

9-10-2010

Nutrient cycling in impacted stream ecosystems: from microbes to watersheds

David van Horn

Follow this and additional works at: https://digitalrepository.unm.edu/biol_etds



Part of the [Biology Commons](#), and the [Terrestrial and Aquatic Ecology Commons](#)

Recommended Citation

van Horn, David. "Nutrient cycling in impacted stream ecosystems: from microbes to watersheds." (2010).
https://digitalrepository.unm.edu/biol_etds/119

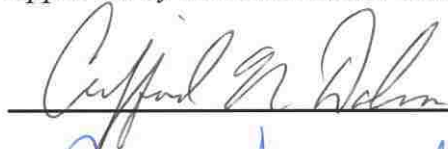
This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biology ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.

David J Van Horn
Candidate

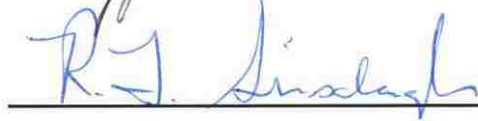
Biology
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:



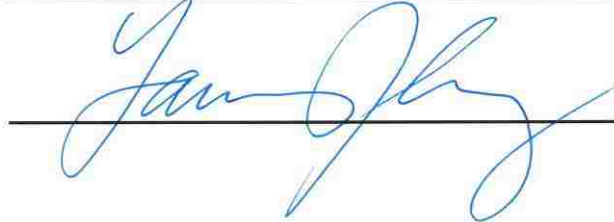
Dr. Clifford N Dahm, Chairperson



Dr. Robert L Sinsabaugh



Dr. Cristina D Takacs-Vesbach



Dr. Laura J Crossey

**NUTRIENT CYCLING IN IMPACTED STREAM
ECOSYSTEMS: FROM MICROBES TO WATERSHEDS**

BY

DAVID JAMES VAN HORN

B.S., Biology, Houghton College, 2001

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biology**

The University of New Mexico
Albuquerque, New Mexico

July, 2010

DEDICATION

For my parents, Herb and Dawn Van Horn, who started my career in ecology by introducing me to the woods and waterfalls of Southern Ohio before I could walk.

ACKNOWLEDGEMENTS

I acknowledge my dissertation advisor Dr. Cliff Dahm, and my committee members, Dr. Robert Sinsabaugh, Dr. Cristina Takacs-Vesbach, and Dr. Laura Crossey, for teaching, mentoring, and supporting me throughout the dissertation process. I also thank my fellow graduate students who assisted me along the way including Lydia Zeglin, Maceo Martinet, Chelsea Crenshaw, Jennifer Follstad Shah, Kendra Mitchell and many others. A special thanks to my community of friends in Albuquerque, my family, and my wife Jennifer DePaolo Van Horn, for supporting me through these years. Your encouragement sustained me throughout this process.

**NUTRIENT CYCLING IN IMPACTED STREAM
ECOSYSTEMS: FROM MICROBES TO WATERSHEDS**

BY

DAVID JAMES VAN HORN

ABSTRACT OF DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biology**

The University of New Mexico
Albuquerque, New Mexico

July, 2010

NUTRIENT CYCLING IN IMPACTED STREAM ECOSYSTEMS: FROM MICROBES TO WATERSHEDS

By

David James Van Horn

B.S., Biology, Houghton College, 2001
Ph.D., Biology, The University of New Mexico, 2009

ABSTRACT

The conditions found in stream ecosystems are an integration of watershed characteristics and processes. Anthropogenic disturbances are part of this integration and include direct inputs to streams, alteration of riparian areas, and modification of catchment properties which affect material inputs. Impacts to discrete portions of terrestrial watersheds combine as water moves down gradient, transporting the byproducts of catchment disturbances to streams including, nutrients, organic materials, particulates, and toxins. This dissertation explores the effects of disturbance on nutrient cycling in stream ecosystems at three spatial scales: the patch scale includes localized processes and assemblages, the reach scale encompasses tens to hundreds of meters of stream length, and the watershed scale consists of the hierarchical network of stream orders found in catchments. Additionally, the gut bacterial communities of three freshwater snail species were investigated to better understand the ecology and physiology of this important group of aquatic grazers. Snails are important regulators of periphyton growth, which plays an integral role in nutrient cycling in stream ecosystems.

Freshwater snails are also intermediate hosts for a variety of parasites of medical and veterinary significance.

Biofilm assemblages are patch scale communities which dominate the metabolism and biogeochemical cycles in stream ecosystems. To determine the effects of eutrophication, one of the most common disturbances to stream ecosystems, on the structure and function of heterotrophic stream biofilms, we created an enrichment gradient by amending darkened stream channel mesocosms with a stoichiometrically balanced solution of sucrose, NH_4 , and PO_4 . A total of ~2000 high quality bacterial partial 16S rRNA gene sequences yielded 381 unique phylotypes (<97% similarity). Significant differences ($p < 0.005$) were detected between communities from all treatments, with increasing enrichment resulting in greater community divergence and decreased diversity. Biofilm community productivity and function responded exponentially to enrichment, with exponents of 1.5 for areal mass, 2.3 for live cell density, and 2.5-3.5 for the activities of 5 extracellular enzymes. The observed nonlinear increase in functional capacity suggests biofilms are highly responsive to resource availability likely due to the physical structures and synergistic social interactions found in biofilm assemblages.

Domestic and native ungulate grazers significantly alter riparian areas, stream reaches, and catchment characteristics. We examined nutrient cycling linkages between riparian soils and adjacent streams and the impacts of ungulate grazing on these ecosystems and processes at six grazing enclosure sites in the Valles Caldera National Preserve, NM, USA. The exclusion of native and domestic ungulate grazers for three years significantly increased the riparian aboveground biomass of standing vegetation

(273 ± 155 vs. 400 ± 178 g m⁻²) and litter (56 ± 75 vs. 107 ± 77 g m⁻²) ($p = 0.005$ and 0.013 , respectively). Soil nutrient values (0 to 15-cm depth) were minimally affected by grazing after five growing seasons, with significant increases in soil total phosphorus at three of the six sites. No connection was found between soil and stream nutrient availability or limitation. Stream geomorphology was not significantly altered by five years of grazing exclusion. The elimination of grazing suppressed instream nutrient processing with significantly longer NH₄ uptake lengths ($p = 0.02$) and non-significant trends toward decreased NH₄ uptake rates observed in enclosure reaches. These results suggest ungulate grazing impacts terrestrial characteristics which are linked to ecosystem services provided by adjacent aquatic ecosystems. Management plans should carefully balance the positive effect of grazing on stream nutrient processing and retention reported here with the well documented grazing related loss of other ecosystem services such as decreased fish and aquatic invertebrate habitat and effects on water quality parameters such as turbidity and water temperature.

Nutrient cycling in aridland catchments and rivers is controlled by a unique set of inputs and retention mechanisms. We investigated spatial and temporal variation in the sources and sinks of nutrients in the middle Rio Grande (MRG), a 300 km reach of aridland river in the southwestern United States that drains an agro-urban catchment experiencing rapid population growth. Wastewater treatment plant inputs were the dominant source of nutrients to the MRG, increasing loads of NO₃-N, SRP, and NH₄-N by 1000-2000% relative to upstream loading. The total retention of NO₃-N and SRP inputs in the MRG corridor ranged from 6-99% and 34-99%, respectively. Retention was strongly and positively correlated with the percentage of water diverted from the MRG

for agricultural irrigation ($R^2 = 0.86$ and 0.80 for $\text{NO}_3\text{-N}$ and SRP, respectively).

Irrigation diversions downstream of the urban wastewater inputs sequestered on average 480, 370 and 40 kg day^{-1} of $\text{NO}_3\text{-N}$, SRP, and $\text{NH}_4\text{-N}$, respectively, during the irrigation season. Within the river channel, retention was $129\text{-}906 \text{ kg day}^{-1}$ for $\text{NO}_3\text{-N}$ and $56\text{-}779 \text{ kg day}^{-1}$ for SRP, values similar to those measured in mesic systems. However, the combination of in-stream and irrigation network nutrient processing in the MRG adds up to catchment scale retention levels that are significantly higher than those found in mesic systems.

Little is known about the microbial gut flora of freshwater snails in spite of the important role gastropod mollusks play as grazers in freshwater ecosystems. Some freshwater snail species are also responsible for the transmission of parasitic diseases including schistosomiasis, which affects ~ 200 million humans worldwide. This study used culture independent methods to describe the community composition and the variability of gut microbes within and among three species of planorbid snails, *Helisoma duryi* (North American species), *Bulinus africanus* (African species), and *Biomphalaria pfeifferi* (African species). Three hundred and fourteen unique bacterial operational taxonomic units (OTUs, DNA sequences with <98% similarity) were found in the guts of the three snail species. This diversity was distributed across 23 bacterial phyla with the largest number of OTUs found in the *Proteobacteria* and *Bacteroidetes* groups. A small percentage of bacterial clones from every snail species were related to opportunistic pathogens that infect a range of hosts including snails and humans. Measures of β diversity revealed minimal divergence among the gut microbial communities both within and among the three planorbid species, with samples differing primarily in the abundance

of sequences within bacterial lineages and not in the presence or absence of lineages. These results suggest the presence of highly diverse and relatively similar gut microbial communities in the three snail species in spite of varying levels of phylogenetic and geographic separation, and highlight the need for additional study to determine the roles gut microbes play in the physiology of these important intermediate hosts for digenetic trematodes of medical and veterinary significance.

This investigation of nutrient cycling in stream ecosystems at three scales and under three disturbance regimes revealed anthropogenic impacts substantially alter this important ecosystem function; however, negative impacts are frequently moderated by other factors. Eutrophication was shown to decrease bacterial diversity at the local level, however, the functional capacity to process organic materials increased exponentially for microbial communities exposed to enrichment. At the reach scale domestic and native ungulate grazers negatively impact numerous stream characteristics but in this study were shown to enhance instream nutrient retention. At the watershed level urban wastewater inputs were the dominate source of stream enrichment, however, the use of nutrient enriched river water for irrigation removed substantial portions of these inputs. These findings highlight the importance of ecosystem based management plans to address the effects of disturbance on nutrient cycling in impacted stream ecosystems.

TABLE OF CONTENTS

Chapter 1: NONLINEAR RESPONSES OF STREAM BIOFILM COMMUNITIES TO A RESOURCE GRADIENT	1
Abstract.....	1
Introduction.....	2
Materials and Methods	4
General Experimental Design	4
Biofilm Productivity and Physical Structure.....	6
Biofilm Function	7
Biofilm Community Structure and Diversity	7
Data Analysis	8
Results	10
Biofilm Physical Structure	10
Biofilm Function	12
Biofilm Community Composition.....	14
Discussion.....	19
Biofilm versus Planktonic Bacterial Responses to Increased Resources	19
Implications of Non-Linear Responses and Enzyme Ratios	22
Response of Bacterial Community Diversity and Composition to Enrichment and Implications for Productivity/Diversity/Function Relationships in Microbial Communities	24
Conclusions	28
Acknowledgements.....	29

Literature Cited.....	30
Chapter 2: THE EFFECTS OF WATERSHED CHARACTERISTICS AND	
UNGULATE GRAZING ON NUTRIENT CYCLING IN MONTANE GRASSLAND	
STREAMS AND RIPARIAN AREAS.....	41
Abstract.....	41
Introduction.....	42
Materials and Methods	45
Site Description	45
Soil Physical and Chemical Measurements.....	49
Above Ground Biomass Measurements	51
Stream Solute Injections.....	51
Statistical Methods	53
Results	53
Soil Physical and Chemical Measurements.....	53
Above Ground Biomass	57
Stream Physical Characteristics	57
Stream Nutrient Cycling Parameters	58
Discussion.....	61
VC Soil, Vegetation, and Stream Characteristics.....	61
Soil to Stream Connections	63
Grazing Impacts on Soil, Vegetation, and Stream Nutrient Cycling.....	64
Conclusions	68
Acknowledgements.....	69

Literature Cited.....	69
Chapter 3: NUTRIENT RETENTION IN ARIDLAND RIVERS.....	76
Abstract.....	76
Introduction.....	77
Materials and Methods	79
Site Description	79
Sampling Methods.....	83
Data Analysis	84
Results	85
Nutrient Sources to the MRG.....	85
Nutrient Sinks and Retention in the MRG	88
Water Loss Associated with Agricultural Irrigation.....	94
Discussion.....	94
Nutrient Sources and Sinks in the MRG	94
Potential Mechanisms for the Agricultural Nutrient Sink in the MRG.....	99
Comparison of Nutrient Retention in the MRG to Other River Ecosystems	102
Conclusions	106
Acknowledgements.....	106
Literature Cited.....	107
Chapter 4: GUT MICROBIAL COMMUNITY COMPOSITION AND VARIABILITY IN THREE PLANORBID SNAIL SPECIES	116
Abstract.....	116
Introduction.....	117

Materials and Methods	120
Collection and Identification of Snails.....	120
Bacterial Gut Flora 16S rRNA Gene Amplification and Sequencing.....	121
Statistical Analysis	122
Phylogenetic Analysis	125
Accession Numbers.....	126
Results	126
Snail Identification and Phylogeny.....	126
General Distribution of Bacterial 16S rRNA Sequences and OTUs.....	127
Bacterial Community Comparison between Individual Snails and Snail Species ...	135
Discussion.....	139
Gut Microbial Community Composition in Planorbid Snails.....	139
Gut Microbial Community Similarities between Samples and Snail Species.....	144
Conclusions	145
Acknowledgments	145
Literature Cited.....	146

Chapter 1: NONLINEAR RESPONSES OF STREAM BIOFILM COMMUNITIES TO A RESOURCE GRADIENT

David J. Van Horn¹, Robert L. Sinsabaugh¹, Cristina D. Takacs-Vesbach¹, Kendra R. Mitchell², Clifford N. Dahm¹

¹Department of Biology, University of New Mexico, 167 Castetter Hall MSC03 2020, 1
University of New Mexico, Albuquerque, NM 87131-0001

²Department of Microbiology & Immunology, University of British Columbia
Life Sciences Centre 2552-2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3

Abstract

The metabolism and biogeochemical cycles of aquatic ecosystems are largely mediated by microbial communities, with biofilm assemblages dominating in stream ecosystems. To determine the effects of resource availability on the structure and function of heterotrophic stream biofilms, we created an enrichment gradient by amending darkened stream channel mesocosms with a stoichiometrically balanced solution of sucrose, NH₄, and PO₄. A total of ~2000 high quality bacterial partial 16S rRNA gene sequences yielded 381 unique phylotypes (<97% similarity). Significant differences (p<0.005) were detected between communities from all treatments, with increasing enrichment resulting in greater community divergence and decreased diversity. Biofilm community productivity and function responded exponentially to enrichment, with exponents of 1.5 for areal mass, 2.3 for live cell density, and 2.5-3.5 for the activities of 5 extracellular enzymes. The observed nonlinear increase in functional capacity suggests biofilms are highly responsive to resource availability likely due to the physical structures and synergistic social interactions found in biofilm assemblages.

Introduction

The metabolism and biogeochemical cycles of aquatic ecosystems are largely mediated by microbial communities. Aquatic microbial communities come in two primary forms, planktonic assemblages that develop in the water columns of marine, lentic and large river environments, and attached biofilm communities whose contributions to ecosystem metabolism are most prominent in small to mid-sized streams, wetlands, and shallow lakes. While plankton and biofilm communities share similar biogeochemical and organic carbon processing functions they have markedly different physical structures and biotic interactions that may lead to differing responses to changing resource supply.

Planktonic microbial communities lack self-imposed physical structure. Metabolism is regulated by top-down (predator/prey) and bottom-up (resource availability) interactions. Bottom-up effects are dominant in oligotrophic systems and top-down interactions are most important in eutrophic environments (Dufour and Torreton 1996, Gasol et al. 2002, Vargas et al. 2007, Thelaus et al. 2008). Within planktonic communities there is little evidence for cooperative or synergistic interactions among populations. Predator-prey interactions create a microbial loop that either packages carbon and nutrients for higher trophic levels or returns dissolved carbon and nutrients to support bacterial production.

In contrast to planktonic microbial communities, attached microbial populations form complex structured associations in biofilms. When planktonic microbial cells adhere to solid surfaces, signal cascades alter gene expression and initiate the formation of a dense layer of extracellular polymeric substances (Watnick and Kolter 2000, Beloin and

Ghigo 2005) that shields biofilm organisms from predators and insulates inhabitants from external variables such as pH, temperature, ultraviolet light, desiccation, and toxic or antimicrobial substances (Webb et al. 2003, Hall-Stoodley et al. 2004). As biofilms thicken, physical and chemical gradients form internally, facilitating cooperative metabolic interaction within and between populations (Costerton et al. 1995, Davey and O'Toole 2000). Populations interact through multiple intercellular communication mechanisms including quorum sensing (Hense et al. 2007), programmed cell death within populations (Webb et al. 2003), and lateral gene transfer within and among species (Watnick and Kolter 2000, Parsek and Fuqua 2004).

The substantial differences between planktonic and biofilm associations likely impact the response of these communities to altered resource supply, and ultimately the ecosystem services provided. Because they are simpler to manipulate, plankton communities have been the focus of most studies that examine microbial community composition and metabolism in relation to resource availability. This extensive body of research suggests planktonic bacterial communities respond predictably to changing resources. Meta-analyses of cross site eutrophication gradients show that primary production increases linearly with nutrient enrichment, stimulating a linear, and less than 1:1, increase in bacterioplankton production (Cole et al. 1988, Thelaus et al. 2008). Bacterial biomass, however, increases more slowly than production as a result of increased predation (Thelaus et al. 2008). Similar patterns have been observed using experimental mesocosms through the manipulation of primary production (Hobbie and Cole 1984, Oviatt et al. 1986) and direct additions of C, N and P (Joint et al. 2002, Smith and Prairie 2004, Jansson et al. 2006).

In contrast, studies of the effects of resource supply on biofilm community structure and function are limited. Evidence from single level enrichment studies in microcosms suggests that modest increases in resource supply in the form of C (sucrose), N and P, lead to substantial, and possibly non-linear, changes in biofilm production and function (Mohamed et al. 1998, Chenier et al. 2003, Chenier et al. 2006).

In this study, we used stream mesocosms to measure the effects of a resource supply gradient on heterotrophic biofilm productivity and function, community diversity and structure. We compared the magnitude and linearity of these responses in relation to resource supply, the implications of these responses for biofilm turnover time and nutrient retention, and contrast these responses to those reported for planktonic microbial communities.

Materials and Methods

General Experimental Design

Heterotrophic microbial biofilms were established in the dark in fifteen experimental stream channel mesocosms (Singer et al. 2006). The channels (depth/width/length: 0.02/0.1/3.0 m) were lined with removable unglazed ceramic tiles, supplied with stream water to ensure colonization by ambient microbial populations, and continuously enriched with a stoichiometrically balanced (C:N:P ratio 106:16:1) solution of dissolved organic carbon (DOC), N and P to create a productivity gradient (Fig. 1). Water was diverted to the mesocosms from an intermittent stream located in Bear Canyon in the foothills of the Sandia Mountains near Albuquerque New Mexico, at a rate of 0.03 l s⁻¹ per mesocosm, generating a nominal flow velocity of ~ 2 cm s⁻¹ within the channels. Enrichment treatments were multiples of the ambient DOC concentration (1.5 mg l⁻¹).

Treatments included no enrichment in control channels, and 2X, 4X, 8X and 10X increases in ambient DOC concentration with supplemental N (NO_3 , ambient concentration = $0.002 \text{ mg l}^{-1} \text{ NO}_3\text{-N}$) and P (PO_4 , ambient concentration = $0.002 \text{ mg l}^{-1} \text{ PO}_4$) added to preserve a C:N:P ratio of 106:16:1. Nutrient solutions were metered into channels behind baffles to assure even mixing. Three replicate channels were used for each level of enrichment. After three weeks of growth, the time necessary to produce a mature biofilm, colonized tiles were collected for biofilm analyses.

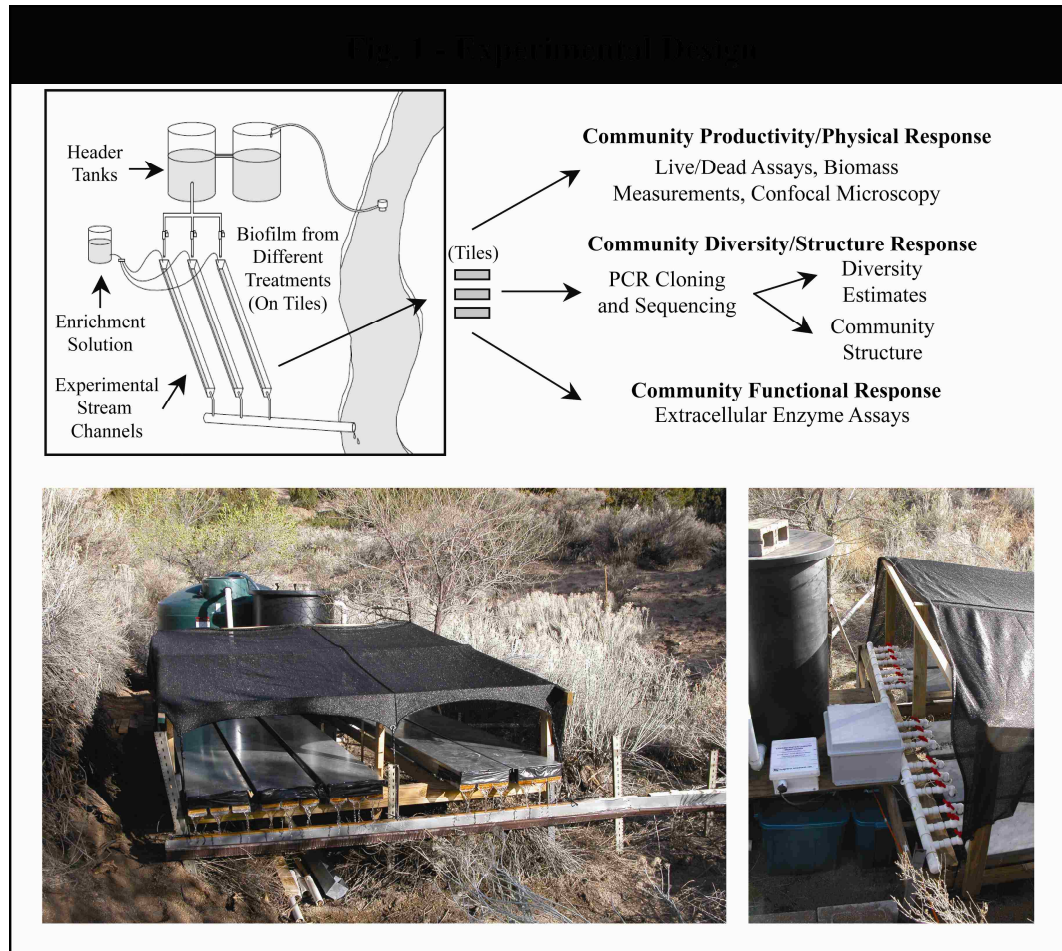


Figure 1: Schematic experimental design for stream-side mesocosm experiment along with photos of the installed mesocosms.

Biofilm Productivity and Physical Structure

Biofilm mass (dry mass - DM, and ash free dry mass - AFDM) was measured for three replicate tiles from each channel. Biofilm was scraped from the tiles and deposited onto ashed, tared glass fiber filters placed in aluminum pans. Filters were dried at 80°C, reweighed to calculate DM, and ashed at 500°C for 3 h. Ashed filters were reweighed and AFDM was calculated as the difference between dry mass and ash mass.

