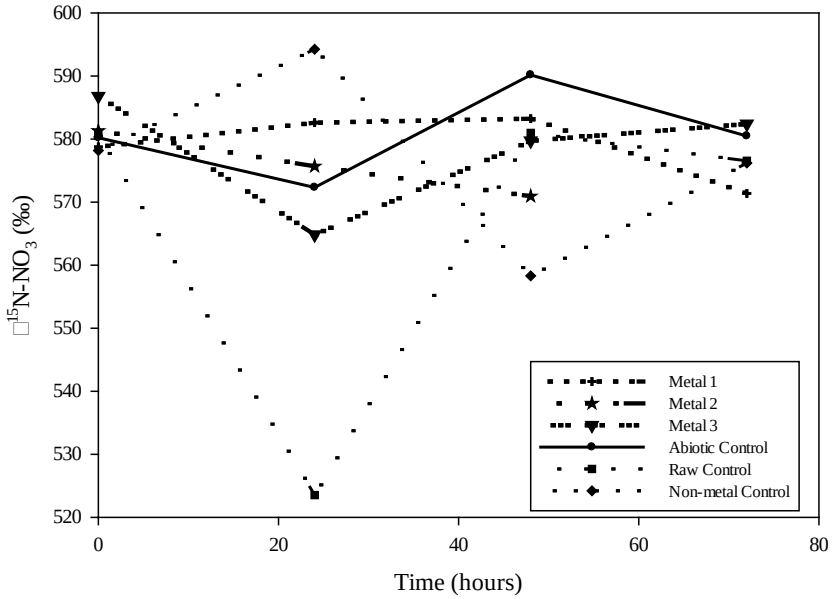

Figure 22 δ^{15}N-NO_3 isotopic composition measured in microcosm water samples.

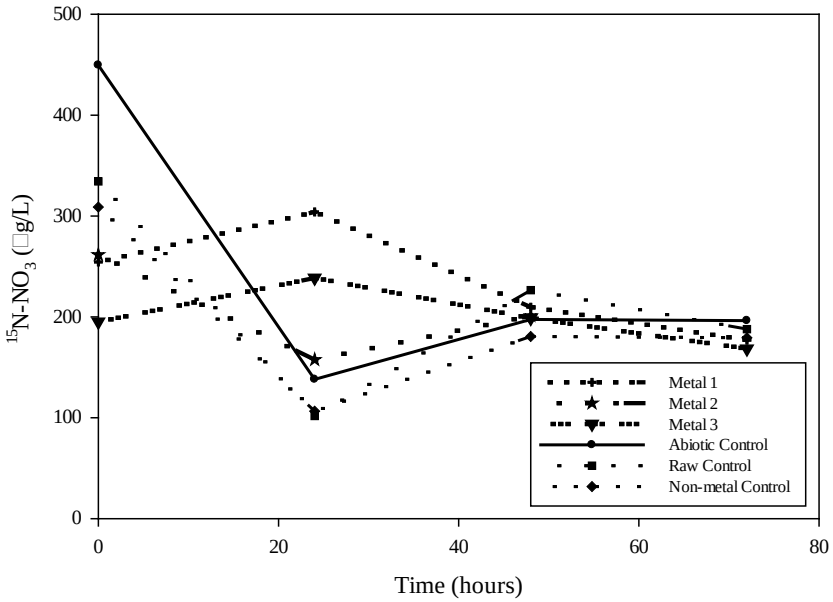

Figure 23 ^{15}N-NO_3 concentrations measured in microcosm water samples.

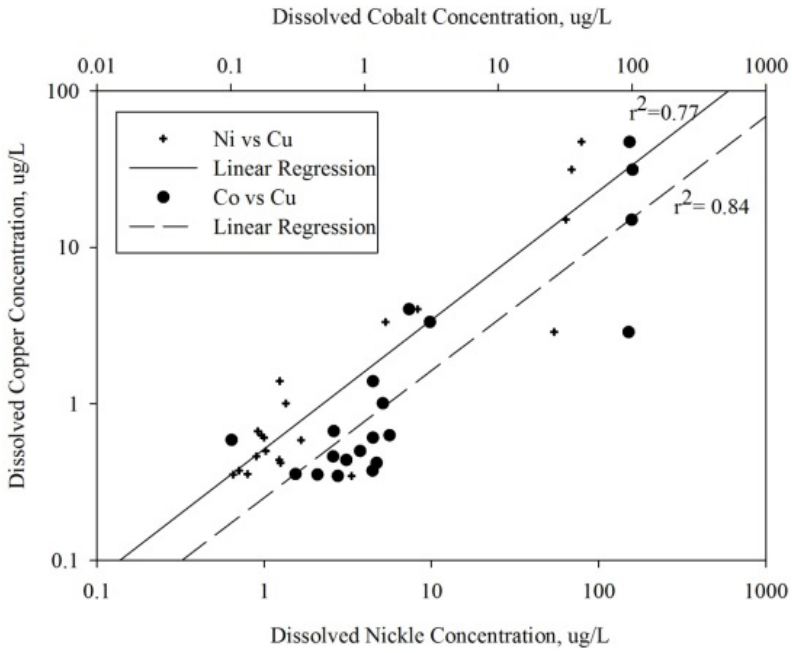

Figure 24 Dissolved copper, nickel, and cobalt concentrations from microcosm water samples.