The relative abundance of live and dead cells was assessed for triplicate samples from each channel using a BacLight Kit (Invitrogen, Eugene, OR). The stains were checked for linearity of fluorescence over the biofilm cell densities to be analyzed. Samples were homogenized in a bicarbonate buffer, eight 250 µl replicates for each sample were pipetted into black 96-well microplates, and 6 µl of an equal mixture of a 1:10 dilution of the stains was added to the microplate wells. Samples were incubated at room temperature in the dark for ~15 min and then read on a fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA) set to an excitation wavelength of 485 nm and an emission wavelength of 538 nm for the SYTO[®] 9 stain, and an excitation wavelength of 485 nm and an emission wavelength of 591 nm for the propidium iodide stain. Results were normalized by the area of the tile sampled and were corrected for dilution where appropriate.

The physical structure of biofilms was assessed using confocal microscopy. Briefly, a single biofilm colonized tile from each channel was placed in a plastic tray while still submerged in the experimental stream channel, and transported on ice to the Keck Confocal Laboratory at the University of New Mexico. Samples were stained with

the live/dead stain described above and imaged using a 5x objective on a LSM 510 confocal microscope (Carl Zeiss).

Biofilm Function

Biofilm function was assessed by measuring the potential activity of five hydrolytic extracellular enzymes: α -glucosidase (AG), β -glucosidase (BG), N-acetylglucosaminidase (NAG), alkaline phosphatase (AP), and leucine aminopeptidase (LAP). Potential activities were measured using methylumbelliferyl linked substrates following protocols similar to those described by Sinsabaugh et al. (1997). Triplicate tiles were analyzed from each channel. Biofilm was scraped from the tile and homogenized in 50 mM bicarbonate buffer (pH 8). 200 μ l aliquots of biofilm homogenate and 50 μ l of 200 μ M substrate were added to black, 96-well microplates with 16 replicate wells per sample. The microplates were incubated in the dark at room temperature. Each plate contained reference standards, substrate controls, and sample controls. Fluorescence was measured periodically for up to 19 h using a fmax Fluorescence Microplate Reader set to an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The fluorescence results were checked for linearity over the incubation period and activities were calculated as nmol substrate converted per hour per cm^2 of tile ($\text{nmol h}^{-1} \text{cm}^{-2}$).

Biofilm Community Structure and Diversity

Bacterial 16S rRNA gene sequences were amplified using the bacteria-specific forward primer 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1492R 5'-GTTTACCTTGTTACGACTT-3' (Lane 1991) in triplicate 50 μ l reactions containing 5 μ l 10X buffer (Promega Buffer B with 1.5 mM MgCl_2), 12.5 mM each dNTP (BioLine

USA, Inc.), 20 pmol each of 8F and 1492R primers, 2.5 U Taq polymerase (Promega U.S.). The PCR thermal cycling (ABI GeneAmp 2700, Applied Biosystems, Foster City, CA) consisted of 30 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. Replicate 16S rRNA gene amplifications were pooled and gel purified using a DNA Purification Kit (MoBio Laboratories, Carlsbad, CA), and cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). One hundred and ninety two clones per library were sequenced using high-throughput Sanger sequencing (ABI 3730 Capillary Sequencer, Applied Biosystems, Foster City, CA), half with M13 and half with 8F primers.

Data Analysis

Differences among enrichment treatments for biomass, enzyme activities, and live/dead cell abundances were assessed using one-way ANOVA and Bonferroni multiple comparisons on log-transformed data using SAS (version 9.2, SAS Institute, Cary, North Carolina). SMATR (Falster et al. 2006, Warton et al. 2006) was used to calculate exponential scaling exponents and 95% confidence intervals for biomass, enzyme activities, and live/dead cell abundances using ordinary least squares regression of log-transformed data. SMATR was also used to fit a line to C:P (BG:AP) and C:N (BG:(LAP+NAG)) enzyme ratios using the standard major axis method (SMA) and to determine significant differences in the slopes of these lines.

Biofilm community 16S rRNA gene sequence data was checked for quality using CodonCode Aligner. High quality sequences (average Phred 20 values > 500) greater than 600 bp were exported to Greengenes (<http://greengenes.lbl.gov>) for alignment (NAST Alignment Tool, DeSantis et al. 2006a), chimera checking (Bellerophon Chimera Check Tool, Huber et al. 2004), identification of the most closely related 16S rRNA gene

sequences previously characterized from cultured and uncultured bacteria (DeSantis et al. 2006b), and determination of the taxonomic affiliation (Hugenholtz classification, Classify Tool). ARB was used to filter the sequences to a uniform length and create a distance matrix of the aligned and filtered sequences (Ludwig et al. 2004). The taxonomic affiliation results and distance matrix were analyzed in mothur (Schloss et al. 2009) to divide sequences into phylotypes, generate rarefaction curves using a 97% DNA sequence similarity cutoff, calculate the Chao1 estimate of richness, and examine the taxonomic affiliation and abundance of each phylotype in each sample using a heatmap.

The phylogeny of the bacterial 16S rRNA genes from the enrichment gradient was analyzed using UniFrac (Lozupone and Knight 2005, Lozupone et al. 2006) and mothur. Briefly, all aligned, high quality sequences were added to a backbone phylogenetic tree of 6634 bacterial 16S rRNA gene sequences (Hugenholtz 2002) using the parsimony add function in ARB (Ludwig et al. 2004). This tree was imported into UniFrac to calculate the UniFrac metric which is defined as the phylogenetic distance between sets of taxa in a tree, calculated as the percentage of branch length that leads to descendants from only one of a pair of environments represented in a single phylogenetic tree (UniFrac Metric) (Lozupone and Knight 2005). Parsimony (mothur) and un-weighted UniFrac hypothesis testing were performed to test whether the communities from the five treatments had the same structure. Environment Distance Matrices (EDM-UniFrac) were calculated to measure distances between all sample pairs in a tree (Lozupone et al. 2006) to hierarchically cluster samples using an Un-weighted Pair Group Method with Arithmetic Mean (UPGMA-UniFrac) algorithm (Lozupone et al. 2006). Jackknife analysis was used to assess confidence in the nodes of the UPGMA tree (Lozupone et al. 2006). The EDM

were also used to perform a principal coordinate analysis (PCoA-UniFrac) (Lozupone et al. 2006).

Results

Biofilm Physical Structure

Mean AFDM values in the control, 2X and 4X enrichments ranged from ~ 0.1 to 0.25 mg cm⁻² while values in the 8X and 10X enrichments ranged from ~1.0 to 1.25 mg cm⁻² (Fig. 2). AFDM values for the control and 2X enrichment were similar and not statistically different. The values for the 8X and 10X enrichments were also similar to each other.

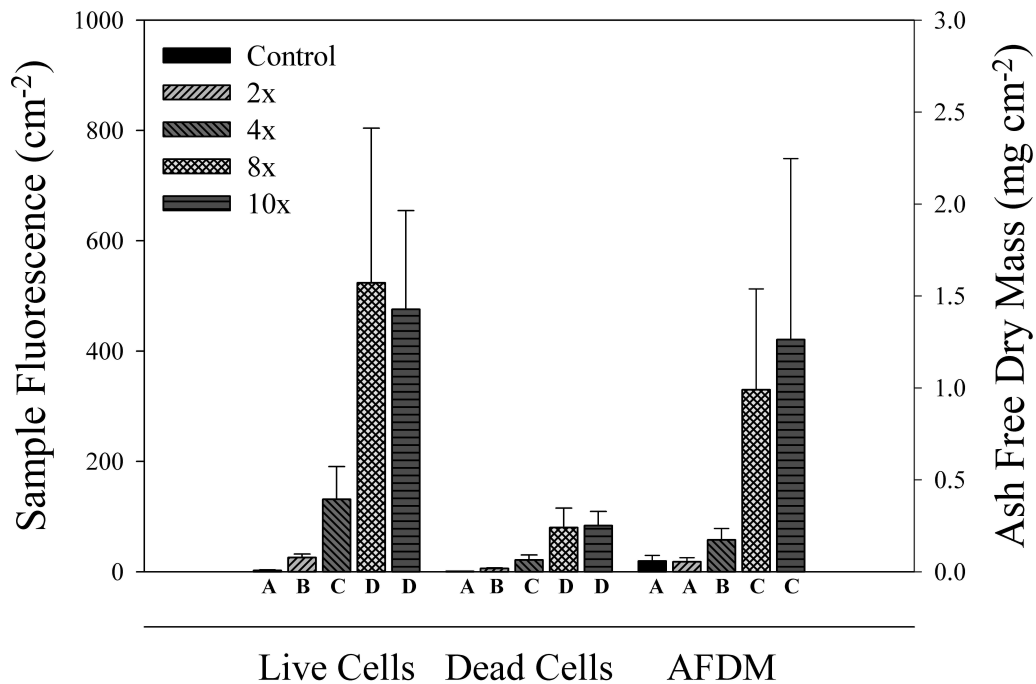


Figure 2: Biomass response to enrichment as measured by the fluorescence signal of live/dead cells (cm²) and ash free dry mass (mg cm⁻²). Values were obtained for all treatments, however, in some cases control values are too low to be seen in the figure.

The areal density of AFDM and DM responded in a roughly linear fashion to the resource gradient with 23 and 18 fold increases (scaling exponents (b) and 95% CI of 1.48 ± 0.28 and 1.31 ± 0.29), respectively, over the 10 fold enrichment gradient (Fig. 3).

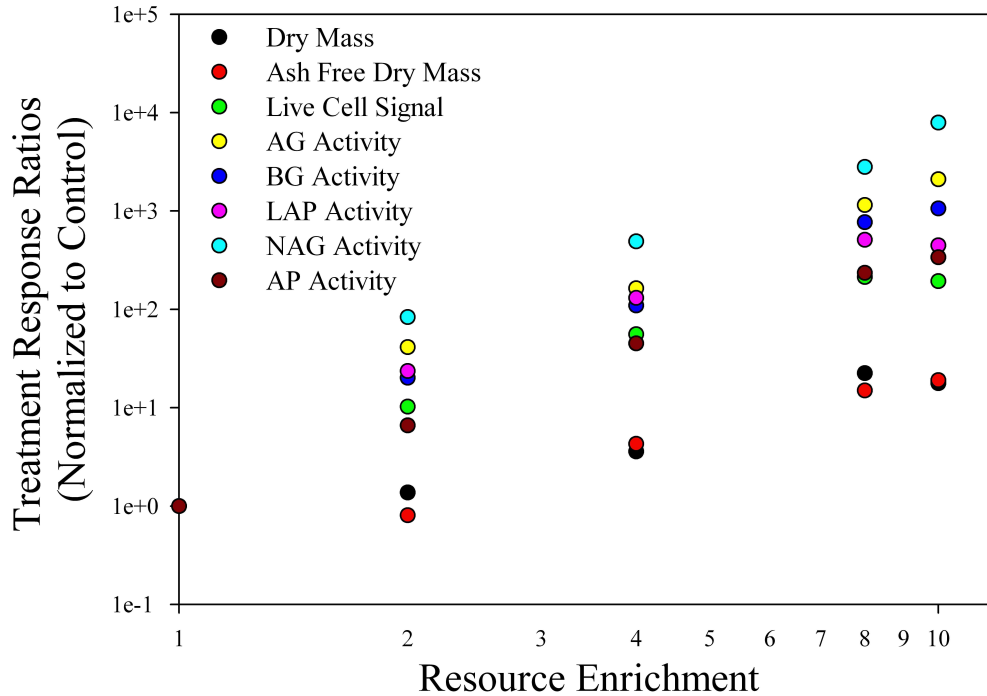


Figure 3: Response ratios (normalized to control) of biofilm biomass and functional parameter responses to an enrichment gradient. The single dot at an enrichment level of 1 represents the control values for all eight parameters.

Confocal laser microscopy showed that biofilm from the control treatment was a dense layer of bacterial cells with minimal vertical development. Images from successive enrichments showed increasing vertical development driven by increasing abundance of filamentous growth forms interspersed with cocci and rod shaped cells (images not shown). As a result, the areal abundance of live and dead cells increased much more than AFDM in response to resource enrichment with increments of 10, 53, 213, and 193 fold

and 10, 34, 126, and 132 fold, respectively, in the 2X, 4X, 8X, and 10X enrichments, respectively (b and 95% CI of 2.25 ± 0.23 and 2.05 ± 0.18) (Fig. 3). The live and dead cell fluorescence values for the control, 2X, and 4X treatments were significantly different from one another and from the 8X and 10X treatments, which were not statistically different (Fig. 2). One of the 15 channels, channel 9 a 4X treatment, was a consistent outlier and was excluded from the biofilm physical structure and function analyses.

Biofilm Function

As biofilm mass cm^{-2} increased in response to enrichment, extracellular enzyme activities (EEA) increased exponentially, with responses even greater than those observed for cell density (Fig. 3). In relative terms, EEA increased in the order AG, NAG, BG, LAP, and AP. For AG, activity in the control and 10X enrichment treatment ranged from 0.004 to $8.0 \text{ nmol h}^{-1} \text{ cm}^{-2}$, respectively; for AP the corresponding range was 0.14 to $47 \text{ nmol h}^{-1} \text{ cm}^{-2}$. Response ratios for the 10X enrichment relative to the control treatment increased in approximately the reverse order with values of 350, 500, 1100, 2100 and 7900 for AP, LAP, BG, AG, and NAG, respectively (Fig. 3). Exponential scaling exponents and 95% CI were 2.42 ± 0.26 , 2.48 ± 0.35 , 2.76 ± 0.40 , 3.01 ± 0.43 , and 3.46 ± 0.52 for AP, LAP, BG, AG, and NAG, respectively (Fig. 3). Only one enzyme, LAP, reached a plateau at the 8X enrichment with a slight decrease in activity found in the 10X enrichment (Fig. 4). For each enzyme, activity in the control treatment was significantly different from that in enrichment treatments while activities in the 8X and 10X enrichment treatments were not significantly different (Fig. 4).

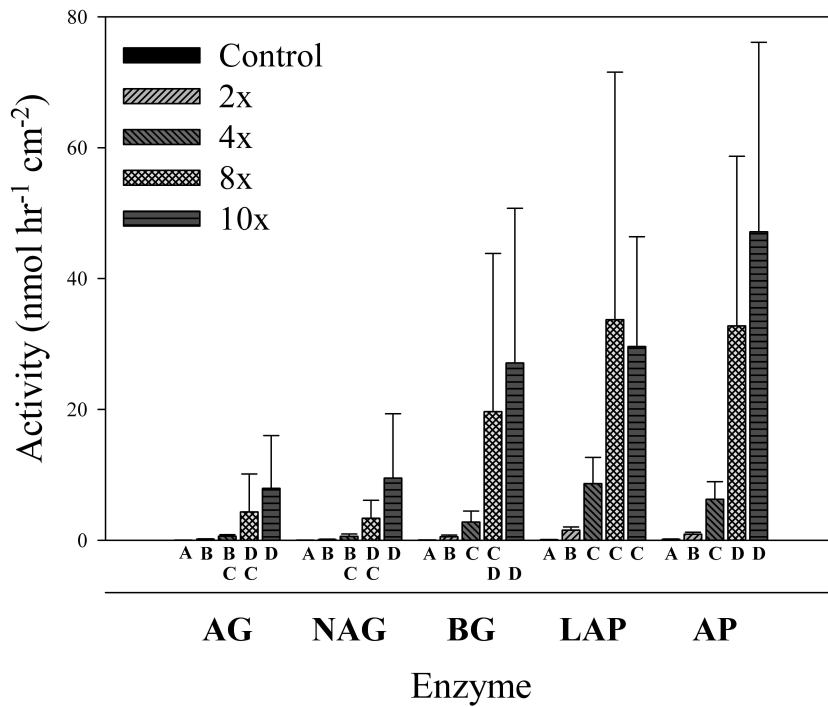


Figure 4: Response of extracellular enzyme activities (nmol hr⁻¹ cm⁻²) to an enrichment gradient. Values were obtained for all treatments, however, control values are too low to be seen in the figure in some cases.

Across the enrichment gradient, ratios of the activities of C, N and P acquiring enzymes, represented as BG:(LAP+NAG):AP remained consistent at ~ 1:1:1 (Fig. 5). This stoichiometric EEA ratio is characteristic of heterotrophic microbial communities in soils and freshwater sediments (Sinsabaugh et al. 2009). SMA of ln(BG) vs. ln(LAP+NAG), and ln(BG) vs. ln(AP) across the enrichment gradient had significantly different slopes ($p < 0.02$) of 1.09 (95% CI ± 0.05) and 1.20 (95% CI ± 0.07), respectively (Fig. 5).

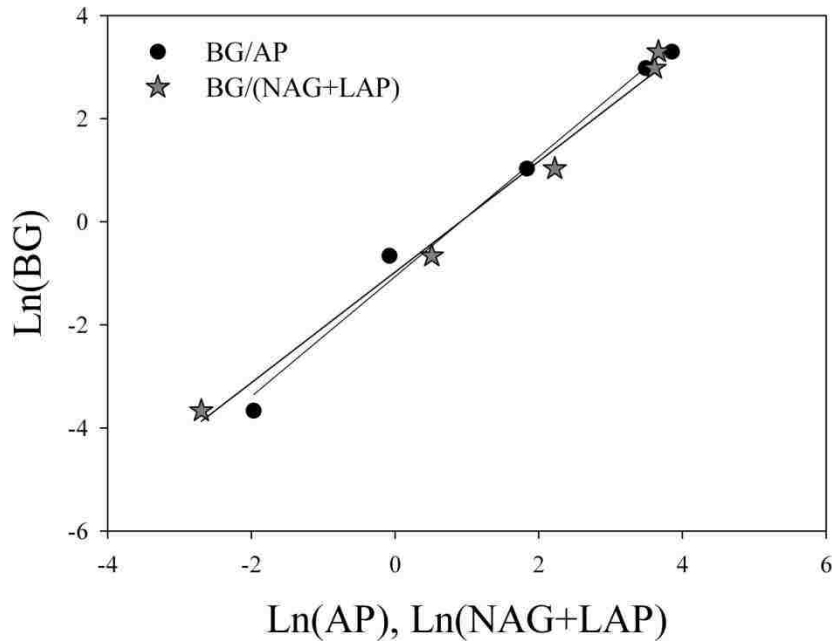


Figure 5: C:P and C:N ratios of the ln normalized activities for enzymes responsible for acquiring carbon (BG), nitrogen (NAG + LAP), and phosphorus (AP).

Biofilm Community Composition

A total of 2014 high quality partial 16S rRNA gene sequences were obtained. These sequences were distributed across the resource gradient treatments with 391, 413, 421, 411, and 378 sequences from the control, 2X, 4X, 8X, and 10X enrichment treatments, respectively. A total of 381 phylotypes were identified at the species level (<97% similarity) with 206, 87, 94, 78, and 71 phylotypes found in the control, 2X, 4X, 8X, and 10X treatments, respectively. Rarefaction curves from the four enrichment treatments (97% similarity cut-off) approached a plateau indicating comprehensive sampling of the 16S rRNA gene diversity amplified in these samples (Fig. 6). The rarefaction curve from the control treatment did not reach a plateau; however, the slope of this curve was decreasing indicating a substantial amount of the 16S rRNA gene diversity

was successfully amplified (Fig. 6). The rarefaction curves indicated that bacterial diversity progressively decreased from the control, 4X, 2X, 8X, and 10X enrichment treatments (Fig. 6) with 95% confidence intervals for Chao1 species richness estimates (<97% identity) of 402-723, 131-278, 107-159, 106-226, and 103-241, respectively.

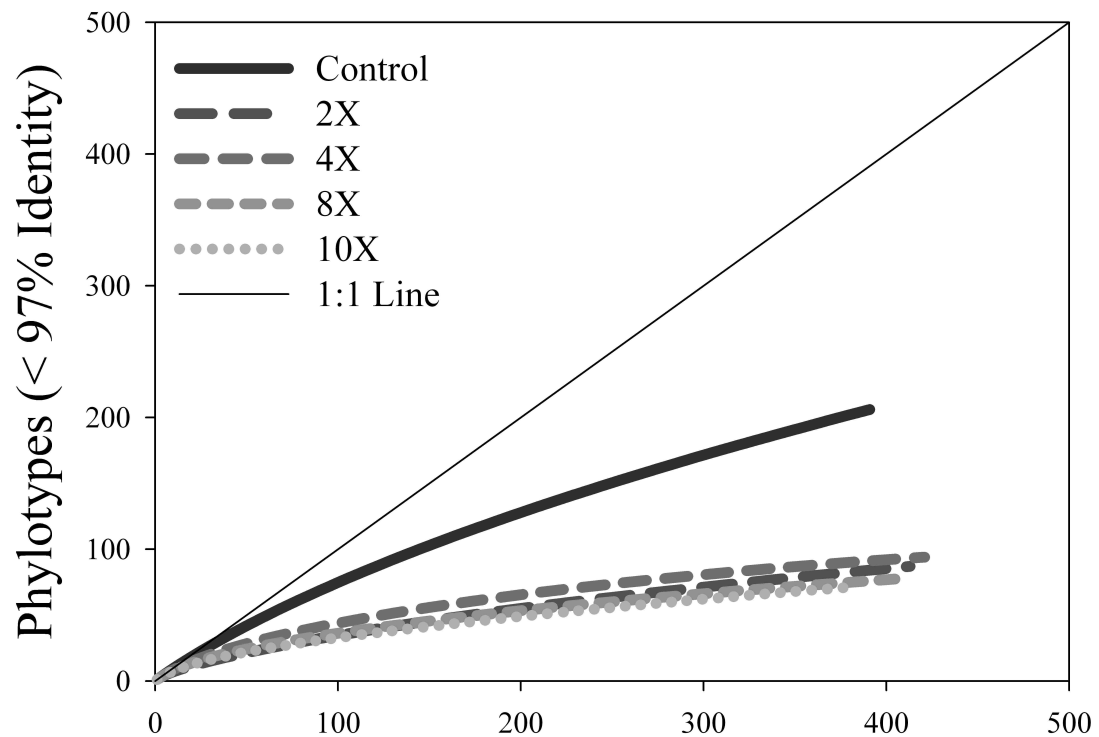


Figure 6: Rarefaction curves created in mothur (<97% similarity) for the bacterial communities from the five enrichment treatments obtained by analysis of a distance matrix created from sequence data in ARB.

Representatives from 10 bacterial phyla and candidate divisions were found in the 2014 partial 16S rRNA gene sequences recovered. Of the 10 phyla detected, 8, 7, 4, 4, and 4 were represented in the control, 2X, 4X, 8X and 10X enrichment treatments, respectively (Fig. 7).