Trace metal interaction and biogeochemical activity are important because of the implications for exposure pathways of microbial communities to potentially toxic metals derived from the overlying soil and groundwater recharge. Inspection of metals data collected from microcosm samples shows clear indications of the affinity of some trace elements to bind to DOC as seen in the relation between dissolved Cu, Ni, and Co concentrations (Figure 24). Affinity for binding sites on organic molecules follows the order 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 moving along 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 are most abundant when the epikarst receives pulses of recharge during storm events. This addition of DOC can increase the potential for metal-microbiota interaction. Cu(II) specifically is a micro-nutrient and has an active role in aerobic metabolic processes in microbial communities, but at higher, 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 (Santo et al., 2008). The results of the microcosm experiment perhaps display a combination of toxicity response and morphological adaptation response by the microbial communities, particularly in microcosms treated with the highest concentrations of metals. Inactivity in the microcosm was observed in the first 24-28 hours, and the lag in response may have been a period in which microbes adapted to the Cu-induced stress, concurrent with increasing Fe(II) concentrations beyond 48 hours. What distinguishes microcosm with relatively large amounts of metals from the other microcosms is microbial inactivity, which is not seen in microcosms with less than 1 μg/L of metals added. Microbial activity was observed for the microcosms with Cu(II) concentrations below 0.4 μg/L.

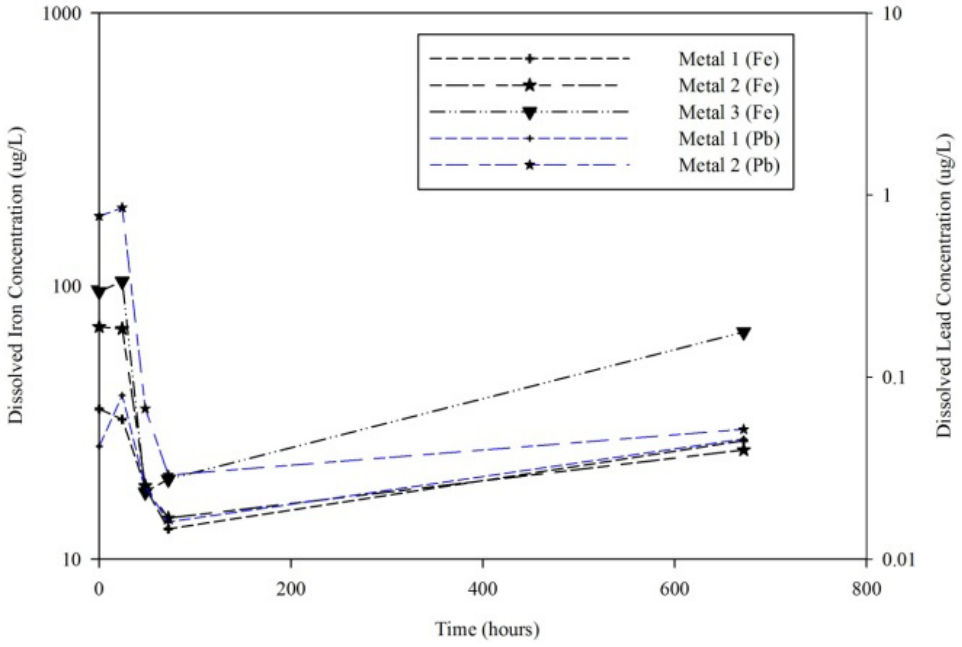

Figure 25 Time-series dissolved iron and lead concentrations in microcosm water samples.

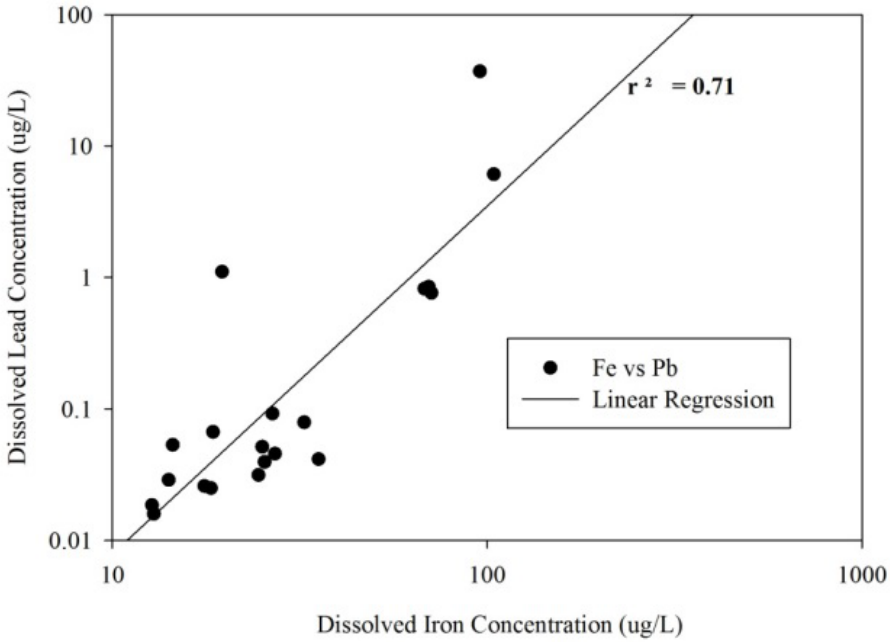

Figure 26 Dissolved iron concentration versus dissolved lead concentration measured in water samples from microcosms.

Lead mobility is closely linked to the prevalence of oxidized iron and magnesium compounds. Aqueous Pb adsorbs to these particulates and is removed from solution. 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 of ferric iron in the microcosms was reduced to ferrous iron, which provides and 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 background concentrations observed in the spring sample, <0.1 μg/L. The only exception to this trend was the microcosm with the greatest amount of Pb added in which the concentration of Pb decreased 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 also 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 researchers has confirmed the adsorption of heavy metals to mineral surfaces as well as to biological surfaces in the sub-surface; for this reason a better understanding of biosorption is necessary. Boyanov et al. (2003), 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 reduced drastically as demonstrated by reduced oxygen use, δ^{13}C-DIC fractionation, and DIC production. The study also successfully demonstrated the ability of bacteria to adapt to acutely toxic heavy metals exposure, as seen in microcosm with a range of heavy metal treatment concentrations containing ranging 10 μg/L to $\leq$1.0 μg/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, complexation of DOC with heavy and transition metals, poses 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 AN EPIKARST SPRING WITH RESPECT TO ERYTHROMYCIN, TTRACYCLINE, AND METALS AS INTEPRETTED 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. One 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 were conducted using acetate, nitrate, and phosphate additions, which 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 for a duration 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 Triptic 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 high concentrations of labile carbon has not received much attention in karst research. Specifically, little is known about the changing dynamics of microbial communities in epikarst biofilms exposed to high concentrations of labile dissolved organic carbon (DOC). Well drained soils and the high porosity of weathered limestone in northern Arkansas give way to extensive surface-water and groundwater interaction. Epikarst, the unsaturated zone underlying soil cover, represents a critical zone in the evolution of water chemistry. Positioned above karst, epikarst represents a physically, chemically and biologically

transient zone. Microbial life in epikarst is largely derived from overlying soils. The chemistry of infiltrating water is altered by biological and chemical processes that are initiated in the soil environment overlying epikarst. Alteration of the soil environment would spell changes for the underlying karst biogeochemistry. Application of liquid manure fertilizers to pasture is an example of a surficial activity with great potential to affect karst biogeochemistry. 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) in association with DOC. Constituents transported via DOC derived from liquid manure fertilizers leach from the soil into the groundwater where DOC is disseminated on a larger scale leading to eutrophication of water ways, human health problems, decreased biodiversity, and economic turmoil particularly when natural resources are economic staples (e.g. national parks).

In the soil environment, organically rich water infiltrating provides key nutrition for a broad range of organisms. The soil environment also provides a matrix for adsorption of organic compounds and metals, effectively removing them from the water as it moves through the soil profile; however, sequestration of organic matter, nutrients, and metals is not without limits in the soil environment. Soils act as primary buffers for the groundwater environment, but when saturated with organic compounds, metals, and nutrients, these constituents move through the soil and directly into groundwater. In epikarst, which is encountered where present, variable hydrology and the range of chemical conditions of the entering water create a unique biogeochemical environment. 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 (Figure 27). A single bacteria cell attaches to a surface and replicates. More cells of various types of bacteria also colonies the surface, and the colonies produce the slime-like substance known as extracellular polymeric substance (EPS). The composition of EPS varies across community structures, but largely is composed of polysaccharides, genetic information, and proteins. The functions of EPS include but are not limited to; protection of the biofilm community, more effective exchange of genetic information, better conditions for nutrient grazing in flowing water. EPS is also believed to be a “highway” for quorum signaling between communities of bacteria within the biofilm. As biofilms mature the shear force from flowing water also increases, and causes 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 characterize the community structure of biofilms grown under a range of varying chemical conditions using their fatty acid (FA) compositions. Fatty acids are phospholipid molecules used to store energy within the cells of organisms. Fatty acids 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) and biological processes. In this study biofilms were harvested from two laboratory studies and one field study. The laboratory studies represented biofilm growth under varying DOC concentrations: along with varying nitrate and phosphate concentrations in one study and varying heavy and trace metal concentrations in the second

study. A field study was conducted that acted as an environmental control, which was used to compare results from laboratory results to an in-situ analog.

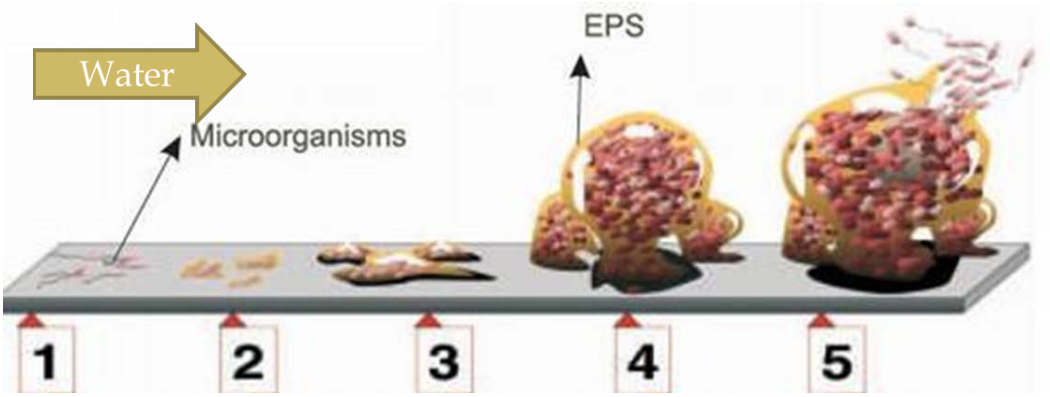

Figure 27 Five-stages of microbial biofilm growth (adapted from Monroe, 2007)

METHODS

Site Description

Field studies were conducted in Newton County, located in the Ozark Highlands physiographic region of northern Arkansas. The topography of the area is rolling hills to mountainous with elevations ranging from 243 m to 457 m. Annual mean temperature in the area ranges from 3 °C during the winter to 27 °C the summer, and mean annual precipitation ranges from 49 mm to 172 mm (Weather.com, 2016). 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 gravely nature of the soils, they are not ideal for crop farming and therefore much of the land is used as pastures for grazing cattle. Mississippian age Boone Formation limestone and chert is the predominant outcropping geology in the area. The Boone Formation is highly weathered and fractured limestone with interbedded chert as much as 70% is some areas. The highly weather 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 numerous well-developed karst

features, which allow for rapid interaction between surface and groundwater interaction via various dissolution features across the landscape.