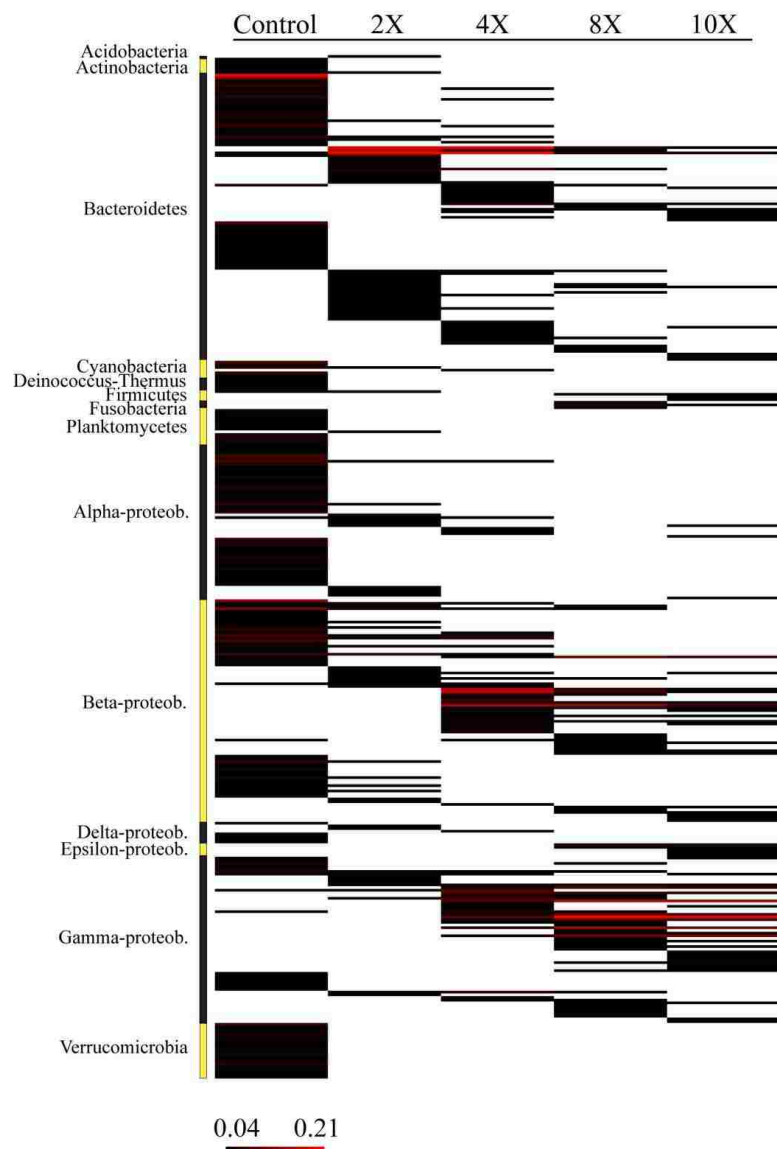


Figure 7: Abundance heatmap created in mothur for bacterial 16S rRNA gene sequences ordered by taxonomy (Hugenholtz classification) and enrichment level. Each row in the heatmap represents a phylotype defined at 97% similarity cutoff. The color of each phylotype bar represents the maximum relative abundance (*i.e.* [relative abundance / maximum relative abundance]) of each phylotype for each enrichment treatment according to the legend in the bottom left corner. The phylum division of each phylotype is indicated on the left.

An analysis of phylotype overlap (<97% identity) between treatments showed the least overlap between the control and 10X treatments (4 phylotypes) and the most overlap

between the 4X and 8X (37 phylotypes) and 8X and 10X (37 phylotypes) treatments (Table 1). In general, samples from treatments with increasingly divergent levels of enrichment shared a decreasing number of phylotypes (Fig. 7).

Table 1: Phylotype overlap between treatments.

Treatment Comparison	Shared Phylotypes (97% Similarity Cut-off)
Cont - 2X	28
Cont - 4X	23
Cont - 8X	8
Cont - 10X	4
2X - 4X	28
2X - 8X	15
2X - 10X	7
4X - 8X	37
4X - 10X	30
8X - 10X	37

The parsimony and un-weighted UniFrac hypothesis testing analysis both indicated that communities from the five treatments were significantly different from one another ($p < 0.005$), with the exception of the 8X and 10X treatments, which were not significantly different using the UniFrac test. UPGMA clustering of the sequence data in UniFrac (1000 permutations, un-weighted) revealed nine sample groupings that were well supported by jackknife analysis (data not shown). Sequences from the control, 2X, and 4X treatments grouped into three distinct clades, indicating that the community structures of these samples were unique from each other and the 8X and 10X samples. The 8X and 10X samples grouped together in a fourth clade with mixed grouping of samples among the two treatments. PCoA results show relatively tight and distinctive grouping of sequences from the control, 2X and 4X enrichment treatments and a grouping that includes sequences from the 8X and 10X treatments (Fig. 8). Principal Coordinate 1

explained 27% of the variation in the samples and appeared to be well correlated with the eutrophication enrichment level as the sample clusters increased in enrichment from the left to the right side of the plot (Fig. 8).

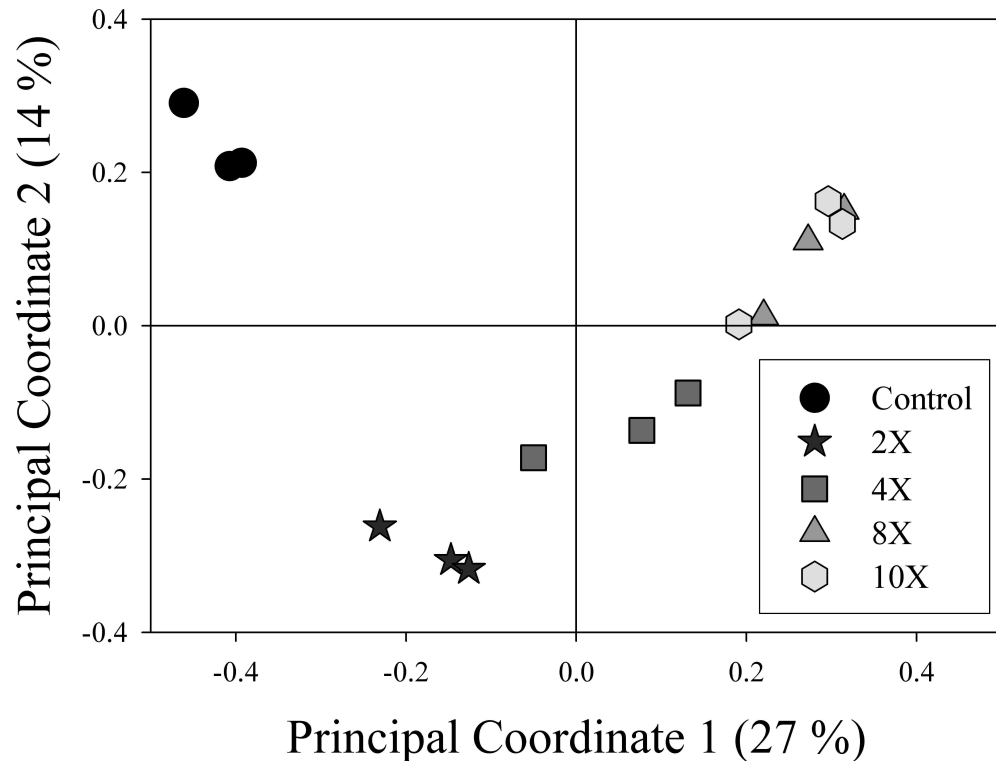


Figure 8: Principal coordinate analysis performed in UniFrac for the bacterial communities of the fifteen individual enrichment channels. Analysis was performed using the UniFrac un-weighted analysis option which provides a qualitative measure of β diversity, disregarding the relative abundance of lineages in the sample tree and focusing on the presence/absence of bacterial lineages within a community.

Classification of the partial 16S rRNA gene sequences (RDP Classification, Greengenes Classification Tool) revealed taxonomic shifts in the most frequently found sequences from each treatment. Sequences from the control treatment were the most evenly distributed between numerous bacterial genera with the greatest percentage of sequences from the *Flavobacterium* (Bacteroidetes), *Leptothrix* (Betaproteobacteria), and

Chitinophaga (Bacteroidetes) genera. Sequences from the 2X enrichment were most commonly found in the *Arcicella* (Bacteroidetes), *Leptothrix* (Betaproteobacteria), and *Rheinheimera* (Gammaproteobacteria) genera, the 4X enrichment was composed of *Arcicella* (Bacteroidetes), *Rhodoferrax* (Betaproteobacteria), and *Rheinheimera* (Gammaproteobacteria) genera, and the 8X and 10X enrichments were most populated with sequences from the *Aeromonas* (Gammaproteobacteria), *Janthinobacterium* (Gammaproteobacteria), and *Arcicella* (Bacteroidetes) genera.

Discussion

Biofilm versus Planktonic Bacterial Responses to Increased Resources

Biofilm community biomass responded nonlinearly to enrichment, with exponents (b) of ~ 1.5 for areal mass and 2.3 for live cell density. Comparable results for cell density were reported by Mohamed et al. (1998) who found that a 15% increment in available DOC led to 3.6X increment in biofilm bacterial density (b ~ 2). In contrast, a synoptic comparison of fifteen marine systems showed that planktonic bacterial biomass and abundance increased only 10X over a 2800X range in primary production (b ~ 0.01) (Thelaus et al. 2008). Bacterial productivity was more dynamic, showing a 1000X response in relation to the 2800X change in primary production (b ~ 0.8). Planktonic responses to experimental enrichments over much smaller ranges show similar results. Jansson et al. (2006) and Joint et al. (2002) reported that 2X and 32X enrichments of C, N, and P in planktonic mesocosms resulted in 2X and 17X increases in bacterial production (b ~ 0.7) and a 1X and 2X increase in bacterial biomass (b ~ 0.1), respectively. These findings show that biofilm development is highly responsive to

resource availability with ultimate limits likely imposed by physicochemical constraints such as shear strength and diffusion gradients (Battin et al. 2003, Besemer et al. 2007).

As biofilm biomass increased in response to resource enrichment the activity of enzymes responsible for C, N and P acquisition increased more quickly (range of $b \sim 2.4-3.5$). These results are generally consistent with previous biofilm studies that used single level enrichments (but see Ylla et al. 2009). Modest 15-20% increases in C, N and P resulted in 3 to 200 fold increases ($b \sim 1.2-3$) in biofilm functional processes including the utilization of carbon and nitrogen compounds (Chenier et al. 2003, Chenier et al. 2006), nitrification and denitrification rates (Chenier et al. 2003, Chenier et al. 2006), and bacterial abundance (Mohamed et al. 1998). In contrast to biofilms, functional processes in planktonic microbial communities exhibit muted responses to increased resources. A study relating EEA to bacterioplankton productivity in three rivers found that EEA lagged resource driven increases in production ($b < 1$) (Sinsabaugh et al. 1997, Sinsabaugh and Shah 2009). Several mesocosm studies have used glucose additions to stimulate nitrogen removal by bacterioplankton [27, 28, 49]; the response ratios appear to be well below one, suggesting that C enrichment minimally impacted these functions (Shiah and Ducklow 1995, Joint et al. 2002, Jansson et al. 2006). These findings are not surprising given the dispersed nature of planktonic communities and their modest biomass response to increased resources. These results suggest the differing biomass/production responses of biofilm and planktonic communities to altered resources lead to differing functional capacities for these aquatic microbial communities.

Plankton and biofilm communities share a common physiological organization in terms of EEA stoichiometry (Sinsabaugh et al. 2009) and the relationship between EEA

and productivity (Sinsabaugh et al. 2010, in revision). The differential responses of these communities to nutrient enrichment reflect the fate of the products as can be seen when biomass turnover times are considered along with predation patterns and functional responses to enrichment. Turnover (% biomass day⁻¹) has been estimated for a wide variety of attached and planktonic bacterial communities. Biofilm turnover for environments including decaying leaf litter, streams, mesocosm wetlands, and tropical coastal lagoons range from ~ 0.02 – 4000 % day⁻¹ with a median value of ~ 48 % day⁻¹ (see summary table in Su et al. 2007, and Thomaz and Esteves 1997a, Thomaz and Esteves 1997b, Törnblom and Søndergaard 1999, Carr et al. 2005, Tao et al. 2007). Bacterioplankton turnover for lakes and the open ocean ranges from ~3-180 % day⁻¹ with median values of ~ 33 % day⁻¹ (Hyun et al. 1998, Torréton et al. 2002, Hyun and Kim 2003, Chen et al. 2005, Hyun and Yang 2005, Gao et al. 2007).

While significant variability exists, median turnover rates for the two types of communities are similar; however, the mechanisms regulating these rates appear to be different. Bacterioplankton biomass increases moderately in response to increased resources while production and predation increase very rapidly, with total predation increasing 1000 fold, and predation per unit of bacterial biomass increasing 100 fold, over a 2800 fold range in primary productivity (Thelaus et al. 2008). Thus, although bacterioplankton production rates are high, predation eliminates much of this biomass and is a primary factor controlling turnover rates. In biofilm communities, biomass increases moderately with increasing resources ($b \sim 1.5$). However, enzymes responsible for the depolymerization of senescent material increase much more rapidly than biomass, resulting in the rapid and efficient internal cycling of materials. While grazing can be

important in regulating biofilms (Huws et al. 2005), attached bacteria produce chemical defenses that protect the communities from eukaryotic predation (Weitere et al. 2005, Matz et al. 2008) and modify their organization to create grazing resistant structures (Wey et al. 2008). These results suggest turnover in biofilms is a result of internal recycling and reuse supported by the structural properties of biofilm communities that concentrate nutrients (Tsuchiya et al. 2009), energy yielding materials, and extracellular enzymes, which are dispersed in planktonic communities.

Implications of Non-Linear Responses and Enzyme Ratios

Results from this study also provide insights into the effects of enrichment on biofilm efficiency, stoichiometric balance, and the potential these communities have for nutrient retention. Ratios of extracellular enzymatic activities can be incorporated into the threshold elemental ratio concept to link the functional stoichiometry of microbial communities with resource lability, growth and assimilation efficiencies, and the metabolic theory of ecology (MTE) (Sinsabaugh et al. 2009). The threshold elemental ratio concept connects stoichiometry and metabolic theory by combining metabolic parameters, such as growth efficiency and respiration, and the elemental composition of organisms and their food sources (Allen and Gillooly 2009). A meta-analysis of soil and sediment extracellular enzyme activities found the ratio of commonly measured activities responsible for C, N, and P acquisition was 1:1:1 and was invariant over a wide range of habitats (Sinsabaugh et al. 2009). These findings suggest disparate microbial communities have similar functional organization. Ratios of close to 1:1:1 in this biofilm study were maintained across a tenfold enrichment gradient, providing further evidence of the universal nature of these resource acquisition ratios regardless of the scale of the

system, ambient nutrient levels, community productivity, or community composition and structure.

This study found extracellular enzyme activities responded more quickly to enrichment than biomass accumulation. Because these enzymes are responsible for the depolymerization of macromolecules, this differential response implies rates of substrate turnover increased with enrichment. Rough estimates of carbon turnover were generated by converting AFDM values to nmoles of carbon and dividing by the sum of the enzyme activities responsible for carbon acquisition (alpha and beta glucosidase). Estimates of biomass turnover were on the low end of turnover rates from other studies, ranging from $\sim 0.03 \text{ \% day}^{-1}$ for the control biofilm to 1.7 \% day^{-1} for the 10X enrichment in spite of a 20 fold increase in areal biomass over this range. The most significant turnover differences were between the control and 2X enrichments. This apparent threshold may be related to a shift in the microbial community composition from cocci and rod shaped to filamentous cells which provide effective physical retention of extracellular enzymes responsible for breaking down senescent biofilm material.

Increased biofilm biomass and turnover rates have implications for the retention of non-conservative solutes (NCS) such as nutrients and organic matter in stream ecosystems because biofilms are the primary processing site for reactive solutes in lotic ecosystems. Spiraling theory in streams describes the downstream transport of NCS in terms of uptake length (S), which is comprised of the uptake length of NCS dissolved in the water column (S_W) and in the particulate or benthic compartment (S_B) (Newbold et al. 1982). S_B and S_W are directly related to NCS supply and inversely related to NCS utilization or uptake (Newbold et al. 1982). Battin et al. (2003) found that as stream

biofilms developed, the transient storage (low velocity storage areas important for nutrient uptake) increased dramatically and there was an increasing trend in the uptake of both a highly (glucose) and moderately (arabinose) labile carbon source. Interestingly, the uptake of arabinose increased more rapidly than that of glucose. The authors attribute this finding to the increasing importance of the mass transfer of solutes into biofilms as they grow and thicken: the lower molecular weight arabinose diffuses more readily into the biofilm at later stages of biofilm development. These findings of exponential biofilm growth and function with enrichment suggest that both biofilm transient storage areas and the uptake rates of NCS are likely to increase as streams undergo eutrophication, potentially compressing the downstream spiraling of NCS.

Response of Bacterial Community Diversity and Composition to Enrichment and Implications for Productivity/Diversity/Function Relationships in Microbial Communities

An extensive body of research has examined the relationships between productivity, diversity, and function of plant and animal communities, however, little is known about these properties in microbial communities. The productivity, diversity, and functional gradients found in this study in response to an eutrophication gradient provide insights into the form of these relationships for bacteria.

Unlike biomass and activity that increased exponentially with resource availability, enrichment led to decreased bacterial 16S rRNA gene richness. Numerous studies have shown enrichment alters the general community structure of bacterial biofilm and planktonic communities (Lebaron et al. 2001, Schäfer et al. 2001, Chenier et al. 2003, Chenier et al. 2006, Haukka et al. 2006), but resolution has been limited. Declines in bacterioplankton richness (Carlson et al. 2002, Bertoni et al. 2008) or

evenness (Sipura et al. 2005) with enrichment have been observed in some planktonic studies, supporting the trend observed in this study. Enrichment also led to dramatic changes in the composition of bacterial communities. In the control treatment Bacteroidetes, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were present in approximately equal numbers, with Verrucomicrobia and Actinobacteria present at lower frequencies. These phyla are typical of those found in other stream biofilm communities (Brümmer et al. 2003, Besemer et al. 2007, Kobayashi et al. 2009, Ylla et al. 2009). In contrast, Betaproteobacteria and Gammaproteobacteria were the dominant phyla in the highest levels of enrichment and phylotypes related to Verrucomicrobia and Actinobacteria were absent.

The dominant genera from each enrichment level also changed dramatically, however, the majority of these genera are classified as chemoorganotrophs. The three dominate genera found in the control treatment, *Flavobacterium*, *Leptothrix*, and *Chitinophaga*, are aerobes. *Chitinophaga* is known to hydrolyze chitin as a primary energy source and may indicate that fungal biomass was a source of organic matter in the control treatment as chitin is present in fungal cell walls. Relatives of both the *Flavobacterium* (Kobayashi et al. 2009) and *Leptothrix* (Brümmer et al. 2003, Ylla et al. 2009) genera have been found in other stream biofilms. The dominant genera in the 8X and 10X enrichments, *Aeromonas*, *Janthinobacterium*, and *Arcicella*, have been found in other stream biofilms (Kobayashi et al. 2009), are chemoorganotrophs, and are aerobes with the exception of *Aeromonas* which is a facultative anaerobe. The dominance of this facultative anaerobe suggests oxygen may have been limiting in the well developed biofilms that resulted from enrichment. Relatives of the most common genera from the

control and enrichment treatments described above have been found in biofilms from globally distributed locations. This ubiquity suggests these genera are members of a cosmopolitan freshwater biofilm cluster.

Changes in bacterial community composition have been related to enrichment in other systems. Ylla et al. (2009) found glucose additions eliminated phylotypes related to Actinobacteria from a community of heterotrophic bacteria, a pattern observed in this enrichment study. Kobayashi et al. (2009) used bacterial community profiling techniques to synoptically sample two rivers in Japan. Significant changes in the patterns of bacterial community composition were most strongly related to anthropogenic nitrogen inputs; however, insufficient sequencing prevented a determination of which bacterial genera were responsive to enrichment. A similar study in Germany of the Elbe River and a highly polluted tributary found pollution primarily impacted the abundance rather than the presence/absence of bacterial genera (Brümmer et al. 2003).

Reviews of studies from plant and animal communities suggest the most common productivity-diversity relationships are unimodal at local scales and monotonically increasing at regional scales (Waide et al. 1999, Mittelbach et al. 2001, Evans et al. 2005). A frequently proposed mechanism to explain this pattern states that in low productivity/available energy systems, insufficient resources exist to support many species at viable population levels. As productivity increases, the number of viable populations and the diversity increase. At local scales when productivity levels are high, interspecific competition increases, driving some species to extinction (Rajaniemi 2003). In this study of bacterial biofilms an enrichment gradient produced a productivity gradient; however, phylotype richness declined at all levels of enrichment. This was

surprising as richness was expected to increase with the number of physical and chemical niches that form as biofilms mature and thicken. These findings suggest that for bacterial communities, an increase in total available energy does not increase the total number of viable bacterial populations, and resource diversity may be more important for structuring these communities than total available resources. This is consistent with results from both a laboratory culturing experiment that found bacterial diversity had a unimodal relationship to productivity in heterogeneous but not in homogeneous environments (Kassen et al. 2000), and from a high carbon soil environment in which resource heterogeneity was thought to drive diversity patterns (Zhou et al. 2002).

The effects of biodiversity on ecosystem functioning have been debated for several decades in an attempt to understand and predict how current human-induced loss of diversity will alter the ability of ecosystems to provide services such as carbon sequestration and nutrient retention and processing. While some uncertainty remains about diversity-function relationships, a positive trend has been found between diversity and ecosystem function for macro-organisms (Loreau et al. 2001, Hooper et al. 2005). Few studies have investigated diversity-function relationships in microbial communities due to logistical difficulties; however, some relevant data exist. Toxic substances have been used to experimentally decrease soil microbial diversity. This reduction had little effect on parameters driven by the entire microbial community such as respiration and decomposition rates. More specific parameters, however, such as nitrification, denitrification and methane oxidation, decreased (Griffiths et al. 2000, Muller et al. 2002, Girvan et al. 2005). A single aquatic study revealed a strong positive relationship between

bacterial diversity and community respiration rates, indicating broad scale functions may also be affected by changes in diversity in some systems (Bell et al. 2005).

In the biofilm communities in this study, decreased diversity resulting from nutrient additions did not translate into a diminished ability to process large organic molecules as measured by extracellular enzyme activities. Instead, a nonlinear increase in function was observed. This response is likely due to physical changes that occurred in the biofilms as a result of enrichment. Increased biofilm thickness protects extracellular enzymes from being washed out of the system by downstream flow, potentially encouraging microbes to secrete higher quantities of enzymes into this protected environment. Furthermore, it is possible that efficient recycling and cooperation within complex biofilms is facilitated by a simplified community which may make intercellular communication more effective.

Conclusions

Stream biofilm communities play a significant role in processing nutrient and organic matter inputs to streams. As streams are enriched with nutrients and dissolved organic carbon the community diversity and structure of these biofilm communities as well as their ability to perform ecosystem functions undergo significant changes. These responses do not follow patterns expected from the extrapolation of results from studies of planktonic microbial or metazoan communities. In planktonic communities, increased production is transferred up the food web where it increases secondary production and respiration. In biofilms, the products of nutrient enrichment (e.g. cells, enzymes, extracellular polymers) tend to accumulate, initiating a positive feedback that exponentially increases biomass and metabolism. This positive feedback is likely further

reinforced by cooperative interactions within the community. As the biofilm develops, the potential for cell signaling and cellular differentiation along internal gradients of resource availability and physicochemical conditions also increases. This positive feedback is eventually truncated by structural failures (sloughing) that export material downstream or by invertebrate grazing. Further study of enrichment effects on biofilms using a complex carbon source, as opposed to the readily labile carbon source used here, would provide additional insights into diversity-function relationships as a more diverse community may be necessary to break down various fractions of a more complex carbon pool.