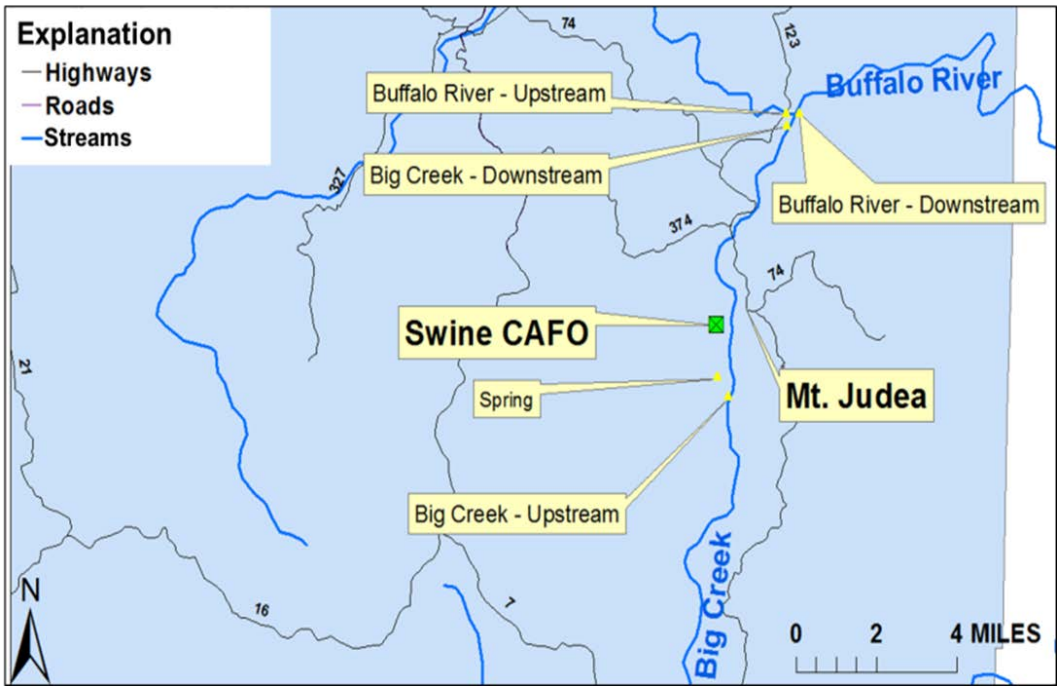

Figure 28 Locations of sampling sites for antibiotic sampling (yellow triangles)

Antibiotic Sampling and Analysis

Three replicate grab samples were collected for the analysis of antibiotics from shown on map (Figure 28). 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 filters (45 µm) on a stainless steel plate filter. Sample water was collected in a PTFE 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 Ziploc plastic bags and stored on ice before being mailed to the USGS Kansas Organic Geochemistry Laboratory for analysis. Samples not shipped the same day of collection were stored 4°C and shipped within 48 hours. 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 25oC. 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) were 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 dark-temperature- controlled environment to simulate epikarst environmental conditions. The microcosms were allowed to incubate at 12°C for 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 fatty acid methyl ester (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 dissolved inorganic carbon (DIC) within the first week of the experiment. An experiment looking at the first week of activity was determined to be beneficial to further understanding the impact of metals on DOC-DIC conversion and denitrification. The second laboratory experiment used a single DOC concentration (100 mg/L) and NO_3 concentration (1.0 mg/L) in a matrix of spring-water and gravel as described in the initial microcosm study. The microcosms were spiked with a suite of metals at three different concentrations levels: 1.0 μg/l, 10 μg/L, and 100 μg/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 cm^2. The ceramic plates were placed at a well and at a spring that discharged downstream of the well. The ceramic plates were placed in wire mesh pouches and tied and weighed to ensure they remained below the water's surface in the well and at the mouth of the spring. Three packets were placed at each location. Biofilm packets were collected concurrently 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 they were eluted with DCM. The solution was heated at 100°C for 1 hour, derivitized using 1 mL of boron triflouride (BF_3), 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 Wilcoxian Rank Sum test which 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 determine by calculating the Spearman Correlation Coefficient. All statistical analysis was conducted using the Sigma Plot software package release 12.

Fluoroquinolines (0.005 µg/L)	Macrolides	Sulfonamides	Tetracyclines (0.010 µg/L)	Pharmaceuticals	Others
Ciprofloxacin	Azithromycin	Sulfachloropyridazine (0.005 µg/L)	Chlortetracycline	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-H20 (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 3 Measured antibiotic and antibiotic degradation compounds measured in water samples.

	DOC 1 (1.0 mg/L); D1	DOC 2 (10.0 mg/L); D2	DOC 3 (100.0 mg/L); D3
NO_3 1 (0.1 mg/L); N1	D1N1	D2N1	D3N1
NO_3 2 (1.0 mg/L); N2	D1N2	D2N2	D3N2
NO_3 3 (10.0 mg/L); N3	D1N3	D2N3	D3N3
NO_3 + PO_4 1 (0.1 mg/L); NP1	D1NP1	D2NP1	D3NP1
NO_3 + PO_4 1 (1.0 mg/L); NP2	D1NP2	D2NP2	D3NP2
NO_3 + PO_4 1 (10.0 mg/L); NP3	D1NP3	D2NP3	D3NP3
PO_4 1 (0.1 mg/L); P1	D1P1	D2P1	D3P1
PO_4 2 (1.0 mg/L); P2	D1P2	D2P2	D2P2
PO_4 3 (10.0 mg/L); P3	D1P3	D2P3	D3P3

Table 4 Nutrient microcosm amendment schedule and treatment abbreviation key

RESULTS

Antibiotic Sampling and Dose-Response Study

No antibiotics or antibiotic degradation products were found at measurable concentrations. Results of the dose response assay indicated resistance to both erythromycin and tetracycline in laboratory cultured bacteria. Excluding samples taken upstream on the Buffalo River, bacteria grew at all concentrations of erythromycin; however, as the erythromycin concentration increased the concentration of bacteria decreased (Figure 29). Bacteria growth was observed at all sites, but in the presence of erythromycin the upstream site on Big Creek and the spring samples displayed the highest bacteria concentrations (Figure 29). Bacteria growth downstream on the Buffalo River showed intermediate bacteria concentration values, followed by the lower bacteria concentrations downstream on Big Creek and upstream on the Buffalo River. At all study sites, the bacteria seemed capable of growing with erythromycin, even at high concentrations. However, 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 (Figure 30).

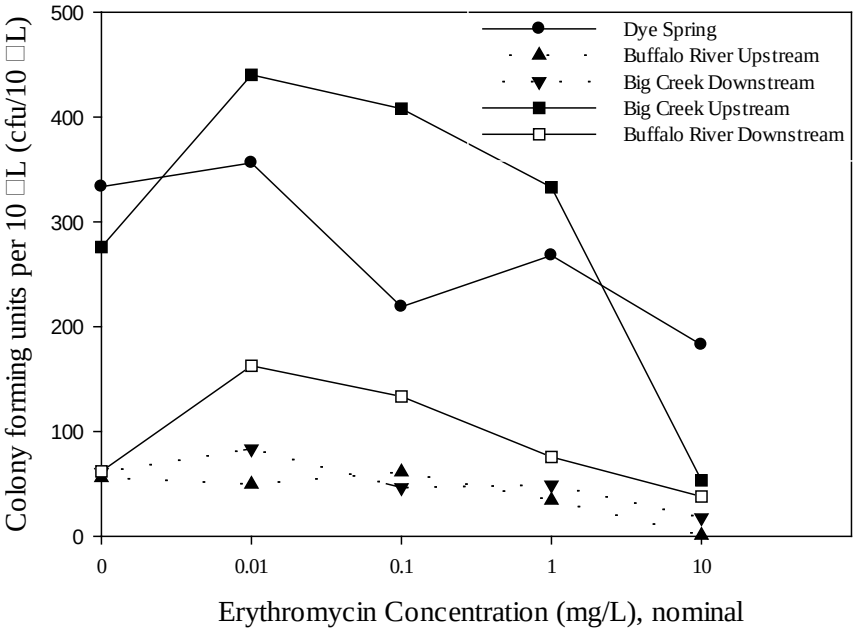

Figure 29 Colony counts of bacteria grown on erythromycin dosed growth media.

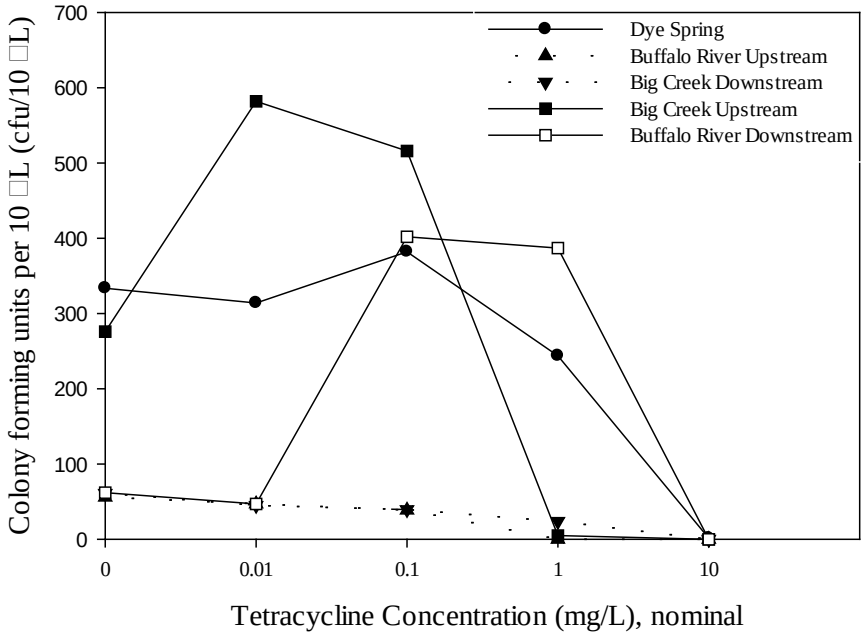

Figure 30 Colony counts of bacteria grown on tetracycline dosed growth media.

DOC, Nitrate, Phosphate Addition Microcosm Study

Samples collected from the microcosm studies using varying concentrations of DOC, NO_3, and

PO_4 showed 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 (Figures 32, 33, and 34). 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%±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 (Figure 34). 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, NO_3, and PO_4 concentrations samples containing only PO_4 had significantly greater amounts of palmitic acid than microcosms containing only NO_3 ($p=0.05$). The fraction of palmitic acid in microcosms containing equal parts of NO_3 and PO_4 was negatively correlated with the amended DOC concentration ($\rho=-0.69$, $p= 0.04$); however, a similar relationship with concentrations of NO_3 and PO_4 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%±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 (Figure 34). Although not statistically significant, a positive trend was observed between the percentage of stearic acid and DOC concentration in microcosms containing only NO_3 and equal parts of NO_3 and PO_4. The stearic acid percentage was, however, negatively correlated with DOC concentration in microcosms containing only PO_4 (ρ=-0.69, p= 0.04). There was no statistical difference in the fraction of stearic acid contained within the biofilms of microcosms augmented with NO_3 only and PO_4 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%) (Figure 34). 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%±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% (Figure 33). 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 important 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%±0.96, and the mean oleic acid fraction was 3.17%±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 (Figure 33 and 32). 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%±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 (Figure 35). 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 (ρ=-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 (Figure 35). 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 ρ=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%±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%±4.94 of the total fatty acid composition, and the mean fraction of stearic acid was 17.39%±4.7 (Figure 31). 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.