Acknowledgements

I acknowledge Erin Driscoll, Sarah Hicks, and Martina Stursova for field and laboratory assistance. The Albuquerque Academy provided the research location for this experiment. Sequencing for this study was performed at the Genome Sequencing Center at Washington University School of Medicine in St. Louis Missouri. Confocal microscopy was made possible through funding from the W.M. Keck Foundation for the UNM Keck Nanofluidics Laboratory. Funding for this project was provided by: The New Mexico Water Resources Research Institute Student Research Grant, T&E Inc. Grants for Conservation Biology Research, and a University of New Mexico Research Allocations Committee Grant. Support was provided to David Van Horn through the Alvin R. and Caroline G. Grove Doctoral Scholarship. The U.S. National Science Foundation provided support to Clifford Dahm through the Experimental Program to Stimulate Competitive Research (EPSCoR) (EPS 0814449), to Robert Sinsabaugh through the NSF EaGER program, and to Clifford Dahm, and David Van Horn through the Sevilleta Long-Term Ecological Research (LTER) Project (DEB-0620482).

Literature Cited

- Allen, A. P. and J. F. Gillooly. 2009. Towards an integration of ecological stoichiometry and the metabolic theory of ecology to better understand nutrient cycling. *Ecology Letters* **12**:369-384.
- Battin, T. J., L. A. Kaplan, J. D. Newbold, and C. M. E. Hansen. 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* **426**:439-442.
- Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner, and A. K. Lilley. 2005. The contribution of species richness and composition to bacterial services. *Nature* **436**:1157-1160.
- Beloin, C. and J. M. Ghigo. 2005. Finding gene-expression patterns in bacterial biofilms. *Trends in Microbiology* **13**:16-19.
- Bertoni, R., C. Callieri, E. Balseiro, and B. Modenutti. 2008. Susceptibility of bacterioplankton to nutrient enrichment of oligotrophic and ultraoligotrophic lake waters. *Journal of Limnology* **67**:120-127.
- Besemer, K., G. Singer, R. Limberger, A. K. Chlup, G. Hochedlinger, I. Hödl, C. Baranyi, and T. J. Battin. 2007. Biophysical controls on community succession in stream biofilms. *Applied and Environmental Microbiology* **73**:4966-4974.
- Brümmer, I. H. M., A. Felske, and I. Wagner-Döbler. 2003. Diversity and seasonal variability of β -Proteobacteria in biofilms of polluted rivers: Analysis by temperature gradient gel electrophoresis and cloning. *Applied and Environmental Microbiology* **69**:4463-4473.

- Carlson, C. A., S. J. Giovannoni, D. A. Hansell, S. J. Goldberg, R. Parsons, M. P. Otero, K. Vergin, and B. R. Wheeler. 2002. Effect of nutrient amendments on bacterioplankton production, community structure, and DOC utilization in the northwestern Sargasso Sea. *Aquatic Microbial Ecology* **30**:19-36.
- Carr, G. M., A. Morin, and P. A. Chambers. 2005. Bacteria and algae in stream periphyton along a nutrient gradient. *Freshwater Biology* **50**:1337-1350.
- Chen, C. C., F. K. Shiah, H. J. Lee, K. Y. Li, P. J. Meng, S. J. Kao, Y. F. Tseng, and C. L. Chung. 2005. Phytoplankton and bacterioplankton biomass, production and turnover in a semi-enclosed embayment with spring tide induced upwelling. *Marine Ecology Progress Series* **304**:91-100.
- Chenier, M. R., D. Beaumier, N. Fortin, R. Roy, B. T. Driscoll, J. R. Lawrence, and C. W. Greer. 2006. Influence of nutrient inputs, hexadecane and temporal variations on denitrification and community composition of river biofilms. *Applied and Environmental Microbiology* **72**:575-584.
- Chenier, M. R., D. Beaumier, R. Roy, B. T. Driscoll, J. R. Lawrence, and C. W. Greer. 2003. Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. *Applied and Environmental Microbiology* **69**:5170-5177.
- Cole, J. J., S. Findlay, and M. L. Pace. 1988. Bacterial production in fresh and saltwater ecosystems: a cross-system overview. *Marine Ecology Progress Series* **43**:1-10.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappinscott. 1995. Microbial biofilms. *Annual Review of Microbiology* **49**:711-745.

- Davey, M. E. and G. A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **64**:847-867.
- DeSantis, T. Z., P. Hugenholtz, K. Keller, E. L. Brodie, N. Larsen, Y. M. Piceno, R. Phan, and G. L. Andersen. 2006a. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research* **34**:W394-W399.
- DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006b. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* **72**:5069-5072.
- Dufour, P. H. and J. P. Torreton. 1996. Bottom-up and top-down control of bacterioplankton from eutrophic to oligotrophic sites in the tropical northeastern Atlantic Ocean. *Deep Sea Research Part I: Oceanographic Research Papers* **43**:1305-1320.
- Evans, K. L., P. H. Warren, and K. J. Gaston. 2005. Species-energy relationships at the macroecological scale: A review of the mechanisms. *Biological Reviews* **80**:1-25.
- Falster, D. S., D. I. Warton, and I. J. Wright. 2006. SMATR: Standardized major axis tests and routines, ver 2.0.
- Gao, G., B. Q. Qin, R. Sommaruga, and R. Psenner. 2007. The bacterioplankton of Lake Taihu, China: Abundance, biomass, and production. *Hydrobiologia* **581**:177-188.
- Gasol, J. M., C. Pedrós-Alió, and D. Vaqué. 2002. Regulation of bacterial assemblages in oligotrophic plankton systems: Results from experimental and empirical approaches *Antonie Van Leeuwenhoek* **81**:435-452.

- Girvan, M. S., C. D. Campbell, K. Killham, J. I. Prosser, and L. A. Glover. 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology* **7**:301-313.
- Griffiths, B. S., K. Ritz, R. D. Bardgett, R. Cook, S. Christensen, F. Ekelund, S. J. Sorensen, E. Baath, J. Bloem, P. C. de Ruiter, J. Dolfing, and B. Nicolardot. 2000. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. *Oikos* **90**:279-294.
- Hall-Stoodley, L., J. W. Costerton, and P. Stoodley. 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology* **2**:95-108.
- Haukka, K., E. Kolmonen, R. Hyder, J. Hietala, K. Vakkilainen, T. Kairesalo, H. Haari, and K. Sivonen. 2006. Effect of nutrient loading on bacterioplankton community composition in lake mesocosms. *Microbial Ecology* **51**:137-146.
- Hense, B. A., C. Kuttler, J. Mueller, M. Rothballer, A. Hartmann, and J. U. Kreft. 2007. Does efficiency sensing unify diffusion and quorum sensing? *Nature Reviews Microbiology* **5**:230-239.
- Hobbie, J. E. and J. J. Cole. 1984. Response of a detrital foodweb to eutrophication. *Bulletin of Marine Science* **35**:357-363.
- Hooper, D. U., F. S. Chapin, J. J. Ewel, A. Hector, P. Inchausti, S. Lavorel, J. H. Lawton, D. M. Lodge, M. Loreau, S. Naeem, B. Schmid, H. Setälä, A. J. Symstad, J. Vandermeer, and D. A. Wardle. 2005. Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecological Monographs* **75**:3-35.

- Huber, T., G. Faulkner, and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**:2317-2319.
- Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. *Genome Biology* **3**:1-8.
- Huws, S. A., A. J. McBain, and P. Gilbert. 2005. Protozoan grazing and its impact upon population dynamics in biofilm communities. *Journal of Applied Microbiology* **98**:238-244.
- Hyun, J. H., J. K. Choi, E. J. Yang, and K. H. Kim. 1998. Biomass and productivity of bacterioplankton related to surface water divergence in the northeast equatorial Pacific Ocean. *The Journal of Microbiology* **36**:151-158.
- Hyun, J. H. and K. H. Kim. 2003. Bacterial abundance and production during the unique spring phytoplankton bloom in the central Yellow Sea. *Marine Ecology Progress Series* **252**:77-88.
- Hyun, J. H. and E. J. Yang. 2005. Meso-scale spatial variation in bacterial abundance and production associated with surface convergence and divergence in the NE equatorial Pacific. *Aquatic Microbial Ecology* **41**:1-13.
- Jansson, M., A. K. Bergström, D. Lymer, K. Vrede, and J. Karlsson. 2006. Bacterioplankton growth and nutrient use efficiencies under variable organic carbon and inorganic phosphorus ratios. *Microbial Ecology* **52**:358-364.
- Joint, I., P. Henriksen, G. A. Fonnes, D. Bourne, T. F. Thingstad, and B. Riemann. 2002. Competition for inorganic nutrients between phytoplankton and bacterioplankton in nutrient manipulated mesocosms. *Aquatic Microbial Ecology* **29**:145-159.

- Kassen, R., A. Buckling, G. Bell, and P. B. Rainey. 2000. Diversity peaks at intermediate productivity in a laboratory microcosm. *Nature* **406**:508-512.
- Kobayashi, Y., C. Kim, C. Yoshimizu, A. Kohzu, I. Tayasu, and T. Nagata. 2009. Longitudinal changes in bacterial community composition in river epilithic biofilms: Influence of nutrients and organic matter. *Aquatic Microbial Ecology* **54**:135-152.
- Lane, D. J. 1991. 16S/23S rRNA sequencing. Pages 115-175 *in* E. Stackebrandt and M. Goodfellow, editors. *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Chichester, UK.
- Lebaron, P., P. Servais, M. Troussellier, C. Courties, G. Muyzer, L. Bernard, H. Schäfer, R. Pukall, E. Stackebrandt, T. Guindulain, and J. Vives-Rego. 2001. Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: Changes in abundances, activity and composition. *FEMS Microbiology Ecology* **34**:255-266.
- Loreau, M., S. Naeem, P. Inchausti, J. Bengtsson, J. P. Grime, A. Hector, D. U. Hooper, M. A. Huston, D. Raffaelli, B. Schmid, D. Tilman, and D. A. Wardle. 2001. Ecology - Biodiversity and ecosystem functioning: Current knowledge and future challenges. *Science* **294**:804-808.
- Lozupone, C., M. Hamady, and R. Knight. 2006. UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7**:371-384.
- Lozupone, C. and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**:8228-8235.

- Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer. 2004. ARB: A software environment for sequence data. *Nucleic Acids Research* **32**:1363-1371.
- Matz, C., J. S. Webb, P. J. Schupp, S. Y. Phang, A. Penesyan, S. Egan, P. Steinberg, and S. Kjelleberg. 2008. Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *Public Library of Science One* **3**:Article No.: e2744.
- Mittelbach, G. G., C. F. Steiner, S. M. Scheiner, K. L. Gross, H. L. Reynolds, R. B. Waide, M. R. Willig, S. I. Dodson, and L. Gough. 2001. What is the observed relationship between species richness and productivity? *Ecology* **82**:2381-2396.
- Mohamed, M. N., J. R. Lawrence, and R. D. Robarts. 1998. Phosphorus limitation of heterotrophic biofilms from the Fraser River, British Columbia, and the effect of pulp mill effluent. *Microbial Ecology* **36**:121-130.
- Muller, A. K., K. Westergaard, S. Christensen, and S. J. Sorensen. 2002. The diversity and function of soil microbial communities exposed to different disturbances. *Microbial Ecology* **44**:49-58.
- Newbold, J. D., R. V. O'Neill, J. W. Elwood, and W. Vanwinkle. 1982. Nutrient spiralling in streams: Implications for nutrient limitation and invertebrate activity. *American Naturalist* **120**:628-652.

- Oviatt, C. A., A. A. Keller, P. A. Sampou, and L. L. Beatty. 1986. Patterns of productivity during eutrophication: A mesocosm experiment. *Marine Ecology-Progress Series* **28**:69-80.
- Parsek, M. R. and C. Fuqua. 2004. Biofilms 2003: Emerging themes and challenges in studies of surface-associated microbial life. *Journal of Bacteriology* **186**:4427-4440.
- Rajaniemi, T. K. 2003. Explaining productivity-diversity relationships in plants. *Oikos* **101**:449-457.
- Schäfer, H., L. Bernard, C. Courties, P. Lebaron, P. Servais, R. Pukall, E. Stackebrandt, M. Troussellier, T. Guindulain, J. Vives-Rego, and G. Muyzer. 2001. Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: Changes in the genetic diversity of bacterial populations. *FEMS Microbiology Ecology* **34**:243-253.
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537-7541.
- Shiah, F. K. and H. W. Ducklow. 1995. Regulation of bacterial abundance and production by substrate supply and bacterivory: a mesocosm study. *Microbial Ecology* **30**:239-255.

- Singer, G., K. Besemer, I. Hodl, A. Chlup, G. Hochedlinger, P. Stadler, and T. J. Battin. 2006. Microcosm design and evaluation to study stream microbial biofilms. *Limnology and Oceanography: Methods* **4**:436-447.
- Sinsabaugh, R. L., S. Findlay, P. Franchini, and D. Fisher. 1997. Enzymatic analysis of riverine bacterioplankton production. *Limnology and Oceanography* **42**:29-38.
- Sinsabaugh, R. L., B. H. Hill, and J. J. F. Shah. 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature* **462**:795-798.
- Sinsabaugh, R. L. and J. J. F. Shah. 2010. Integrating resource utilization and temperature in metabolic scaling of riverine bacterial production. *Ecology* **91**:1455-1465.
- Sipura, J., K. Haukka, H. Helminen, A. Lagus, J. Suomela, and K. Sivonen. 2005. Effect of nutrient enrichment on bacterioplankton biomass and community composition in mesocosms in the Archipelago Sea, northern Baltic. *Journal of Plankton Research* **27**:1261-1272.
- Smith, E. M. and Y. T. Prairie. 2004. Bacterial metabolism and growth efficiency in lakes: The importance of phosphorus availability. *Limnology and Oceanography* **49**:137-147.
- Su, R., R. N. Lohner, K. A. Kuehn, R. Sinsabaugh, and R. K. Neely. 2007. Microbial dynamics associated with decomposing *Typha angustifolia* litter in two contrasting Lake Erie coastal wetlands. *Aquatic Microbial Ecology* **46**:295-307.
- Tao, W. D., K. J. Hall, and S. J. B. Duff. 2007. Microbial biomass and heterotrophic production of surface flow mesocosm wetlands treating woodwaste leachate: Responses to hydraulic and organic loading and relations with mass reduction. *Ecological Engineering* **31**:132-139.

- Thelaus, J., P. Haecky, M. Forsman, and A. Andersson. 2008. Predation pressure on bacteria increases along aquatic productivity gradients. *Aquatic Microbial Ecology* **52**:45-55.
- Thomaz, S. M. and F. A. Esteves. 1997a. Bacterial dynamics in periphyton from different regions of a tropical coastal lagoon. *Archiv für Hydrobiologie* **139**:495-507.
- Thomaz, S. M. and F. D. Esteves. 1997b. Secondary productivity (H-3-Leucine and H-3-Thymidine incorporation), abundance and biomass of the epiphytic bacteria attached to detritus of *Typha domingensis* pers. in a tropical coastal lagoon. *Hydrobiologia* **357**:17-26.
- Törnblom, E. and M. Søndergaard. 1999. Seasonal dynamics of bacterial biomass and production on eelgrass *Zostera marina* leaves. *Marine Ecology Progress Series* **179**:231-240.
- Torréton, J. P., J. Pagès, and V. Talbot. 2002. Relationships between bacterioplankton and phytoplankton biomass, production and turnover rate in Tuamotu atoll lagoons. *Aquatic Microbial Ecology* **28**:267-277.
- Tsuchiya, Y., M. Ikenaga, A. Kurniawan, A. Hiraki, T. Arakawa, R. Kusakabe, and H. Morisaki. 2009. Nutrient-rich microhabitats within biofilms are synchronized with the external environment. *Microbes and Environments* **24**:43-51.
- Vargas, C. A., R. A. Martinez, L. A. Cuevas, M. A. Pavez, C. Cartes, H. E. Gonzalez, R. Escribano, and G. Daneri. 2007. The relative importance of microbial and classical food webs in a highly productive coastal upwelling area. *Limnology and Oceanography* **52**:1495-1510.

- Waide, R. B., M. R. Willig, C. F. Steiner, G. Mittelbach, L. Gough, S. I. Dodson, G. P. Juday, and R. Parmenter. 1999. The relationship between productivity and species richness. *Annual Review of Ecology and Systematics* **30**:257-300.
- Warton, D. I., I. J. Wright, D. S. Falster, and M. Westoby. 2006. Bivariate line-fitting methods for allometry. *Biological Reviews* **81**:259-291.
- Watnick, P. and R. Kolter. 2000. Biofilm, city of microbes. *Journal of Bacteriology* **182**:2675-2679.
- Webb, J. S., M. Givskov, and S. Kjelleberg. 2003. Bacterial biofilms: Prokaryotic adventures in multicellularity. *Current Opinion in Microbiology* **6**:578-585.
- Weitere, M., T. Bergfeld, S. A. Rice, C. Matz, and S. Kjelleberg. 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. *Environmental Microbiology* **7**:1593-1601.
- Wey, J. K., A. Scherwass, H. Norf, H. Arndt, and M. Weitere. 2008. Effects of protozoan grazing within river biofilms under semi-natural conditions. *Aquatic Microbial Ecology* **52**:283-296.
- Ylla, I., C. Borrego, A. M. Romani, and S. Sabater. 2009. Availability of glucose and light modulates the structure and function of a microbial biofilm. *FEMS Microbiology Ecology* **69**:27-42.
- Zhou, J. Z., B. C. Xia, D. S. Treves, L. Y. Wu, T. L. Marsh, R. V. O'Neill, A. V. Palumbo, and J. M. Tiedje. 2002. Spatial and resource factors influencing high microbial diversity in soil. *Applied and Environmental Microbiology* **68**:326-334.

**Chapter 2: THE EFFECTS OF WATERSHED CHARACTERISTICS AND
UNGULATE GRAZING ON NUTRIENT CYCLING IN MONTANE
GRASSLAND STREAMS AND RIPARIAN AREAS**

David J. Van Horn¹, Carleton S. White¹, Edward A. Martinez², Christina Hernandez²,
Josh Merrill², Robert R. Parmenter^{1,3}, and Clifford N. Dahm¹

¹Department of Biology, University of New Mexico, 167 Castetter Hall MSC03 2020, 1
University of New Mexico, Albuquerque, NM 87131

²Department of Natural Resources, New Mexico Highlands University, PO Box 9000,
Las Vegas, NM 87701

³Valles Caldera Trust, PO Box 359, Jemez Springs, NM 87025

Abstract

Catchment characteristics and disturbances control the conditions and processes found in stream ecosystems. We examined nutrient cycling linkages between riparian soils and adjacent streams and the impacts of ungulate grazing on these ecosystems and processes at six grazing exclosure sites in the Valles Caldera National Preserve, NM, USA. The exclusion of native and domestic ungulate grazers for three years significantly increased the riparian aboveground biomass of standing vegetation (273 ± 155 vs. 400 ± 178 g m⁻²) and litter (56 ± 75 vs. 107 ± 77 g m⁻²) ($p = 0.005$ and 0.013 , respectively). Soil nutrient values (0 to 15-cm depth) were minimally affected by grazing after five growing seasons, with significant increases in soil total phosphorus at three of the six sites. No connection was found between soil and stream nutrient availability or limitation. Stream geomorphology was not significantly altered by five years of grazing exclusion. The elimination of grazing suppressed instream nutrient processing with significantly longer

NH₄ uptake lengths ($p = 0.02$) and non-significant trends toward decreased NH₄ uptake rates observed in enclosure reaches. These results suggest ungulate grazing impacts terrestrial characteristics that are linked to ecosystem services provided by adjacent aquatic ecosystems. Management plans should carefully balance the positive effect of grazing on stream nutrient processing and retention reported here with the well-documented grazing related loss of other ecosystem services such as decreased fish and aquatic invertebrate habitat and effects on water quality parameters such as turbidity and water temperature.

Introduction

Terrestrial characteristics and disturbances are largely responsible for the conditions and processes found in stream ecosystems. This principle was eloquently presented by H. B. N. Hynes who wrote, ‘In every respect the valley rules the stream’ (Hynes 1975), a statement that has since guided a significant body of research and the development of theories connecting terrestrial and stream ecosystems. Streams have been described as four-dimensional: longitudinal connections link upstream to downstream segments, lateral exchanges connect terrestrial and aquatic environments, vertical flows link ground and surface water, and the fourth dimension of time encompasses seasonal and long term geomorphic fluctuations (Ward 1989, Wiens 2002). The lateral and vertical connections between streams and catchments operate at a variety of spatial and temporal scales. Climate, topography, and geology place broad-scale constraints on stream hydrology, geomorphology, sediment delivery, and water chemistry (Allan and Johnson 1997, Wiens 2002, Allan 2004). Finer scale patch characteristics, seasonal cycles, episodic events, and connectivity between patches are superimposed upon catchment-

scale attributes and are important determinants of energy and nutrient exchange between terrestrial and aquatic environments (Wiens 2002).

Stream water chemistry is tightly coupled to catchment parent geology, soil chemistry, and disturbance. This coupling is maintained through lateral inputs of surface water with dissolved and particulate constituents, and groundwater inputs comprised of water in equilibrium with catchment soils and underlying parent geology. Stream water chemistry sampling and spatially explicit geologic and land use data sets have been used in combination to determine the relative importance of catchment variables in determining stream chemistry values. Underlying catchment parent geology was an important and seasonally consistent predictor of stream water chemistry in each of these studies (Johnson et al. 1997, Cresser et al. 2000, Dow et al. 2006), however, its relative importance varied between sub-regions (Dow et al. 2006). Additionally, geology/land use interactions were responsible for explaining significant portions of water chemistry variability, indicating land use and geology covary (Johnson et al. 1997, Dow et al. 2006).

Recent work has also shown that altered catchment and riparian attributes affect in-stream processing and regulation of the bioavailable components of water chemistry. Restoration of an incised stream channel resulted in changes in stream geomorphology and hydrology, and a 50% and 2000% increase in phosphorus and nitrate uptake, respectively (Bukaveckas 2007). Paired stream reaches with intact and deforested riparian zones showed undisturbed reaches exhibited greater organic material processing and nutrient cycling and retention when these parameters were assessed on a per-unit-stream-length basis (Sweeney et al. 2004). Johnson et al. (2009) found significantly higher ammonium demand in urban streams as compared to agricultural and forested systems.

This result was partially explained by increased light availability in urban streams with reduced canopy cover. These results highlight the importance of stream ecosystems for regulating water chemistry and how these services are affected by altered catchment characteristics.

Overgrazing by native and domestic ungulates is a specific watershed disturbance that alters catchment soil characteristics (Kauffman et al. 2004, Piñeiro et al. 2010) and has negatively impacted up to 80% of the streams in the arid western United States through degraded riparian and in-stream vegetation, water quality, stream channel morphology, and hydrology (Kauffman and Krueger 1984, Belsky et al. 1999, Sarr 2002). Grazing is reported to have a range of effects on soils that vary with soil and vegetation type, grazing intensity and duration, and sampling period (Piñeiro et al. 2010). Piñeiro et al. (2010) reported in a review that ecosystems with annual precipitation between 400 and 850 mm showed similar patterns, with grazing lowering soil root content, increasing soil organic matter (SOM) C:N ratios (suggesting potential N limitation for SOM decomposition), and either increasing or not changing soil bulk density. Grazing affects streamside vegetation, with significant decreases in the total aboveground biomass of riparian vegetation observed in as little as two years of moderate grazing pressure (Clary and Kinney 2002). Long-term grazing exclusion (30 years) resulted in a near doubling of litter cover, a fourfold reduction of bare ground, a fivefold increase in shrub cover, and a 30% increase in graminoid cover (Schulz and Leininger 1990). Changes in riparian vegetation in turn affect stream geomorphology in grasslands where vegetation encroachment on the active channel and sediment trapping ultimately narrow and deepen stream channels (Magilligan and McDowell 1997, Nagle and Clifton 2003). Stream

geomorphic changes have been observed in response to grazing in as little as two years of heavy grazing impacts (Clary and Kinney 2002, Ranganath et al. 2009), however, two to four years of enclosure were insufficient to produce significant recovery results in other systems (George et al. 2002, Lucas et al. 2009).