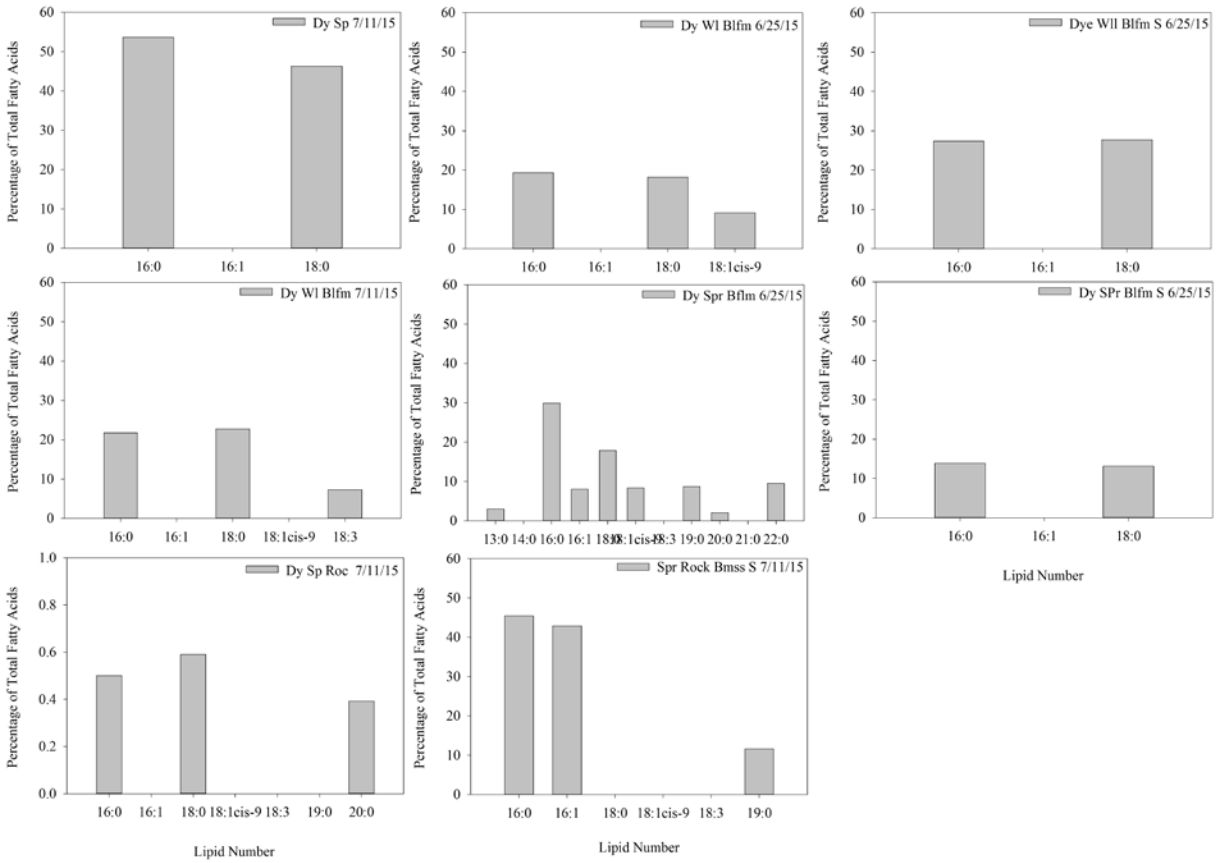

Figure 31 Fame distributions of biofilm samples collected from field biofilm study