While grazing has been shown to impact terrestrial, aquatic, and riparian areas, few studies have attempted to connect these impacts and link them to instream ecosystem processes such as nutrient processing and retention. The goals of this project were to 1) determine if riparian soil nutrient characteristics in the Valles Caldera are reflected in nutrient cycling processes in streams, 2) quantify the effects of ungulate grazing on soil characteristics, terrestrial vegetation biomass, and nutrient cycling in adjacent aquatic ecosystems, and 3) assess whether eliminating grazing pressure for relatively short periods is sufficient to impact these variables.

Materials and Methods

Site Description

The Valles Caldera National Preserve (VCNP) is comprised of ~36,000 ha located in northern New Mexico (35°50'-36°00' N, 106°24'-106°37' W) encompassing a volcanic caldera formed ~1.2 million years BP (Fig. 1).

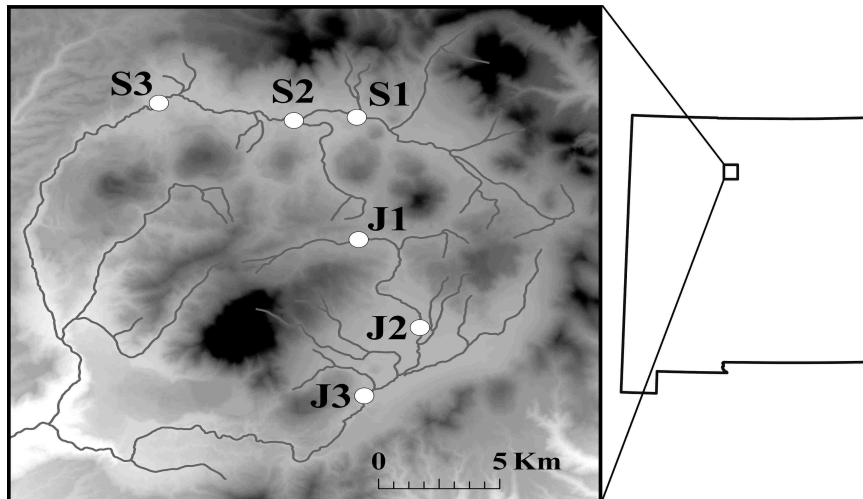


Figure 1: Map of exclusion sites in the VCNP. Exlosures are designated by white circles, streams are gray lines, and topography grades from white at low elevations to black at high elevations.

The VCNP ranges in elevation from ~2,500 m in the valley floors to 3,430 m at Redondo Peak, a resurgent volcanic dome (Heiken et al. 1990). Approximately 10,000 ha of montane grassland (Muldavin and Tonne 2003), 700 ha of wetlands (Muldavin and Tonne 2003), and 100 km of stream length are found in the valley floors of the Valles Caldera (VC). The soils of the VC have been described as either forest or grassland soils, with rocky, loamy-textured, forest soils classified as Andisols, Alfisols and Inceptisols derived from volcanic rocks on the hillslopes, and deep, organic rich grassland Mollisols found in the valley bottoms (Muldavin and Tonne 2003). Streams in the VC are low gradient, high sinuosity systems, with no woody riparian vegetation present in meadow areas. Precipitation in the VCNP during this study ranged from 500 to 700 mm yr⁻¹ (Fig. 2). Typical precipitation patterns include snow during winter months, dry spring and early summer conditions, and significant rainfall inputs during mid to late summer from North American monsoon events (Fig. 2).

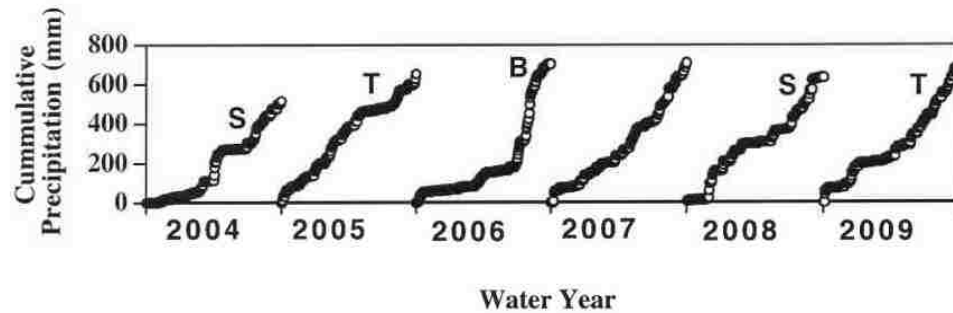


Figure 2: Cumulative precipitation (mm) for the indicated water years (Oct. 1 to Sept. 30) measured at the VCNP headquarters (near site J3). Symbols indicate collection periods for soils (S), vegetation biomass (B), and stream injection studies (T).

Domestic grazing in the VC is limited to summer months due to low winter temperatures and substantial snow accumulation. Livestock grazing in the VC began in the mid-1800's initially supporting small sheep herds (Martin 2003, Anschuetz and Merlan 2007). By the early 1900's an estimated 100,000 sheep were grazed annually in VC (Martin 2003, Anschuetz and Merlan 2007). A decline in wool prices in the 1940's led to the replacement of sheep with cattle, resulting in the annual grazing of ~12,000 cattle by the late 1950's (Martin 2003, Anschuetz and Merlan 2007). From 1960-2000 ~3,000-7,000 cattle were grazed in the VC during summer months. Since the designation of the VCNP in 2000, cattle grazing levels have been dramatically reduced with ~600 cattle grazed in 2004 and 2005 (Cibils et al. 2008). Grazing by native ungulates including elk and deer was eliminated by the early 1900's by hunting (Martin 2003). In 1947 elk were reintroduced to the Jemez Mountains by the New Mexico Department of Game and Fish. Elk populations in 2004-2008 were estimated at 2000-3000 animals (Anderson 2009).

Six sets of grazing exclosure sites were established in 2003 in the VCNP (Fig. 1). Each set of exclosures consists of an open control plot (C) and an ~160 by 160 m square area (~2.5 hectares) with a three meter high chain linked fence serving as an ungulate exclosure (E). Exclosures contain ~ 300 meters of stream length. Three sets of exclosures were constructed in each of the two watersheds draining the VCNP, the East Fork of the Jemez (EFJ) and San Antonio watersheds (Fig. 1). Sites in the EFJ watershed are designated as J1, J2, and J3 in the upstream to downstream direction. Two of the sites in the EFJ (J1 and J2) are on the Jaramillo stream, a tributary to the EFJ. All three sites in the San Antonio watershed are on the main-stem of the stream and are designated as the S1 (upstream), S2 (mid), and S3 (downstream) sites. At the S2 site a location upstream of the exclosure was used as the control area for the soil and vegetation collections while a downstream location was used to measure stream uptake parameters. The study sites span a range of stream sizes from small, second order streams (discharge of ~ 10 – 15 l sec⁻¹) at J1, to larger third order streams (discharge greater than 100 l sec⁻¹) at J3 and S3 reaches. Background concentrations of NH₄-N and PO₄ were less than 50 µg l⁻¹ at all sites (Table 1).

Table 1. Background Concentrations: 2005 and 2009.

Site-Year	Ammonium ($\mu\text{g l}^{-1}$)	Phosphate ($\mu\text{g l}^{-1}$)
J2-05	12 \pm 0.06	33 \pm 0.03
S2-05	10 \pm 0.04	17 \pm 0.03
S3-05	10 \pm 0.05	13 \pm 0.03
J1-09	9 \pm 0.84	46 \pm 7.02
J2-09	14 \pm 0.07	42 \pm 1.25
J3-09	7 \pm 0.02	20 \pm 0.03
S1-09	7 \pm 0.28	13 \pm 2.90
S2-09	7 \pm 0.10	14 \pm 2.51
S3-09	3 \pm 1.20	19 \pm 0.37

Soil Physical and Chemical Measurements

Soil collections were concentrated within the riparian zone, operationally defined as within 10 m of the stream bank. At each plot, five samples were taken at approximately 25-m intervals beginning at least 25 m from the fencing on each side of the stream, resulting in 10 sample locations per plot (180 total sample locations). At each sample location, separate samples were taken for determination of bulk density and for chemical analyses.

Bulk density was determined by making a vertical cut into the soil and extracting an intact 5.08-cm diameter by 5.08-cm depth core from the face of the cut at a depth of 10 cm. The cores were placed in soil cans, oven-dried to constant weight in the lab, and weighed for determination of bulk density (g cm^{-3}).

Samples for chemical analyses included the 0 – 15-cm depth of the mineral soil (organic horizons, if present, were removed). The reasons for taking cores to this depth are: (1) this depth corresponds to the depth of expected impact by ungulate hooves and

should be more closely correlated with potential changes in bulk density; and (2) past sampling indicated that this depth includes the A horizon yet minimizes the portion of lower soil horizons within the sample. Soil cores (5.08 by 15-cm length) were extracted, placed in sterile bags, labeled and recorded, and returned to the lab where they were sieved to pass 2 mm (>2 mm portion weighed and recorded). Field moisture of the samples collected in 2004 was very low and the sieved portion was stored at field moisture content prior to chemical analyses. The samples collected in 2008 were air-dried for storage until chemical analyses are performed. All subsequent chemical analyses were corrected to oven-dry weight to account for the moisture retained by each sample.

Extractable inorganic nitrogen (N) was determined by extraction of a measured amount of soil with 100 ml 2M KCl. The soil solution was shaken thoroughly and allowed to settle for 14 – 18 h. The clarified solution was decanted and analyzed using a Technicon AutoAnalyzer II for $\text{NH}_4\text{-N}$ using an alkaline phenol method and for $\text{NO}_3\text{-N} + \text{NO}_2\text{-N}$ using a cadmium reduction method (reported as mg N kg^{-1} soil). Total N and C were determined using a ThermoQuest CE Instruments NC2100 Elemental Analyzer (ThermoQuest Italia S.P.A., Rodano, Italy) by high temperature combustion and the resulting gases eluted on a gas chromatography column and detected by thermal conductivity. Total N and C are expressed as percent of soil sample. Modification of the method of Stelzer and Lamberti (2001) was used to determine total phosphorus (P). The weighed portion of soil was combusted at 500 °C for one hour, followed by addition of 1 M HCl and incubation at 80 °C for 30 min to dissolve the phosphorus. After dilution and settling, the clarified solution was analyzed for $\text{PO}_4\text{-P}$ using a molybdate method on a Technicon AutoAnalyzer II and was expressed as percent of soil sample. All total C, N,

and P values were converted to mmol kg^{-1} for stoichiometric comparisons and for expression of all elemental ratios (C:N, N:P, C:P, and C:N:P).

Above Ground Biomass Measurements

The above ground biomass of upland and riparian vegetation was measured once in fall, 2006. Five randomized linear stream distances were chosen at each site and grazing treatment using a 10-m buffer from all fences to eliminate edge effects. At each linear distance a riparian (within 10 m of the stream) and upland (greater than 10 m from the stream) location were randomly selected. Standing vegetation was clipped to 1 cm of the ground and litter was raked and collected separately in one quadrat (1 m^2) per location. Samples were oven dried to constant weight and weighed to determine standing aboveground biomass and litter (g m^{-2}).

Stream Solute Injections

Stream flow characteristics and uptake parameters for NH_4 and PO_4 were measured in both the ungulate and control reaches at three sites in 2005 and six sites in 2009. Solute injections were performed using the Stream Solute Workshop (1990) protocols. Prior to the start of injections, background samples were collected at six sites downstream of the injection point to determine background concentrations of Br, NH_4 and PO_4 . Immediately following background sampling, Br (conservative solute) and NH_4 and PO_4 (non-conservative solutes) were injected simultaneously for 100-140 min at a constant rate calculated to elevate background concentrations by 800-1000 ppb for Br and 50 ppb for NH_4 and PO_4 . Following the start of the injection, three samples were collected at each station during the solute plateau as determined visually by the clearing of Rhodamine-WT tracer dye added to the stream at the beginning of the injection.

Samples were filtered immediately in the field using 0.7- μm pore-sized Whatman Glass Fiber Filters ® and were stored at 4 °C until frozen (within 10 h of collection).

Uptake lengths (S_w) for NH_4 and PO_4 were estimated from the change in concentration between the background and plateau samples at each of the six sampling stations downstream of the injection point ($\Delta C(x,t) = C(x,t) - C(x,t_0)$, where t = time, x = station distance in meters, and $C(x,t)$ and $C(x,t_0)$ are the concentrations of the solute measured at the plateau and before the injection began, respectively). The ratio of non-conservative to conservative solutes ($r_c = \Delta C/\Delta \text{Br}$) was used to correct for changes in solute concentrations due to dilution of solutes rather than biological uptake. The longitudinal loss rate, k_l , of the non-conservative solutes was estimated by non-linear regression from the relationship $r_c(x) = r_0 \exp(-k_l x)$ where r_0 is the non-conservative to conservative solute ratio at $x = 0$. Longitudinal loss rates were converted to uptake lengths using $S_w = -1/k_l$ (Newbold et al. 1982). Because S_w is strongly influenced by stream scale, uptake lengths were converted to a mass transfer coefficient or uptake velocity (V_f , mm min^{-1}) and areal uptake rates (U , $\mu\text{g m}^{-2} \text{min}^{-1}$) to compare uptake between streams of different sizes. V_f and U are calculated as $V_f = k_l u d$ (where u = water velocity and d = water depth) and $U = V_f C$ (where C = background nutrient concentration).

Analysis of the conservative solute data (Br) was conducted using a 1-dimensional advection-dispersion model that includes a transient storage component (OTIS, Runkel et al. 1998) to estimate discharge (Q) and cross sectional area (A). Using Q (width x depth x velocity) and A (width x depth), average water velocity (v_w) was calculated as $v_w = Q/A$. Average water depth was calculated as $d = A/\text{width}$ using the mean reach widths from twenty evenly spaced cross-sectional geomorphology transects.

Ammonium samples were analyzed using the phenylhypochlorite method and a 10-cm flow path modified from Hansen and Koroleff (1983). Phosphate was measured using the stannous chloride method (Standard Method 4500-P D; Clesceri et al. 1998). Bromide was analyzed by ion chromatography (Dionex, Standard Method EPA 300.1).

Statistical Methods

Analysis of variance (ANOVA) was used to determine the significance of treatment, between and within sites, and between year effects on soil properties. When necessary, data were log transformed to meet criteria for ANOVA. When significant differences were determined with ANOVA, differences between the individual factors were determined with Scheffe F-test. All figures of soil properties show mean and standard error (when visible). Level of significance is $p < 0.05$ unless otherwise indicated. Student's t-tests were used to determine the significance of grazing treatment on aboveground biomass and litter (data from all six sites was pooled by treatment). A mixed model ANOVA was used to determine the effects of grazing on nutrient spiraling metrics and stream physical characteristics. This model included site as a random effect which allowed data from the same sites but different years to be used in the same model. Linear regression was used to examine relationships between nutrient uptake and stream physical characteristics.

Results

Soil Physical and Chemical Measurements

Soils of the riparian areas in the VC show a range in chemical properties (Figs. 3 and 4) with significant differences between sample sites for most of the soil properties in both sampling periods. However, the differences between sampling periods often

exceeded the differences between sites within a sampling period. Of particular importance was the increase in soil total P, C, and N at 4 of the 6 sites in the 2008 samples relative to the 2004 samples (Fig. 4a-c), along with a general decline in the C:P and N:P ratios between these samplings (C:P from 263 (± 5.0) to 241 (± 3.0) and N:P from 20.0 (± 0.5) to 18.3 (± 0.3), Fig. 4d and 4f). There were no significant differences between the enclosure and open grazing areas within all sites for all soil properties for samples collected in 2004, which was shortly after complete removal of grazing. The differences between sample periods can be attributed to the effects of grazing for only one soil property in 2008. The only effect of the enclosures on soil properties occurred for total P with the enclosures significantly lower than the grazed areas at three of the six sites in 2008 (J1, J2 and S2; Fig. 4c).

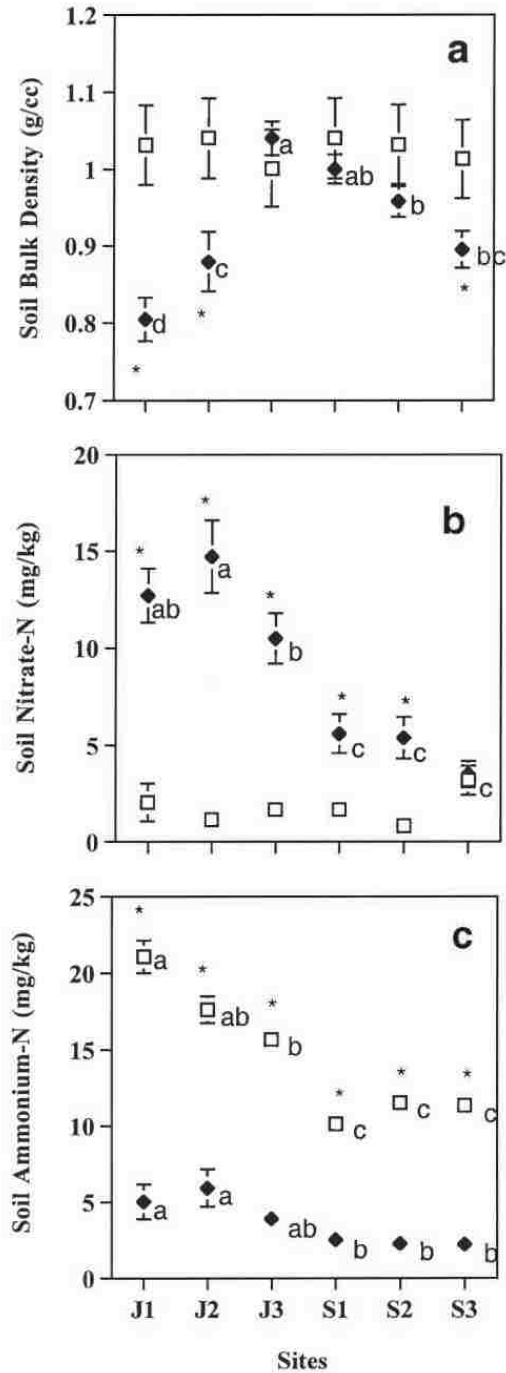


Figure 3: Soil bulk density (a), nitrate-N (b), and ammonium-N (c) in riparian grassland soils from three sites within the East Fork of the Jemez watershed (J1, J2, J3) and the San Antonio watershed (S1, S2, S3) in the VCNP. Samples were collected in 2004 before the summer growing season (open squares) and in the fall of 2008 (solid diamonds; symbols sometimes are behind squares). Within a collection, sites that are significantly different have different letters (*italic* for 2008). Within a site, significant differences between years are indicated by *.

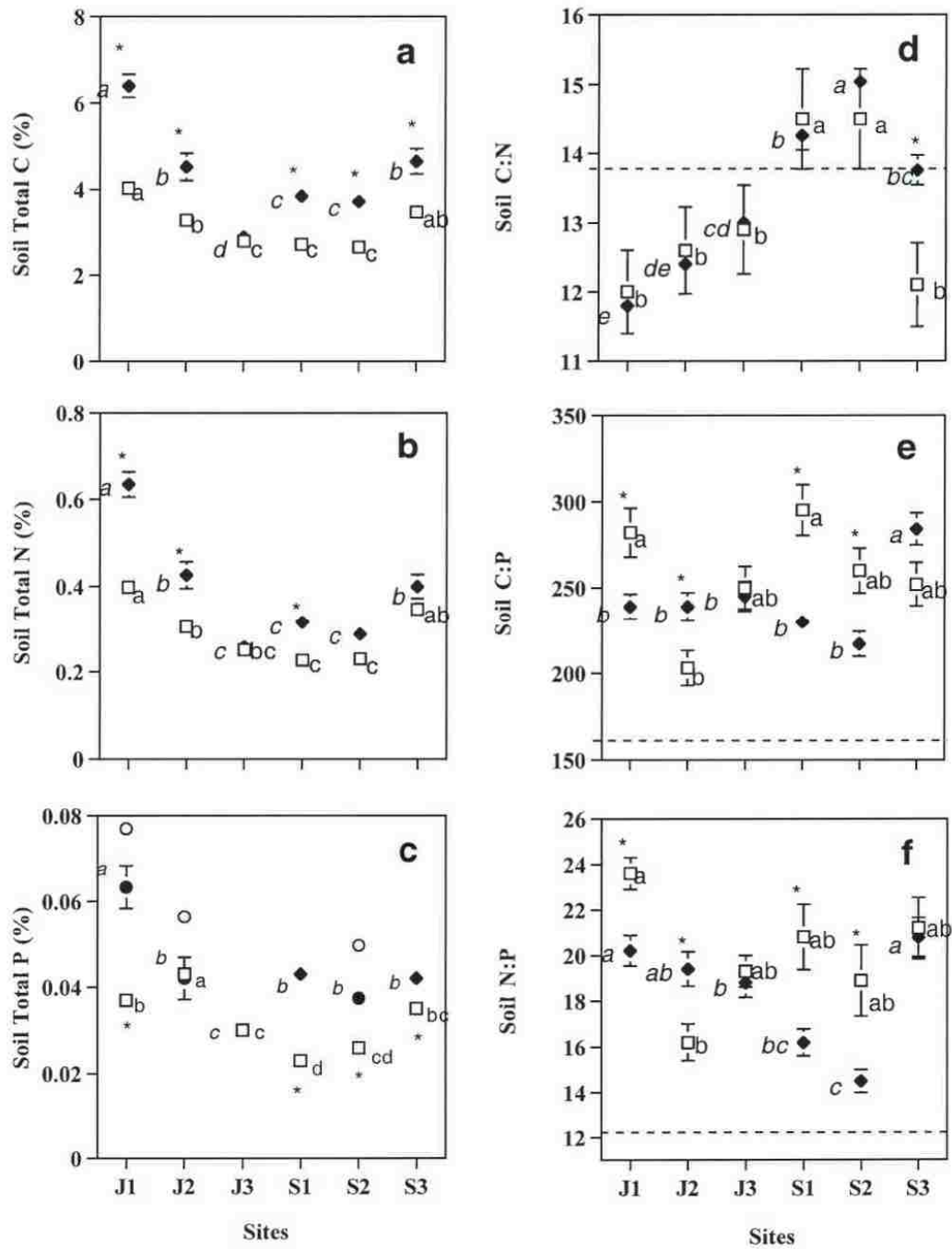


Figure 4: Soil characteristics of riparian grassland soils in the VCNP (see Fig. 3 legend for descriptions). Dashed lines (d, e, and f) indicate grassland averages (Piñeiro et al. 2010). Significant effects of closures occurred at 3 sites for total P in 2008 (c; closures - fill circles, control areas - open circles).