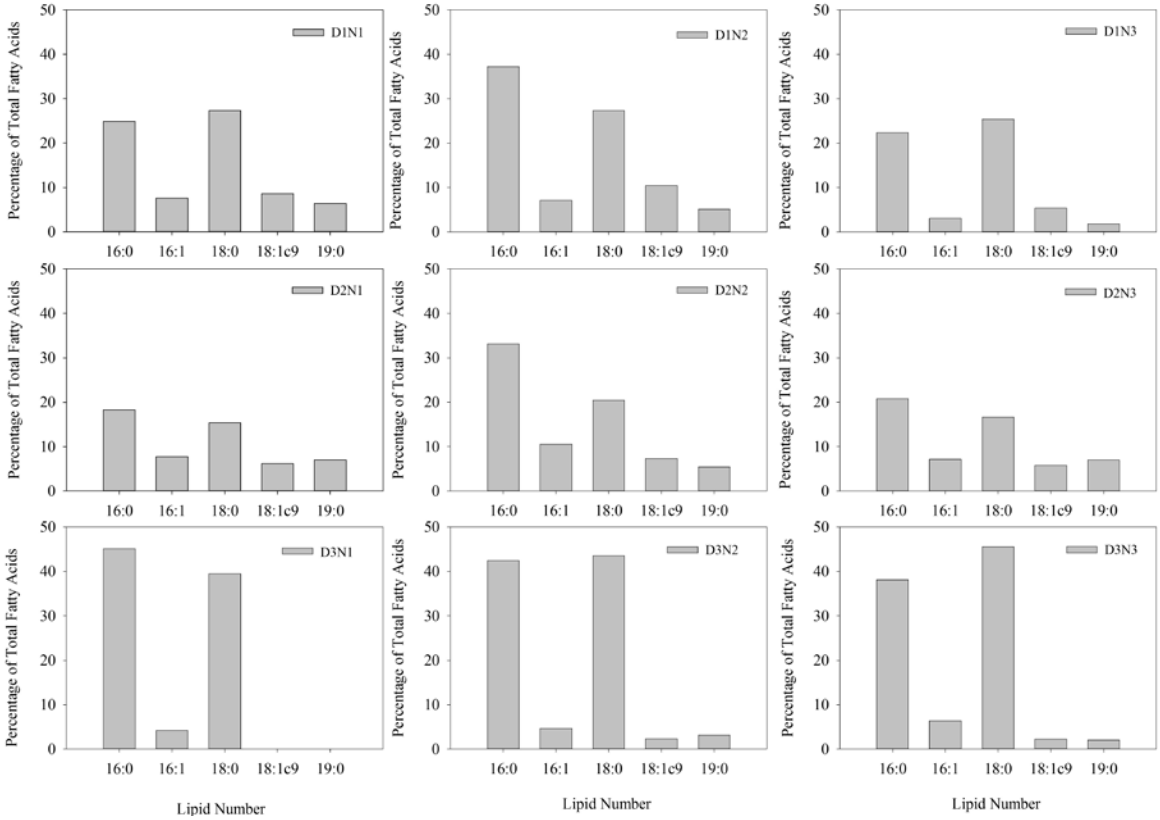

Figure 32 FAME distributions of biofilms harvested from nutrient study.

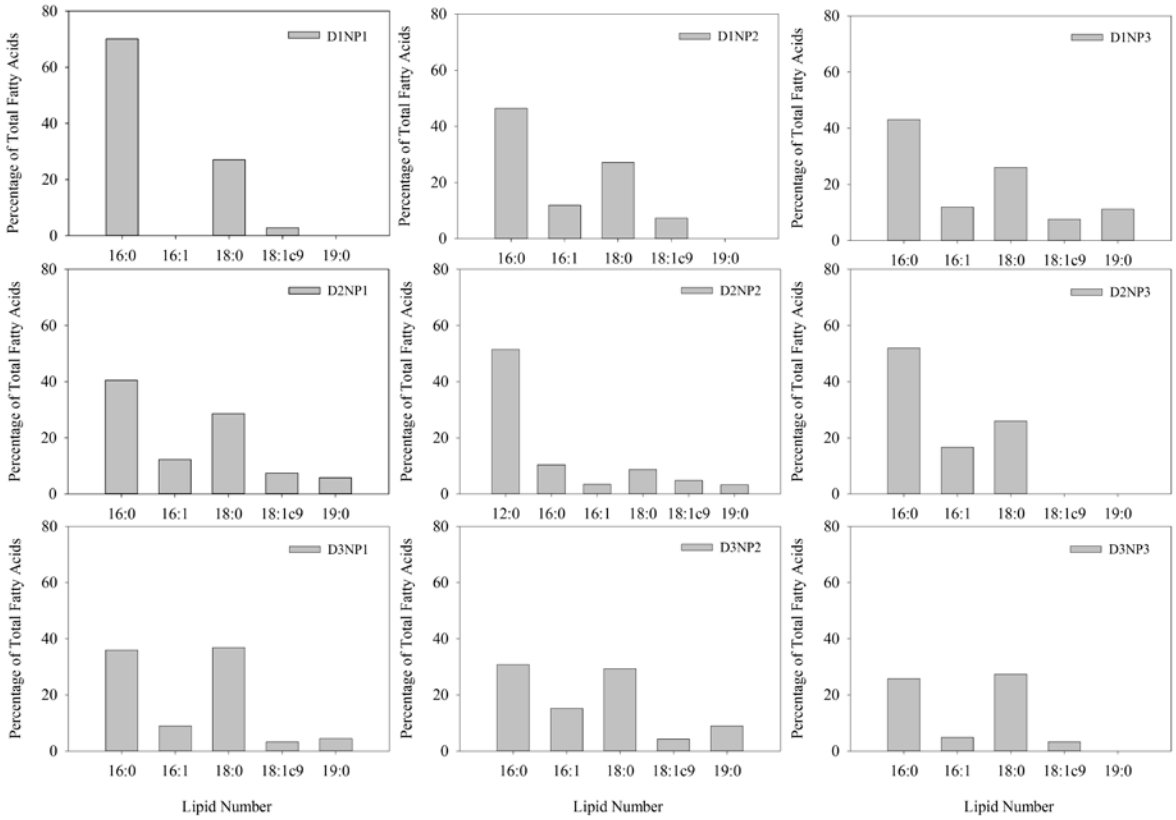

Figure 33 FAME distributions of biofilms harvested from nutrient study.