Above Ground Biomass

Above ground biomass of vegetation in the VCNP had a mixed response to grazing impacts by the end of three growing seasons. Upland standing vegetation and litter values from the combined sites both increased in ungrazed areas; however, these values were not significantly different from grazed sites. In contrast, standing aboveground biomass and litter biomass in the riparian zone from the combined six sites were significantly greater in enclosure areas than in the grazed treatments ($p = 0.005$ and 0.013 , respectively). Standing biomass averaged 273 (std. = 155) and 400 (178) g m^{-2} for the grazed and enclosed sites, respectively, while litter values were 56 (75) and 107 (77) g m^{-2} for the grazed and ungrazed areas, respectively.

Stream Physical Characteristics

A wide range of physical characteristics were documented in VC streams during 2005 and 2009. The most variable parameters were: width 0.71 (J1-C '09) to 5.05 m (J3-E '09), discharge 17 (J2-C '09) to 123 l sec^{-1} (S3-C '09), and width to depth ratios 3 (J1-E '09) to 44 (S3-E '09) (Table 2). Depth and velocity were more consistent between sites with a range of 0.10 (J2-E '09) to 0.27 m (J1-E '09) for depth, and 0.07 (J2-C '09) to 0.22 m sec^{-1} (S1-E '09) for velocity (Table 2). In both watersheds during both years, stream width, discharge, and width to depth ratios generally increased in the downstream direction, however, velocity and depth did not change predictably with location (Table 2). Some between treatment trends were apparent in the physical parameters measured when 2005 and 2009 data were considered together. The mean stream width was greater in the control reaches and the average reach velocity was greater in the enclosure reaches for six of the nine experiments, however, the mean control and enclosure widths (3.15 ± 1.52 m,

2.89 ± 1.50 m) and velocities (0.137 ± 0.041 m sec⁻¹, 0.159 ± 0.037 m sec⁻¹) were not significantly different ($p > 0.05$).

Table 2. VCNP Stream Physical Characteristics: 2005 and 2009.

Site	Treatment	Year	Wetted Width (m)	Depth (m)	Cross-Sectional Area (m ²)	Velocity (m sec ⁻¹)	Discharge (l sec ⁻¹)
J1	C	2009	0.71	0.22	0.15	0.12	18
J1	E	2009	0.73	0.27	0.20	0.12	24
J2	C	2005	2.25	0.16	0.36	0.10	36
J2	E	2005	1.82	0.12	0.23	0.14	32
J2	C	2009	1.69	0.15	0.26	0.07	17
J2	E	2009	1.46	0.10	0.14	0.15	21
J3	C	2009	5.05	0.15	0.77	0.12	95
J3	E	2009	3.85	0.20	0.78	0.12	96
S1	C	2009	2.93	0.16	0.47	0.19	89
S1	E	2009	2.31	0.16	0.37	0.22	82
S2	C	2005	3.04	0.18	0.56	0.17	97
S2	E	2005	2.87	0.20	0.59	0.17	101
S2	C	2009	2.97	0.20	0.61	0.18	108
S2	E	2009	2.96	0.23	0.67	0.16	108
S3	C	2005	4.89	0.20	0.96	0.12	116
S3	E	2005	4.98	0.18	0.88	0.14	118
S3	C	2009	4.81	0.16	0.75	0.16	123
S3	E	2009	5.01	0.11	0.57	0.21	120

Stream Nutrient Cycling Parameters

The uptake of N and P in VC streams varied between nutrients, sites, grazing treatments and years. Injections of NH₄ revealed strong N sequestration during each of the 18 experiments. Ammonium S_w ranged from 52 (J2-C '05) to 559 μM (S1-E '09), V_f ranged from 3.8 (S1-E '09) to 20.5 mm min⁻¹ (S3-C '05), and U ranged from 28 (S1-E '09) to 224 μg m⁻² min⁻¹ (J2-C '05) (Fig. 5). Injections of PO₄ revealed sequestration during 13 of the experiments and PO₄ release during 5 experiments. In general, when PO₄ uptake occurred, it was an order of magnitude lower than NH₄ uptake. For experiments with positive uptake values, PO₄ S_w ranged from 350 (J2-C '05) to 5800 μM (J3-C '09), V_f

ranged from 0.19 (J2-C '09) to 4.84 mm min⁻¹ (S1-E '09), and U ranged from 4 (J3-C '09) to 61 μg m⁻² min⁻¹ (S1-E '09). The extremely low uptake of PO₄ in VC streams hindered the accurate determination of PO₄ spiraling parameters in these systems as minimal longitudinal declines in PO₄ were difficult to measure accurately.

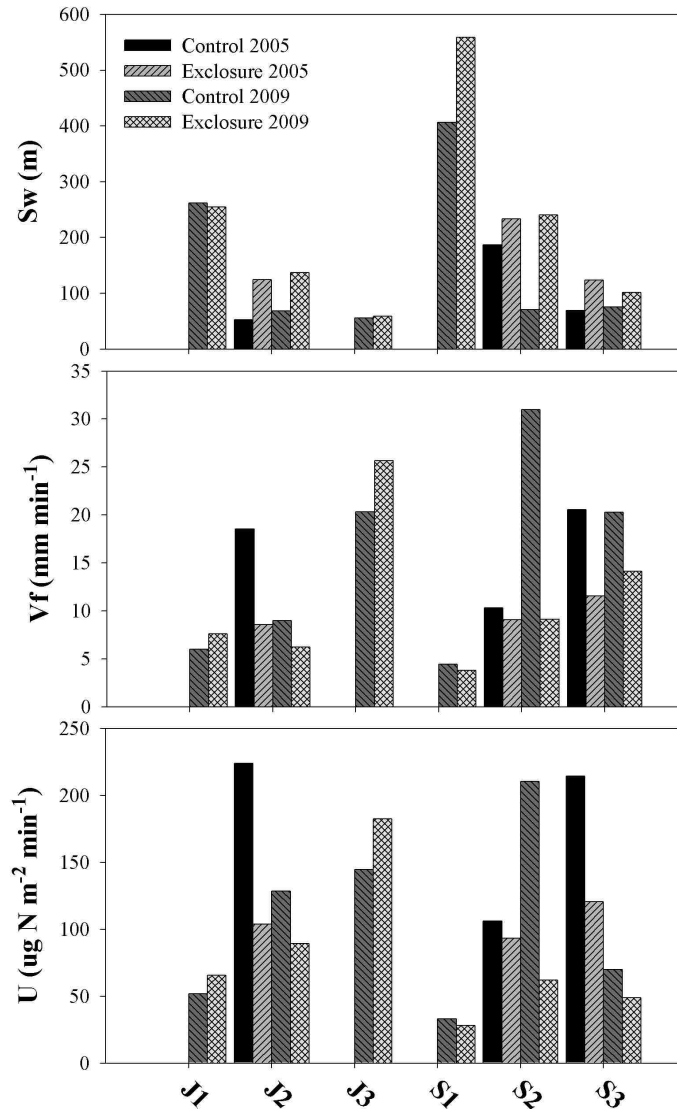


Figure 5: Uptake length (S_w), velocity (V_f) and rate (U) metrics for NH_4 in VCNP stream enclosure and control plots from 2005 and 2009.

Within individual watersheds, $\text{NH}_4 \text{S}_w$ declined at downstream sites regardless of year or grazing treatment. In 2009, J1 and S1 $\text{NH}_4 \text{S}_w$ were approximately 5 times longer than those at J3 and S3 (Fig. 5). Within watershed patterns were less clear for $\text{NH}_4 \text{V}_f$ and U. In general, V_f and U increased at downstream sites. The lowest $\text{NH}_4 \text{V}_f$ and U for each treatment and year were observed at the upstream site, with the exception of the V_f for the J2-E site in 2009 (Fig. 5).

When uptake parameters from the grazing treatments were compared, one significant difference and two non-significant trends were apparent. For $\text{NH}_4 \text{S}_w$, values were greater in the enclosure reaches for eight out of the nine comparisons (Fig. 5). In the single instance in which S_w was greater in the enclosure reach, J1 '09, the J1-E and J1-C S_w values were similar. Ammonium V_f and U values were greater in control reaches for seven out of the nine comparisons, with the exception of J1 and J3 in 2009 (Fig. 5). Mean uptake metric values for grazing treatments with both years combined show S_w values were greater in the enclosure reaches (204 ± 150 vs. 138 ± 124 m), while V_f (16 ± 9 vs. 11 ± 6 mm sec⁻¹) and U (131 ± 73 vs. 82 ± 45 $\mu\text{g m}^{-2} \text{min}^{-1}$) were greater in control reaches. S_w values were significantly longer in the grazing enclosure reaches ($p = 0.02$); however, V_f and U values from the two grazing treatments were not significantly different ($p = 0.16$, and 0.16 , respectively).

Linear regression was used to investigate relationships between stream physical characteristics and nutrient cycling metrics using the combined data from both years. Statistically significant ($p < 0.05$) relationships were found for four of these comparisons. Velocity was a significant positive predictor of $\text{NH}_4 \text{S}_w$ and was negatively related to U, while $\text{NH}_4 \text{V}_f$ was positively related to stream width and cross-sectional area (Fig. 6).

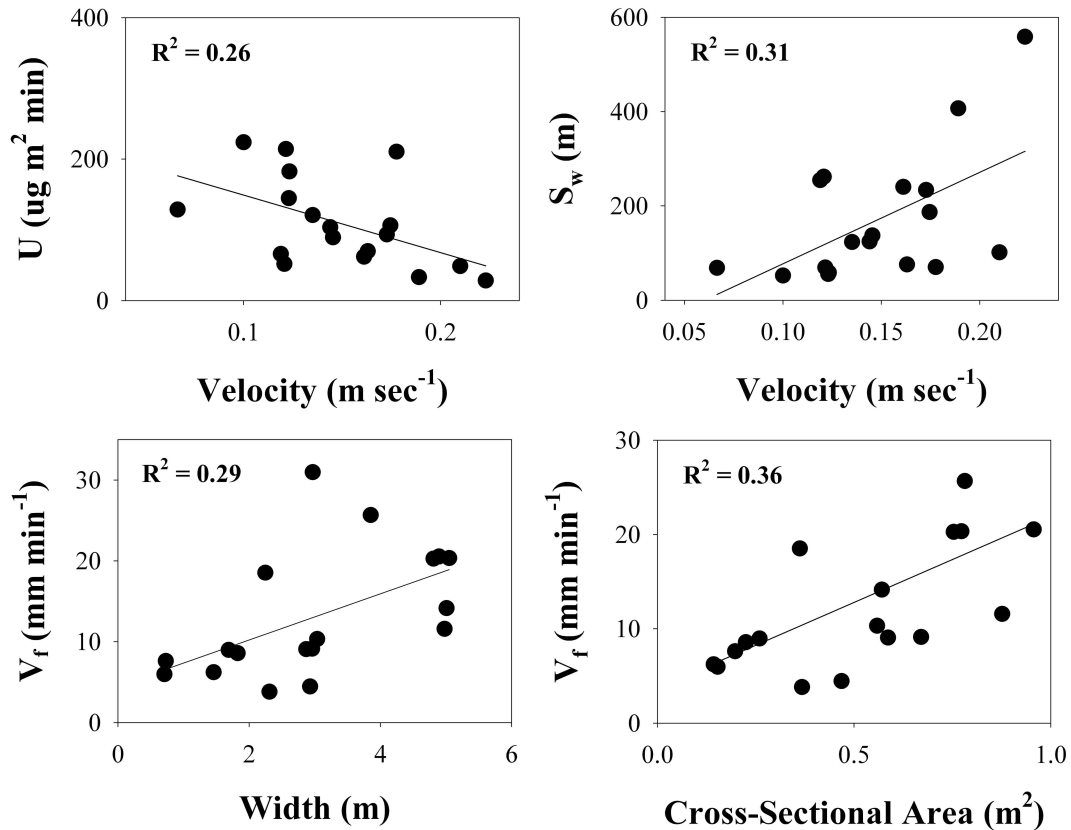


Figure 6: Regression analysis of stream geomorphology and nutrient uptake parameters with 2005 and 2009 data combined. All regression relationships shown are significant ($p < 0.05$).

Discussion

VC Soil, Vegetation, and Stream Characteristics

The VC riparian grassland soils have C:P and N:P ratios higher than the average reported for grasslands worldwide (C:P of 200 to 300 and N:P of 14 to 24 for VC compared to 166 and 12.3, respectively; Cleveland and Liptzin 2007). The range in VC C:N ratios are around the grassland average (12 to 15 for VC compared to average of 13.8; Cleveland and Liptzin 2007). VC soil nutrient ratios indicate that the riparian soils are low in P relative to the other elements and indicate that P could be limiting soil

microbial (Sinsabaugh et al. 2009) and/or plant production (Cleveland and Liptzin 2007). Aboveground biomass of $275 - 400 \text{ g m}^{-2}$ in the VC is comparable to that found in other grassland systems in New Mexico (Brockway et al. 2002) and in the midwestern (Abrams et al. 1986) and western (Frank and McNaughton 1993) United States.

The uptake of NH_4 in VC streams is within the range of uptake values reported for other 1st to 3rd order streams (Ensign and Doyle 2006). However, the inverse relationship between NH_4 uptake and stream size in the VC appears to be different from that found in other systems in which uptake decreases in 1st to 3rd order streams (Ensign and Doyle 2006). This is likely due to a combination of topographical and geomorphic characteristics of VC streams. Low order streams in the VC have higher velocities due to higher stream gradients in the upland reaches where these streams originate. Additionally, smaller streams in the VC are more susceptible to sedge and grass encroachment resulting in narrow, deep channels. In contrast, higher order VC streams are wide, relatively shallow, and exposed to significant solar radiation inputs, characteristics that likely increase benthic primary production and nitrogen uptake. These geomorphic/nutrient uptake relationships are evident in the statistically significant negative relationship between U and stream velocity, and the positive relationships between S_w and velocity, and between V_f and both stream width and cross-sectional area (Fig. 6). The PO_4 uptake observed in VC streams is lower than that from other stream ecosystems (Ensign and Doyle 2006). Additionally, PO_4 uptake lengths were on average approximately ten times longer than ammonium uptake lengths, resulting in $S_{W-N}:S_{W-P}$ ratios of ~ 0.1 . Spiraling theory predicts that ratios of less than one indicate N limitation (Cross et al. 2005), providing strong evidence that during stable summer baseflow N is the limiting nutrient

in VC streams. This finding is consistent with a study of nutrient limitation in 157 streams in Arizona that found N limitation at 72% of all of the sites studied and at 89% of the sites sampled at baseflow (Grimm and Fisher 1986). Similar results were also found at sites in western New Mexico (Coleman and Dahm 1990), suggesting N limitation may be a widespread characteristic of streams in the southwestern United States.

Soil to Stream Connections

No connection was found between 0-15 cm depth soil chemistry and instream nutrient cycling. Nitrogen was abundant in VC near stream soils as compared to other grassland systems (Cleveland and Liptzin 2007), while nitrogen was clearly limiting in adjacent stream ecosystems as seen in the efficient NH_4 uptake and the very long PO_4 uptake lengths. This apparent decoupling of terrestrial and aquatic processes is likely a result of a disconnect between soils at 15-cm depth and stream recharge during mid to late summer when the stream nutrient uptake parameters were measured. An investigation of stream flow pathways in the VC found near-surface runoff that flushes nutrients from upper organic-rich soil horizons was important during spring snow melt but was not a significant contribution during the summer or fall except during extreme monsoon events (Liu et al. 2008). Summer and fall stream inputs were dominated by deeper subsurface flow and groundwater recharge (Liu et al. 2008). The chemistry of these inputs appears to be influenced by subsoil/parent geology rather than near surface soil chemistry. This is consistent with the result of Cresser et al. (2000) who found near stream parent geology was a better predictor of instream water chemistry than near stream soil type. Phosphorus is likely abundant in VC subsoils and parent geology as these materials are relatively young geologically and volcanic ash is a significant source of phosphorus (Felitsyn and

Kirianov 2002). Additionally, when soil N becomes incorporated into aboveground production, it remains sequestered in decomposing grassland litter for an average of ten years before returning to the soil pool (Parton et al. 2007). This tight retention of N in grassland soils may also help explain the apparent disconnect between N rich soils and N limited streams.

Grazing Impacts on Soil, Vegetation, and Stream Nutrient Cycling

Grazing related variables had mixed responses to five years of grazing exclusion ranging from significant changes and trends to no response. We used the extensive body of literature describing grazing effects on grassland soils and streams to place the VC riparian and stream ecosystems along a recovery continuum and make predictions regarding future changes (Fig. 7).

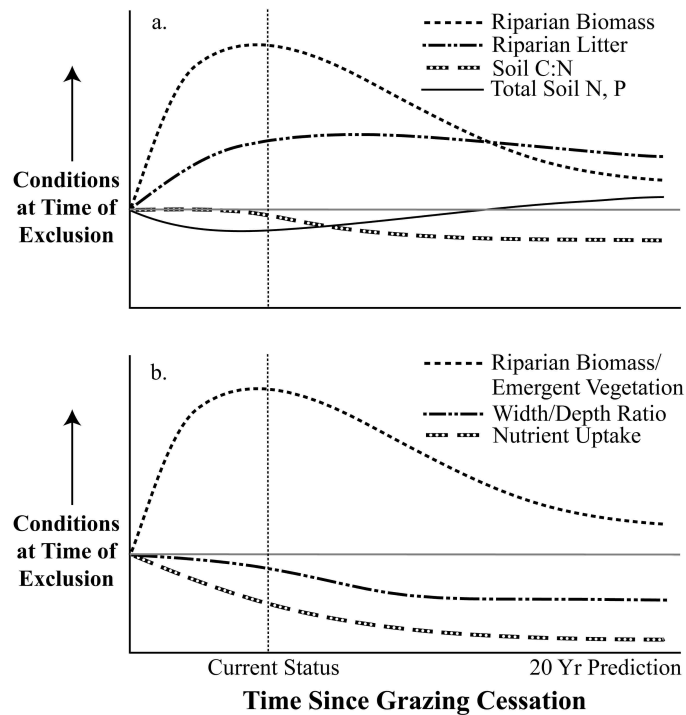


Figure 7: Conceptual diagram of the long term effects of grazing cessation on vegetation and nutrient cycling in VCNP riparian soils (a) and stream ecosystems (b).

Riparian standing biomass and litter were the variables predicted to respond most rapidly to grazing exclusion (Fig. 7a and 7b). The predicted response was observed within three growing seasons, with significant increases in both variables. Similar results have been documented in both short (Clary and Kinney 2002) and long-term (Schulz and Leininger 1990) riparian grazing enclosure experiments; however, grazing in some cases stimulates production in grasslands (McNaughton 1983, Frank and McNaughton 1993). This apparent contradiction may be due to my sampling design that did not measure biomass consumed by grazers. Also, the processes that decrease biomass in ungrazed areas, such as thatch development and N immobilization, may not have developed in the three years of exclusion prior to vegetation sampling. We predict that riparian above ground biomass will begin to decline in 8 to 10 years in response to negative-feedback mechanisms such as thatch development that shades soils, prevents sprouting, and immobilizes N (Fig. 7a and 7b).

The effects of grazing on soil chemistry are tightly coupled to changes in vegetation biomass. If the observed increase in aboveground biomass and litter represent an increase in aboveground net primary production, then demand on soil N and P resources are expected to increase, resulting in lower soil N and P (Fig. 7a). Further decline in soil N could result from immobilization of N during litter decomposition (Parton et al. 2007), which causes a lag between N uptake and return to soil N pools and a greater decline in soil N (and P). Future biomass production is expected to decline in response to lower soil N and the negative effects of shading by increased litter. As decomposition balances production, conservation of N within the riparian soils will

eventually increase soil N and lower soil C:N relative to grazed soils that experience loss of N from herbivore removal (Piñeiro et al. 2010)(Fig. 7a).

Although the reference condition at the time of exclusion is presented as constant in the conceptual model (Figure 7), temporal variation in soil characteristics was observed. At the six exclosure study sites, the greatest differences in most soil parameters occurred between the 2004 and 2008 collections. These changes are attributed to sediment deposition between collections (personal observation of fresh sediment deposition). The only significant effect of grazing on soil nutrients was an increase in total soil P at three (J1, J2, and S2) of the 6 grazed sites with P-rich sediment deposition the logical explanation for the increase. Similar increases in total C and N also occurred, indicating concurrent deposition of organic detritus and/or organic-rich sediment, however, a general decline in the C:P and N:P ratios from 2004 to 2008 indicates that P was in greater relative abundance than C or N in the newly deposited sediment than in the existing soil. Greater overbanking in areas with trampled and degraded stream banks, along with decreased overbanking in exclosures with greater riparian vegetation that acts to laterally constrain high flows are potential mechanisms to explain increased total P in soils of the three grazed areas. Expected effects of grazing on soil nutrients are likely masked by sediment contributions at some sites. The site with the least variation between years and no evidence of significant sediment deposition is J3 (total C, N, P and their ratios were nearly identical in 2004 and 2008, Figure 4), the lowest site on the East Fork of the Jemez. This site showed no significant effects of grazing on any soil characteristics.

The geomorphology of grassland stream ecosystems often responds predictably to grazing pressure, with exclusion resulting in decreased width, increased depth, and decreased width to depth ratios (Magilligan and McDowell 1997, Belsky et al. 1999, Nagle and Clifton 2003, Ranganath et al. 2009). However, the rate at which these changes occur varies between systems with some responding to grazing pressure in as little as two (Ranganath et al. 2009) to five years (Clary 1999), while others show little response in four to fourteen years (Ranganath et al. 2009). VC streams fall into the latter category with no significant changes in wetted width or width to depth ratios detected in the five years following exclosure. We predict that as bank vegetation is reestablished and emergent macrophyte biomass increases, lateral sediment accretion and channel downcutting will cause significant channel narrowing and deepening in 10 to 20 years (Fig. 7b).

This study was the first to examine the effects of ungulate grazing on nutrient cycling in adjacent stream ecosystems. Grazing exclusion significantly increased NH_4 uptake lengths and produced a trend of decreased uptake rates in VC streams. These changes occurred rapidly (increased uptake lengths were observed after one and a half growing seasons) and are likely due to increased riparian and emergent vegetation that constrain and shade the channel and by initial, but not yet statistically significant, geomorphic changes. Shading decreases nutrient uptake in streams by reducing primary production, which is an important mechanism for nutrient retention (Mosisch et al. 2001). Decreased width to depth ratios and increase stream velocities both decrease interactions between the water column and benthos where most nutrient retention occurs. This effect can be produced by either geomorphic changes in which the shape of the channel is

changed (Bukaveckas 2007) or by increased instream vegetation which can narrow and latterly constrain channels in relatively short time periods (Wilcock et al. 2002).

The relative importance of shading versus geomorphic changes can be assessed by comparing the effects of grazing on uptake lengths versus uptake rates. Uptake lengths vary in response to both physical changes, which alter stream velocity, and changes which alter biological uptake. In contrast, U and V_f are normalized for different velocities and thus are designed to measure changes in biological uptake only. Significant increases in S_w and the non-significant trends of decreasing V_f and U in ungrazed reaches suggest grazing impacts nutrient uptake through altering both physical and biological parameters in VC streams. We predict that nutrient retention in VC streams will continue to decrease as continuing changes in geomorphology significantly reduce stream width to depth ratios and shading from riparian and emergent vegetation remains elevated compared to reference conditions (Fig. 7b).

Conclusions

Five years of ungulate grazing exclusion in montane grasslands in the VCNP increased riparian aboveground standing biomass and litter, minimally impacted soil nutrient levels and ratios, and produced both statistically significant and non-significant trends of decreased nutrient retention in adjacent stream ecosystems. These results highlight the long-term nature of ecosystem recovery from grazing pressure and the need for detailed ongoing long-term monitoring to differentiate interannual variability from true long-term trends.

Ungulate grazing has been implicated in the degradation of many stream characteristics; however, this study provides evidence that grazing may enhance nutrient

retention in some streams, a process which is commonly considered a valuable ecosystem service. Managers must consider the balance between the value of instream nutrient retention with grazing related losses of other ecosystem services and products such as decreased fish and aquatic invertebrate habitat and effects on water quality parameters such as turbidity, temperature, and dissolved oxygen.

Acknowledgements

I acknowledge Michael Shank, Laura Shank, Jennifer Van Horn, Christopher White, Sandra White, Shawn West, and VCNP summer interns for field assistance. Funding for this project was provided by The Valles Caldera Trust. Support was provided to David Van Horn through the Alvin R. and Caroline G. Grove Doctoral Scholarship. The U.S. National Science Foundation provided support to Clifford Dahm through the Experimental Program to Stimulate Competitive Research (EPSCoR) (EPS 0814449) and to Clifford Dahm, Carleton White, and David Van Horn through the Sevilleta Long-Term Ecological Research (LTER) Project (DEB-0620482).

Literature Cited

- Abrams, M. D., A. K. Knapp, and L. C. Hulbert. 1986. A ten-year record of aboveground biomass in a Kansas tallgrass prairie: Effects of fire and topographic position. *American Journal of Botany* **73**:1509-1515.
- Allan, J. D. 2004. Landscapes and riverscapes: The influence of land use on stream ecosystems. *Annual Review of Ecology Evolution and Systematics* **35**:257-284.
- Allan, J. D. and L. B. Johnson. 1997. Catchment-scale analysis of aquatic ecosystems. *Freshwater Biology* **37**:107-111.

- Anderson, M. C. 2009. Livestock and elk grazing effects on stream morphology, brown trout population dynamics, movement, and growth rate, Valles Caldera National Preserve, New Mexico. Master of Science. New Mexico State University, Las Cruces, New Mexico.
- Anschuetz, K. F. and T. Merlan. 2007. More than a scenic mountain landscape: Valles Caldera National Preserve land use history. General Technical Report RMRS-GTR-196, Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO.
- Belsky, A. J., A. Matzke, and S. Uselman. 1999. Survey of livestock influences on stream and riparian ecosystems in the western United States. *Journal of Soil and Water Conservation* **54**:419-431.
- Brockway, D. G., R. G. Gatewood, and R. B. Paris. 2002. Restoring fire as an ecological process in shortgrass prairie ecosystems: Initial effects of prescribed burning during the dormant and growing seasons. *Journal of Environmental Management* **65**:135-152.
- Bukaveckas, P. A. 2007. Effects of channel restoration on water velocity, transient storage, and nutrient uptake in a channelized stream. *Environmental Science & Technology* **41**:1570–1576.
- Cibils, A. F., J. A. Miller, A. M. Encinias, K. G. Boykin, and B. F. Cooper. 2008. Monitoring heifer grazing distribution at the Valles Caldera National Preserve. *Rangelands* **30**:19-23.
- Clary, W. P. 1999. Stream channel and vegetation responses to late spring cattle grazing. *Journal of Range Management* **52**:218-227.

- Clary, W. P. and J. W. Kinney. 2002. Streambank and vegetation response to simulated cattle grazing. *Journal of Range Management* **22**:139-148.
- Clesceri, L. S., A. E. Greenberg, and A. D. Eaton, editors. 1998. *Standard Methods for the Examination of Water and Wastewater* 20th ed. 20 edition. American Public Health Association, Washington DC., USA., United Book Press, Baltimore, Maryland, USA.
- Cleveland, C. C. and D. Liptzin. 2007. C : N : P stoichiometry in soil: Is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* **85**:235-252.
- Coleman, R. L. and C. N. Dahm. 1990. Stream geomorphology: Effects on periphyton standing crop and primary production. *Journal of the North American Benthological Society* **9**:293-302.
- Cresser, M. S., R. Smart, M. F. Billett, C. Soulsby, C. Neal, A. Wade, S. Langan, and A. C. Edwards. 2000. Modelling water chemistry for a major Scottish river from catchment attributes. *Journal of Applied Ecology* **37**:171-184.
- Cross, W. F., J. P. Benstead, P. C. Frost, and S. A. Thomas. 2005. Ecological stoichiometry in freshwater benthic systems: Recent progress and perspectives. *Freshwater Biology* **50**:1895-1912.
- Dow, C. L., D. B. Arscott, and J. D. Newbold. 2006. Relating major ions and nutrients to watershed conditions across a mixed-use, water-supply watershed. *Journal of the North American Benthological Society* **25**:887-911.
- Ensign, S. H. and M. W. Doyle. 2006. Nutrient spiraling in streams and river networks. *Journal of Geophysical Research* **111**:doi:10.1029/2005JG000114.

- Felitsyn, S. B. and V. Y. Kirianov. 2002. Mobility of phosphorus during the weathering of volcanic ashes. *Lithology and Mineral Resources* **37**:275-278.
- Frank, D. A. and S. J. McNaughton. 1993. Evidence for the promotion of aboveground grassland production by native large herbivores in Yellowstone National Park. *Oecologia* **96**:157-161.
- George, M. R., R. E. Larsen, N. K. McDougald, K. W. Tate, J. D. Gerlach, and K. O. Fulgham. 2002. Influence of grazing on channel morphology of intermittent streams. *Journal of Range Management* **55**:551-557.
- Grimm, N. B. and S. G. Fisher. 1986. Nitrogen limitation potential of Arizona streams and rivers. *Arizona-Nevada Academy of Sciences* **21**:31-43.
- Hansen, H. P. and F. Koroleff. 1983. Determination of Nutrients. Pages 159-226 in K. Grasshoff, K. Kremling, and M. Ehrhardt, editors. *Methods of Seawater Analysis*. Weinheim: Verlag Chemie.
- Heiken, G., F. Goff, J. N. Gardner, W. S. Baldrige, J. B. Hulen, D. L. Nielson, and D. Vaniman. 1990. The Valles Toledo caldera complex, Jemez volcanic field, New-Mexico. *Annual Review of Earth and Planetary Sciences* **18**:27-53.
- Hynes, H. B. N. 1975. The stream and its valley. *Verhandlungen Internationale Vereinigung fuer Theoretische und Angewandte Limnologie* **19**:1-15.
- Johnson, L. B., C. Richards, G. E. Host, and J. W. Arthur. 1997. Landscape influences on water chemistry in Midwestern stream ecosystems. *Freshwater Biology* **37**:193-208.
- Johnson, L. T., J. L. Tank, and C. P. Arango. 2009. The effect of land use on dissolved organic carbon and nitrogen uptake in streams. *Freshwater Biology* **54**:2335-2350.

- Kauffman, J. B. and W. C. Krueger. 1984. Livestock impacts on riparian ecosystems and streamside management implications...a review. *Journal of Range Management* **37**:430-438.
- Kauffman, J. B., A. S. Thorpe, and E. N. J. Brookshire. 2004. Livestock exclusion and belowground ecosystem responses in riparian meadows of Eastern Oregon. *Ecological Applications* **14**:1671-1679.
- Liu, F. J., R. Parmenter, P. D. Brooks, M. H. Conklin, and R. C. Bales. 2008. Seasonal and interannual variation of streamflow pathways and biogeochemical implications in semi-arid, forested catchments in Valles Caldera, New Mexico. *Ecohydrology* **1**:239-252.
- Lucas, R. W., T. T. Baker, M. K. Wood, C. D. Allison, and D. M. VanLeeuwen. 2009. Streambank morphology and cattle grazing in two montane riparian areas in western New Mexico. *Journal of Soil and Water Conservation* **64**:183-189.
- Magilligan, F. J. and P. F. McDowell. 1997. Stream channel adjustments following elimination of cattle grazing. *Journal of the American Water Resources Association* **33**:867-878.
- Martin, C. 2003. Valle Grande: History of the Baca Location Number 1. All Seasons Publishing, Los Alamos, NM, US.
- McNaughton, S. J. 1983. Compensatory plant growth as a response to herbivory. *Oikos* **40**:329-336.
- Mosisch, T. D., S. E. Bunn, and P. M. Davies. 2001. The relative importance of shading and nutrients on algal production in subtropical streams. *Freshwater Biology* **46**:1269-1278.

- Muldavin, E. and P. Tonne. 2003. A vegetation survey and preliminary ecological assessment of Valles Caldera National Preserve, New Mexico. Natural Heritage, Albuquerque.
- Nagle, G. N. and C. F. Clifton. 2003. Channel changes over 12 years on grazed and ungrazed reaches of Wickiup Creek in Eastern Oregon. *Physical Geography* **24**:77-95.
- Newbold, J. D., R. V. O'Neill, J. W. Elwood, and W. Vanwinkle. 1982. Nutrient spiralling in streams - Implications for nutrient limitation and invertebrate activity. *American Naturalist* **120**:628-652.
- Parton, W., W. L. Silver, I. C. Burke, L. Grassens, M. E. Harmon, W. S. Currie, J. Y. King, E. C. Adair, L. A. Brandt, S. C. Hart, and B. Fasth. 2007. Global-scale similarities in nitrogen release patterns during long-term decomposition. *Science* **315**:361-364.
- Piñeiro, G., J. M. Paruelo, M. Oesterheld, and E. G. Jobbágy. 2010. Pathways of grazing effects on soil organic carbon and nitrogen. *Rangeland Ecology & Management* **63**:109-119.
- Ranganath, S. C., W. C. Hession, and T. M. Wynn. 2009. Livestock exclusion influences on riparian vegetation, channel morphology, and benthic macroinvertebrate assemblages. *Journal of Soil and Water Conservation* **64**:33-42.
- Runkel, R. L., D. M. McKnight, and E. D. Andrews. 1998. Analysis of transient storage subject to unsteady flow: Diel flow variation in an Antarctic stream. *Journal of the North American Benthological Society* **17**:143-154.

- Sarr, D. A. 2002. Riparian livestock exclosure research in the western United States: A critique and some recommendations. *Environmental Management* **30**:516-526.
- Schulz, T. T. and W. C. Leininger. 1990. Differences in riparian vegetation structure between grazed areas and exclosures. *Journal of Range Management* **43**:295-299.
- Sinsabaugh, R. L., B. H. Hill, and J. J. F. Shah. 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature* **462**:795-798.
- Stelzer, R. S. and G. A. Lamberti. 2001. Effects of N : P ratio and total nutrient concentration on stream periphyton community structure, biomass, and elemental composition. *Limnology and Oceanography* **46**:356-367.
- Stream Solute Workshop. 1990. Concepts and methods for assessing solute dynamics in stream ecosystems. *Journal of the North American Benthological Society* **9**:95-119.
- Sweeney, B. W., T. L. Bott, J. K. Jackson, L. A. Kaplan, J. D. Newbold, L. J. Standley, W. C. Hession, and R. J. Horwitz. 2004. Riparian deforestation, stream narrowing, and the loss of stream ecosystem services. *Proceedings of the National Academy of Sciences* **101**:14132-14137.
- Ward, J. V. 1989. The four-dimensional nature of lotic ecosystems. *Journal of the North American Benthological Society* **8**:2-8.
- Wiens, J. A. 2002. Riverine landscapes: taking landscape ecology into the water. *Freshwater Biology* **47**:501-515.
- Wilcock, R. J., M. R. Scarsbrook, K. J. Costley, and J. W. Nagels. 2002. Controlled release experiments to determine the effects of shade and plants on nutrient retention in a lowland stream. *Hydrobiologia* **485**:153-162.

Chapter 3: NUTRIENT RETENTION IN ARIDLAND RIVERS

David J. Van Horn¹, Lydia H. Zeglin^{1, 2}, Clifford N. Dahm¹

¹Department of Biology, University of New Mexico, 167 Castetter Hall MSC03 2020, 1
University of New Mexico, Albuquerque, NM 87131-0001

²Present Address: Department of Crop and Soil Science, Oregon State University, 3017
Agricultural and Life Science Building, Corvallis, OR 97331-7306

Abstract

Nutrient cycling and retention mechanisms in rivers draining xeric catchments deserve further study to predict how rapid population growth that these areas are experiencing worldwide will impact aridland rivers. We investigated spatial and temporal variation in the sources and sinks of nutrients in the middle Rio Grande (MRG), a 300 km reach of aridland river in the southwestern United States that drains an agro-urban catchment experiencing rapid population growth. Wastewater treatment plant inputs were the dominant source of nutrients to the MRG, increasing loads of NO₃-N, SRP, and NH₄-N by 1000-2000% relative to upstream loading. The total retention of NO₃-N and SRP inputs in the MRG corridor ranged from 6-99% and 34-99%, respectively. Retention was strongly and positively correlated with the percentage of water diverted from the MRG for agricultural irrigation ($R^2 = 0.86$ and 0.80 for NO₃-N and SRP, respectively). Irrigation diversions downstream of the urban wastewater inputs sequestered on average 480, 370 and 40 kg day⁻¹ of NO₃-N, SRP, and NH₄-N, respectively, during the irrigation season. Within the river channel, retention was 129-906 kg day⁻¹ for NO₃-N and 56-779 kg day⁻¹ for SRP, values similar to those measured in mesic systems. However, the combination of in-stream and irrigation network nutrient processing in the MRG add up

to catchment scale retention levels that are significantly higher than those found in mesic systems.

Introduction

Over the past century human activities have dramatically increased the quantity of nutrients transported in streams and rivers (Carpenter et al. 1998, Caraco and Cole 1999). Excess nutrients can lead to toxic algal blooms, increased turbidity, and dissolved oxygen depletion, all of which disrupt ecosystem functions in downstream reservoirs, estuaries and coastal marine environments (Rabalais 2002). Research conducted in a wide variety of streams and rivers has demonstrated that lotic systems are not simple conduits for transported materials but are important sites for processing, transforming, and retaining inputs (Webster and Patten 1979, Peterson et al. 2001, Mulholland et al. 2008). While river network nutrient retention and processing is relatively well understood in mesic watersheds (Alexander et al. 2000, Seitzinger et al. 2002), it is unclear whether these patterns and mechanisms are similar in arid regions.

Studies quantifying nutrient retention in mesic rivers have used several approaches. Estimated inputs to river systems have been compared to measured outputs at the mouth of the watershed for a variety of basins in Europe (Behrendt 1996, Behrendt and Bachor 1998, Behrendt and Opitz 2000). Nutrient retention in these studies ranged from 9 - 80% and 4 - 88% for nitrogen (N) and phosphorus (P), respectively, representing upper and lower limits for retention in entire river networks (Behrendt, 1996; Behrendt and Bachor, 1998). Predictive models have also been used to estimate in-stream retention of N (Howarth et al. 1996, Alexander et al. 2000, Seitzinger et al. 2002, Wollheim et al. 2006). These models relate N removal via denitrification to water body depth, residence

time, and a mass transfer coefficient that depends on denitrification rates and water column NO_3 concentrations. Model outputs predicted that N removal within individual stream order segments ranges from 1 - 35% of inputs, with decreased retention in higher order rivers. Total N removal within entire river networks ranges from 35 - 75% of inputs. A direct measurement of NO_3 retention in a 645 km reach of the Ohio River that measured loads at upstream and downstream sites supported model predictions with 10 - 30% NO_3 removal within this river reach (Bukaveckas et al. 2005). In highly impacted systems below major point source inputs such as wastewater treatment plants, however, retention has been shown to be low (Chesterikoff et al. 1992, Gibson and Meyer 2007). Low retention may be due to stress placed on river ecosystems by constant wastewater loading, an effect seen in smaller streams receiving wastewater inputs (Haggard et al. 2001, Marti et al. 2004, Haggard et al. 2005). These studies show that the total export of anthropogenically augmented nutrient loads from mesic catchments is limited by significant instream retention, however, retention efficiency often declines with enrichment (Mulholland et al. 2008).

Nutrient retention in aridland rivers is less well understood, despite the rapid development and urbanization occurring in many arid watersheds. The limited data that exist suggest that there may be significant differences between nutrient retention in arid and mesic river ecosystems. A study that used N inputs and stream and watershed retention coefficients to estimate NO_3 export from 35 major catchments worldwide found the four aridland rivers included in the study exported significantly less NO_3 than predicted by the model (Caraco and Cole 1999). This study also found that NO_3 exports were much lower than expected based on watershed population densities. A more detailed

investigation that included data from xeric catchments in the United States revealed that although N exports from xeric catchments were lower than those from mesic watersheds, human activity increased the concentration and loads by ~ 30 percent in both types of systems (Caraco and Cole 2001). Caraco and Cole (2001) proposed the most likely mechanism driving this low N export from aridland rivers was high rates of instream or floodplain N retention (Caraco and Cole 2001). A similar study of P export from major river catchments worldwide found that the Orange River, one of the two aridland rivers included in the study, had extremely low P export (Caraco 1995). A separate investigation of longitudinal N levels in the South Platte River, an aridland river in eastern Colorado, found N retention in this 105 km reach ranged from 53 to 100% of inputs (Sjodin et al. 1997). The two mechanisms responsible for N retention were denitrification (50%) and diversion of water from the river for municipal and agricultural use (35%) (Sjodin et al. 1997).

The Middle Rio Grande (MRG) of New Mexico is a major reach of a large aridland river (the Rio Grande) located in a region with rapidly increasing population and a number of management interests competing for water use. The goals of this study were to determine how the sources and sinks of nutrients in the MRG vary spatially and temporally, and assess whether nutrient retention rates and mechanisms in the MRG are similar to those found in other river ecosystems worldwide.

Materials and Methods

Site Description

The MRG is defined as the ~300 km section of the Rio Grande in New Mexico below Cochiti Reservoir and above Elephant Butte Reservoir (Fig. 1). This reach of the

Rio Grande is ~450 km below the headwaters in Colorado and ~2,250 km above the terminus at the Gulf of Mexico. The majority of New Mexico's population resides along the MRG, including the City of Albuquerque and environs. The ~64,000 km² MRG watershed ranges in elevation from 1,300 meters at Elephant Butte Reservoir to 3,255 meters in the Sandia Mountains. The geology of the watershed is a complex mixture of granitic, volcanic, and sedimentary deposits, with the MRG running through a rift valley. Watershed vegetation varies with elevation, with desert grasslands at low elevations followed by pinyon and juniper woodlands, ponderosa pine, and mixed conifer forests at higher elevations. Vegetation and land use in the historic floodplain of the MRG is dramatically different from that found in the rest of the watershed. According to the most recent available data (Bureau of Reclamation, 1991 Rio Grande Land Use Inventory) approximately 54% of the floodplain is not directly impacted by humans. The vegetation in this area is composed of desert scrubland (58%), cottonwood gallery forests (33%), and grasses (9%). Agricultural land use occurs on 32% of the MRG floodplain and is primarily comprised of alfalfa (39%), pasture grasses (27%) and fallow fields (22%). The remaining 14% of the flood plain has undergone residential, commercial, or urban development.

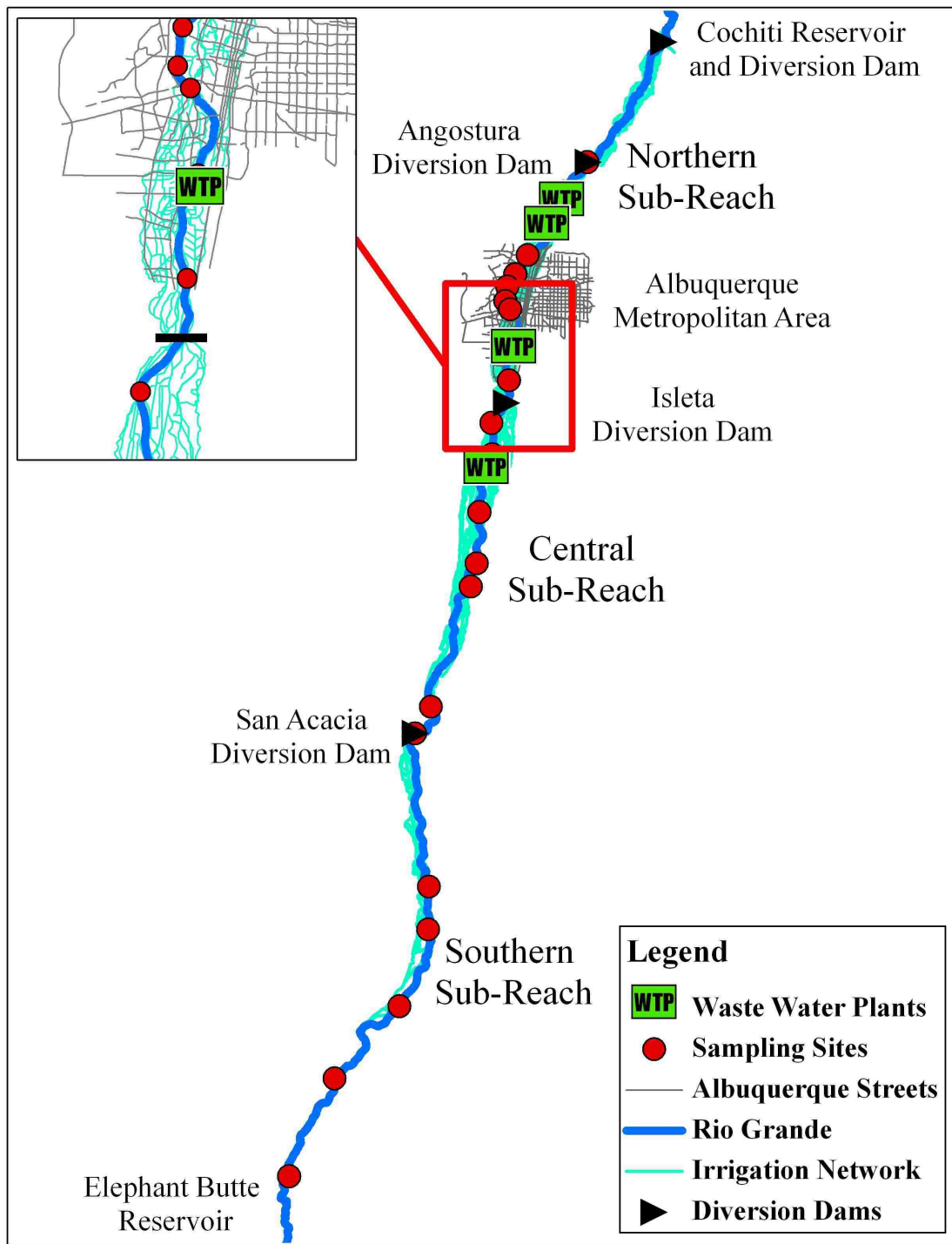


Figure 1. Map of the MRG with four waste water treatment plants that discharge directly into the MRG, mainstem sampling site locations, major streets in the Albuquerque metropolitan area, the mainstem of the river, a detail of part of the irrigation network, and four main diversion dams.