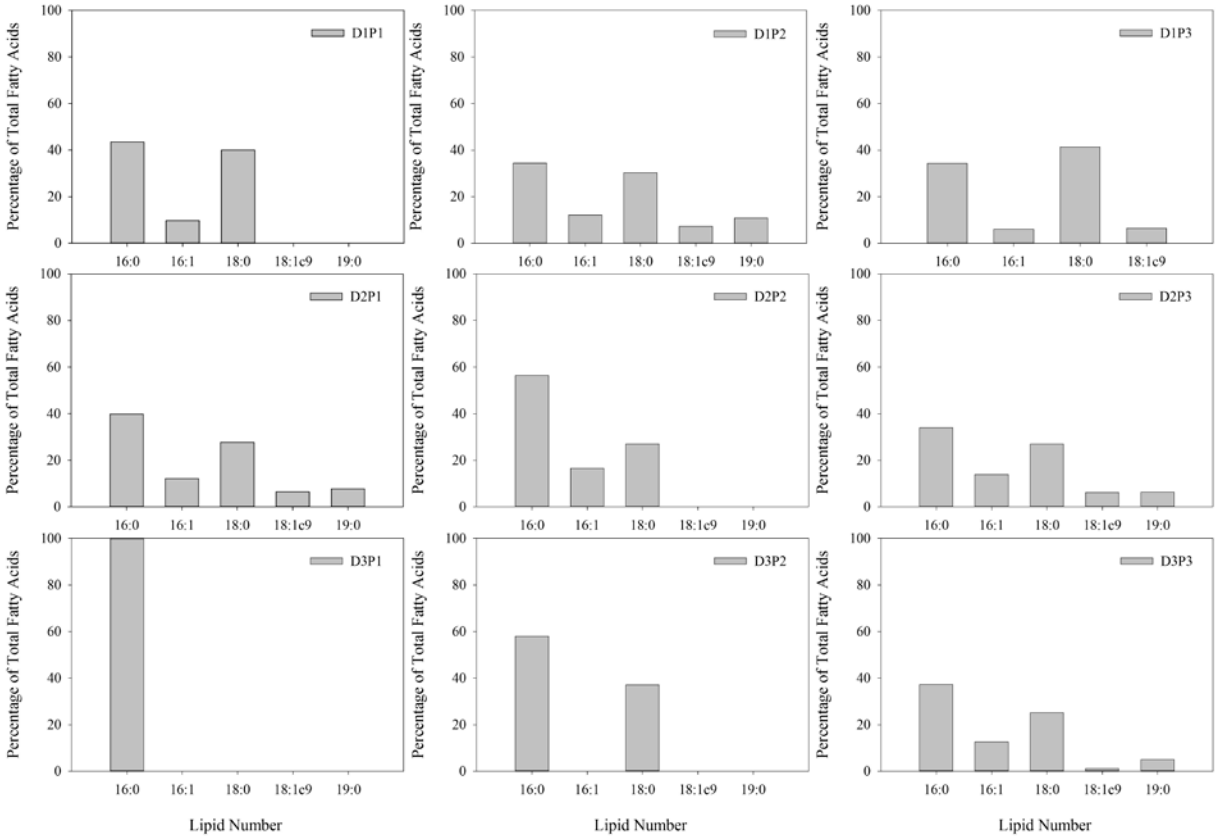

Figure 34 FAME distributions of biofilms harvested from nutrient microcosm study.

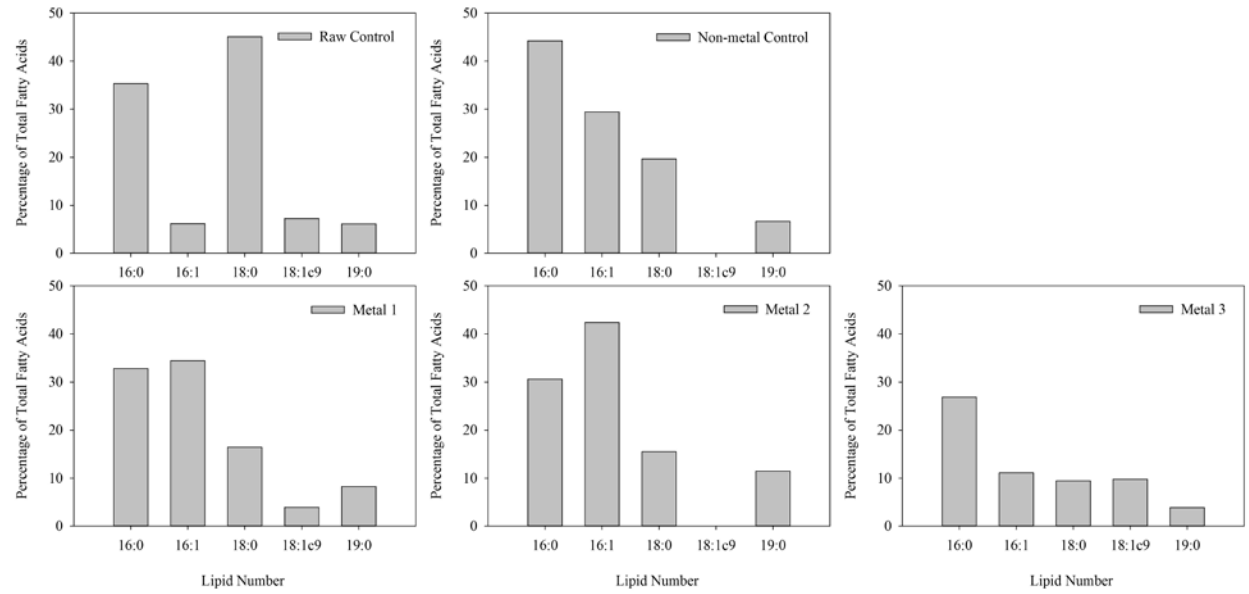

Figure 35 FAME distributions of biofilms harvested from metal microcosm study.

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 aeruginosas* (*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 NO_3 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. Haldemen et al. (1995) report a similar finding, where community FA structure and biomass was statistically indeterminist, 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 the community changes and culture counts was not connected via FA data. The Green & Scow critique of the Haldeman study provide 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 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 epikarst microbial community is much more diverse that what is reflected in observations of biomarkers from laboratory experiments. However, the results of the experiments do provide some indication physiochemical and community changes that potential could occur if conditions in the epikarst 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. 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 affecting 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 bactericidal, antiviral, and bacteriostatic characteristics which have 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 fry 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 in 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, NO_3, 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 NO_3, and in microcosms with the same treatment of DOC and NO_3 with an additional 1 μg/L of trace and heavy metals. After approximately 48 hours these microcosms with DOC, NO_3, and metals began producing biologically derived DIC, isotopic fractionation of DIC showed enrichment of the δ^{13}C-DIC in the heavier ^{13}C 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

(Fe^{2+}) is oxidized to ferric iron (Fe^{3+}). 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.