Discharge in the Rio Grande is dominated by snow melt from the mountainous headwaters in Colorado and northern New Mexico. Analysis of a 105 year record of discharge found peak flows typically occurred in May as a result of snow melt. However, some annual peak flows occurred from July to November and were associated with monsoon precipitation or remnants of tropical storms (Passell et al. 2004). A water budget showed the MRG is overall a losing reach on an annual basis with approximately 30% losses due to irrigation withdrawals, aquifer recharge, riparian zone evapotranspiration, and open water evaporation (Dahm et al. 2002, Passell et al. 2004). Inputs to the MRG include storm and irrigation drains, ephemeral tributaries that on an annual basis contribute ~8% of the total MRG discharge during winter snowmelt and storm events (Dahm et al. 2002), and municipal wastewater inputs. The largest of the four wastewater inputs, the Albuquerque Southside Water Reclamation Plant, discharges on average $2.4 \text{ m}^3 \text{ s}^{-1}$. This contribution is approximately one tenth and one third of total river discharge during high and low flow months, respectively.

During winter months when there are no withdrawals for agricultural irrigation, the hydrology of the MRG is focused within the main channel, with water released from Cochiti Reservoir flowing largely unimpeded to Elephant Butte Reservoir. During the growing season, water is diverted into the irrigation network immediately below Cochiti Reservoir and at three low-head dams located 38, 103 and 188 km below Cochiti Reservoir (Fig. 1). This water is routed through a complex series of ditches and is used to flood irrigate ~ 25,000 ha of cropland per month (personal communication, David Gensler, hydrologist for the Middle Rio Grande Conservancy District). There are ~ 2,100

km of irrigation ditches and drains in the network, approximately seven times the length of the mainstem of the MRG (Fig. 1). Irrigation water percolates through the soils into drains that are lower in elevation than the irrigation ditches. Water also moves via shallow groundwater flow from the main channel of the MRG, under riparian areas, and into the low lying drains (Tibbets and Molles 2005). These drains return water to the mainstem of the MRG many kilometers downstream of where the water was removed from the river.

Sampling Methods

Sampling for nitrate ($\text{NO}_3\text{-N}$), soluble reactive phosphate (SRP), and ammonium ($\text{NH}_4\text{-N}$) concentrations was conducted 28 times (approximately monthly) from September 2005 to January 2008. Major storm events interrupted two of the 28 samplings and partial results from these months were excluded from the data analysis. For each sampling event data were collected from 23 mainstem sites distributed along the 300 km MRG reach. During a few summer months some of the downstream mainstem sites had no discharge so no samples were taken. Mainstem sites were located ~5 km downstream of all of the wastewater and irrigation return flows to the river to allow complete mixing of these inputs with the mainstem. For 15 out of the 26 full sampling events, data were also collected from the major wastewater and irrigation return flow inputs. All samples were collected as close to the stream thalweg as flows permitted. Samples were collected in 130 ml syringes and immediately filtered in the field through ashed 0.7 μm pore size Whatman® GFF filters. Filtered samples were stored at 4°C until analysis for $\text{NO}_3\text{-N}$ and SRP within 72 hours. Ammonium samples were frozen until analysis. Ammonium samples were analyzed using the phenol hypochlorite method with a 10 cm flow path

modified from Hansen and Koroleff (1983). Nitrate and SRP samples were analyzed by ion chromatography (Dionex, Standard Method EPA 300.1, 2).

Data Analysis

Discharge data were obtained from 17 Middle Rio Grande Conservancy District (MRGCD) gages, 10 USGS gages, and 4 wastewater treatment plants. Nutrient concentration data and discharge estimates were used to calculate nutrient loads at each site. Water loss was calculated for the entire MRG by subtracting river discharge exiting the MRG reach from incoming discharge. Water loss was also calculated for three sub-reaches of the MRG (Fig. 1): the northern sub-reach from Cochiti Reservoir to the Isleta Diversion Dam (103 km), the central sub-reach from the Isleta to the San Acacia Diversion Dam (85 km), and the southern sub-reach from the San Acacia diversion dam to Elephant Butte Reservoir (118 km).

The total load of nutrients retained within the MRG corridor was calculated by subtracting the load exiting the MRG reach at the southernmost site from the load of nutrients in the river entering the MRG reach plus the wastewater inputs. During all months $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ concentration data were obtained for the largest contributor of nutrients to the MRG, the Albuquerque Southside Water Reclamation Plant, either through direct sampling of effluent or from data collected daily by the wastewater facility. SRP concentrations were not available for six of the sampling dates so mean data from a total of 20 samplings were used to estimate SRP loading during these months. Mean data values were also used to estimate nutrient loading from the three smaller wastewater plants for six to eight months, depending on the plant.

For the months when nutrient data were collected for all major inputs to the MRG, the nutrient loads from irrigation return flows were subtracted from the loads being diverted for irrigation to determine the load sequestered in or released from the agricultural system. This removal was then subtracted from the total nutrient removal within the MRG to estimate instream retention. Nutrient concentration data from irrigation return flows were also compared to concentration data from diversion water to determine the effects of agricultural irrigation on both nutrient concentrations and loads. This analysis was performed separately for the three sub-reaches of the MRG described above. Natural tributary inputs to the MRG were insignificant during all months as sampling events were scheduled during periods of stable flow and the natural tributaries to MRG only have significant discharge during snowmelt and heavy precipitation events.

Results

Nutrient Sources to the MRG

The primary sources of nutrients to the MRG were wastewater treatment plant effluent, water entering the MRG from the Upper Rio Grande (URG), and agricultural irrigation return drains. Wastewater inputs from the four treatment plants that discharge directly into the MRG were major contributors of $\text{NO}_3\text{-N}$, SRP, and $\text{NH}_4\text{-N}$, resulting in significant increases in $\text{NO}_3\text{-N}$ and SRP downstream of the largest plant during all sampling dates (Fig. 2).

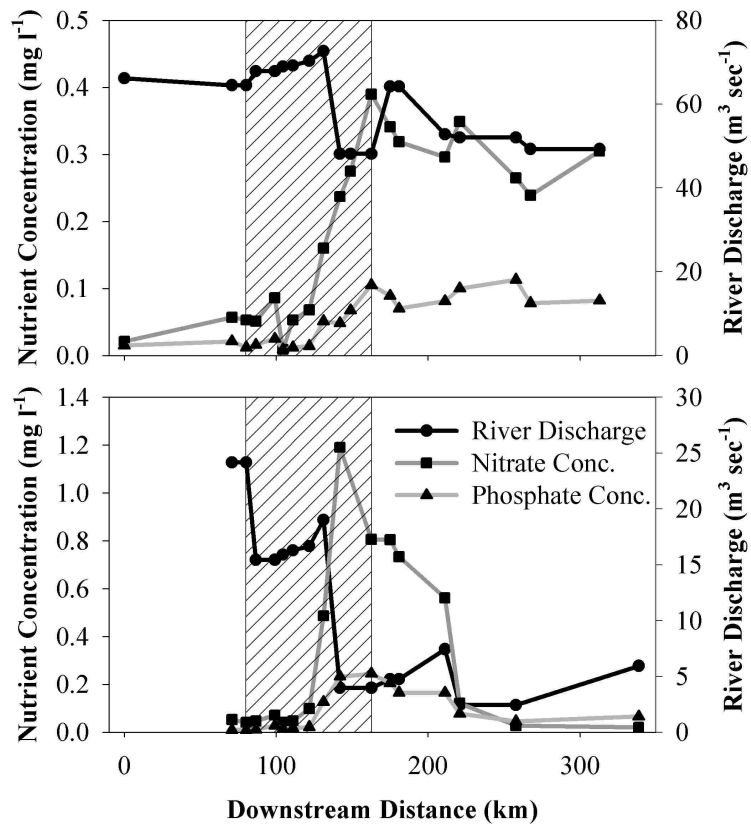


Figure 2. Longitudinal NO_3 and SRP concentrations (mg l^{-1}) during typical low (May 2007 – upper figure) and high (June 2007 – lower figure) diversion months. The shaded area indicates the portion of the MRG reach with urban wastewater inputs.

Average wastewater nutrient concentrations were greater than river water concentrations for each sampling of wastewater effluent: $\text{NO}_3\text{-N}$ concentrations ranged from 0.7 ± 1.7 to $13.9 \pm 6.3 \text{ mg l}^{-1}$, SRP concentrations ranged from 1.0 ± 1.0 to $3.7 \pm 0.9 \text{ mg l}^{-1}$ and $\text{NH}_4\text{-N}$ concentrations ranged from 0.2 ± 0.2 to $11.6 \pm 9.4 \text{ mg l}^{-1}$ (Table 1). Average wastewater loads calculated for the wastewater effluent for $\text{NO}_3\text{-N}$ ($1073 \pm 323 \text{ kg day}^{-1}$), SRP ($659 \pm 163 \text{ kg day}^{-1}$), and $\text{NH}_4\text{-N}$ ($106 \pm 64 \text{ kg day}^{-1}$) were at least an order of magnitude greater than loads carried by the river water entering the MRG (Table 1). We

observed no seasonal trend in wastewater loads or concentrations for any of the wastewater treatment plants.

Table 1. Means and standard deviations for concentrations and loads of wastewater inputs and water entering the MRG from 26 monthly sampling events from September 2005 to February 2008.

Site	Mean NO ₃ -N Conc. (mg l ⁻¹)	Mean NO ₃ -N Ld. (kg day ⁻¹)	Mean SRP Conc. (mg l ⁻¹)	Mean SRP Ld. (kg day ⁻¹)	Mean NH ₄ -N Conc. (mg l ⁻¹)	Mean NH ₄ -N Ld. (kg day ⁻¹)
Water Entering MRG	0.02 ± 0.03	62 ± 105	0.01 ± 0.01	33 ± 31	0.003 ± 0.004	8 ± 15
Bernalillo WWTP	0.7 ± 1.7	2 ± 4	1.0 ± 1.0	3 ± 3	11.6 ± 9.4	29 ± 27
Rio Rancho WWTP	10.2 ± 3	127 ± 40	3.1 ± 1.2	38 ± 14	0.2 ± 0.2	2 ± 2
Albuquerque WWTP	4.5 ± 1.5	916 ± 311	2.9 ± 0.7	601 ± 159	0.4 ± 0.3	74 ± 64
Los Lunas WWTP	13.9 ± 6.3	53 ± 25	3.7 ± 0.9	14 ± 4	1.8 ± 1.3	7 ± 6

Water entering the MRG from the upper Rio Grande (URG) had low nutrient concentrations and loads with average NO₃-N, SRP and NH₄-N concentrations of 0.02 ± 0.03 mg l⁻¹, 0.01 ± 0.01 mg l⁻¹, and 0.003 ± 0.004 mg l⁻¹, and average NO₃-N, SRP and NH₄-N loads of 62 ± 105 kg day⁻¹, 33 ± 31 kg day⁻¹, and 8 ± 15 kg day⁻¹, respectively (Table 1). No seasonal trends for incoming NO₃-N or NH₄-N concentrations or loads were observed. A weak seasonal trend for incoming SRP concentrations and loads with slight increases during spring and summer months was found (data not shown).

Agricultural return drain nutrient concentrations and loads varied widely between irrigation and non-irrigation months and among the three sub-reaches of the MRG. During irrigation months, agricultural return drains in the northern sub-reach had significantly lower NO₃-N and SRP concentrations and loads than the drains in the two southern sub-reaches. Ammonium concentrations and loads, however, were similar in all three sub-reaches (Table 2). Nutrient concentrations and loads in the agricultural return drains were similar in all three sub-reaches during non-irrigation months. Additionally, concentrations of NO₃-N and SRP in agricultural return drains in the two southern sub-

reaches were ~ 60 – 80 % lower during non-irrigation months than concentrations during irrigation months, and NO₃-N and SRP loads decreased by ~ 75 – 90 % (Table 2).

Table 2. Mean (+/- SD) nutrient concentrations and loads for drains entering three sub-reaches of the MRG during both the irrigation and non-irrigation seasons and for water diverted to the irrigation network during the irrigation season.

Sub-Reach	Diversion Water (Out), Return Drain Water (In)	Season	NO ₃ Conc. (mg l ⁻¹)	PO ₄ Conc. (mg l ⁻¹)	NH ₄ Conc. (mg l ⁻¹)	NO ₃ Ld. (kg day ⁻¹)	PO ₄ Ld. (kg day ⁻¹)	NH ₄ Ld. (kg day ⁻¹)
Northern	Out	Irrig.	0.03 ± 0.03	0.02 ± 0.01	0.003 ± 0.004	7 ± 6	17 ± 11	0.000
Central	Out	Irrig.	0.77 ± 0.28	0.50 ± 0.25	0.05 ± 0.05	768 ± 300	487 ± 232	46 ± 44
Southern	Out	Irrig.	0.70 ± 0.28	0.28 ± 0.15	0.01 ± 0.01	191 ± 90	76 ± 34	1 ± 2
Northern	In	Irrig.	0.07 ± 0.07	0.09 ± 0.07	0.01 ± 0.01	37 ± 9	45 ± 10	5 ± 4
Central	In	Irrig.	0.57 ± 0.32	0.24 ± 0.12	0.01 ± 0.01	280 ± 138	113 ± 64	3 ± 3
Southern	In	Irrig.	0.22 ± 0.09	0.11 ± 0.04	0.01 ± 0.01	126 ± 75	65 ± 34	4 ± 5
Northern	In	Non-Irrig.	0.11 ± 0.11	0.11 ± 0.08	0.04 ± 0.06	35 ± 5	32 ± 4	16 ± 6
Central	In	Non-Irrig.	0.13 ± 0.06	0.10 ± 0.07	0.01 ± 0.02	22 ± 13	16 ± 13	1 ± 2
Southern	In	Non-Irrig.	0.04 ± 0.01	0.04 ± 0.03	0.004 ± 0.004	16 ± 7	17 ± 13	2 ± 2

Nutrient Sinks and Retention in the MRG

The primary sinks of nutrients in the MRG identified in this study were instream processing and retention and sequestration within the agricultural irrigation network. The total retention of NO₃-N in the MRG ranged from 6 to 99 % of the inputs from wastewater treatment plants and upstream sources, while the total retention of SRP ranged from 34 to 99 % of the inputs (Table 3, Fig. 2).

Table 3. Total monthly water diversions below the urban portion of the MRG, retention of MRG nutrient loads, and percent removal of MRG nutrient loads. A * indicates months with unstable river discharge and ** indicates a sampling conducted immediately after the irrigation network was activated for the irrigation season.

Sampling Dates	Percent Water Diverted from Reach Below Urban Area	Total MRG Nitrate Load (kg NO ₃ -N day ⁻¹)	Total MRG SRP Load (kg SRP day ⁻¹)	Percent NO ₃ Removed	Percent SRP Removed
10-11 Jan. 07	0	948	598	30	46
3-4 Jan. 08	0	1259	714	36	54
3-4 Feb. 07	0	975	715	28	51
2-3 Feb. 08	0	940	698	9	55
2-3 Dec. 06	0	2013	420	45	37
10-11 Nov. 07	4	1053	960	43	82
9-11 Feb. 06	4	660	504	13	63
18-20 Nov. 05*	6	2210	683	68	73
16-18 Dec. 05	8	1392	677	35	47
13-14 Jan. 06	9	1859	697	58	51
30 May-1 Jun. 07	15	1780	723	32	48
31 Mar.-1 Apr. 07	22	1848	735	35	60
2-4 Mar. 07**	27	726	614	12	71
28-29 Oct. 06	40	1281	944	52	65
26-27 Jun. 06	56	1185	872	98	97
27-29 Apr. 07*	58	930	602	25	76
26-28 May 06	62	1382	659	98	99
3-4 Sep. 06*	71	954	679	29	81
29-30 Apr. 06	76	1577	690	97	96
24-26 Mar. 06	85	1285	680	98	96
28-30 Oct. 05	87	1959	677	91	91
30 Sep.-1 Oct. 06	88	905	638	95	96
20-21 Oct. 07	94	731	436	89	88
15-16 Sep. 07	100	913	731	88	93
3-4 Jul. 07	100	1669	470	99	93
17-19 Sep. 05	100	1144	1120	94	98

Retention of NH₄-N was difficult to measure accurately due to the relatively low loading of NH₄-N to the MRG (Table 1). During the 22 sampling months in which there was stable flow unaffected by storm events or irrigation system flushing (see below), total retention of NO₃-N and SRP was strongly and positively correlated with the amount of water diverted from the reach for use in the agricultural system (Fig. 3).

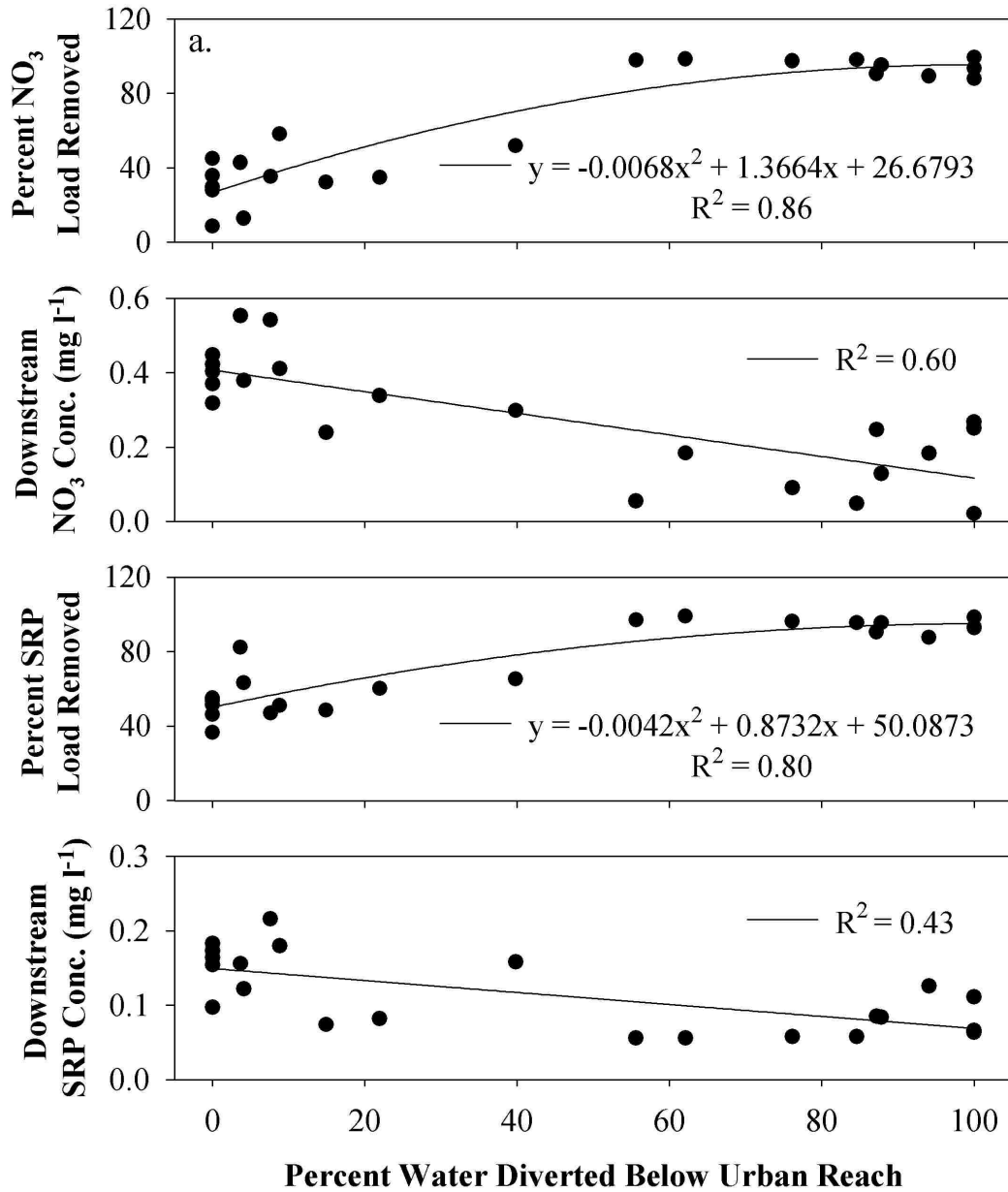


Figure 3. The percentage of water diverted from the MRG below the urban reach versus the percent of NO₃ and SRP inputs removed within the MRG (panels a. and c.), and the downstream concentration of nitrate and SRP. 100% diversion indicates complete dewatering of the main river channel (panels b. and d.).

The relationship between the percentage of the NO₃-N and SRP load retained and the percentage of the incoming water diverted for irrigation below the urban inputs explained

significant portions of the variation in $\text{NO}_3\text{-N}$ ($R^2 = 0.86$) and SRP retention ($R^2 = 0.80$) (Fig. 3). Retention of $\text{NO}_3\text{-N}$ and SRP approached 100 % during summer months when significant portions of the discharge were removed from the river (Table 3). Six to 60 % of the $\text{NO}_3\text{-N}$ and 34 to 72 % of the SRP were retained during spring and winter months when discharge was high and water withdraws were minimal (Table 3). The amount of water diverted from the reach was negatively correlated with $\text{NO}_3\text{-N}$ and SRP concentrations at the most downstream sampling site ($R^2 = 0.60$ and 0.43 , respectively) (Fig. 3) showing that the diversion of water removes nutrients from the MRG with respect to both concentration and load.

Data from sampling events where collections were made from all of the major inputs to and diversions from the river were analyzed to determine whether the concentrations and loads of nutrients in return flow water were elevated or depleted compared to diversion water during irrigation months, and to partition $\text{NO}_3\text{-N}$ and SRP removal into percentages and loads retained by the agricultural system and instream processes. Return flow concentrations and loads were analyzed separately for the three sub-reaches. The concentration of $\text{NO}_3\text{-N}$, SRP, and $\text{NH}_4\text{-N}$ in drains from the northern sub-reach were on average 0.04 , 0.07 , and 0.007 mg l^{-1} higher, respectively, than water diverted at northern diversions (Cochiti and Angostura) (Table 2). Additionally drains in the northern sub-reach contributed on average 30 kg day^{-1} of $\text{NO}_3\text{-N}$, 27 kg day^{-1} of SRP and 5 kg day^{-1} of $\text{NH}_4\text{-N}$ to the MRG during the irrigation season (Table 2). The drains from the central sub-reach had on average 0.23 , 0.26 , and 0.04 mg l^{-1} lower $\text{NO}_3\text{-N}$, SRP, and $\text{NH}_4\text{-N}$ concentrations, respectively, than water diverted at the Isleta Diversion (Table 2). Drains returning to the MRG in the central reach delivered dramatically lower loads of

nutrients than had been diverted to the irrigation network, with average sequestration of 488, 374, and 43 kg day⁻¹ of NO₃-N, SRP, and NH₄-N, respectively (Table 2). The drains from the southern sub-reach had on average 0.48 and 0.17 mg l⁻¹ lower NO₃-N and SRP concentrations, respectively, while NH₄-N concentrations in diversion and return water were similar (Table 2). Drains in the southern reach delivered back to the MRG on average 65 and 11 kg day⁻¹ less NO₃-N and SRP, respectively, than had been diverted to the irrigation network (Table 2).

Partitioning of nutrient retention revealed that the agricultural system was a slight source of NO₃-N (1 to 16 % of total load) and SRP (4 to 7 % of total load) to the river during six of the 15 months and a sink of NO₃-N (1 to 74 % of total load) and SRP (1 to 84 % of total load) during the remaining sampling dates (Fig. 4). Nutrient retention loads associated with the agricultural system ranged from a source of 124 kg NO₃-N day⁻¹ and 51 kg SRP day⁻¹ to a sink of 1091 kg NO₃-N day⁻¹ and 936 kg SRP day⁻¹. The 18,240 hectares of irrigated farmland downstream of the major urban wastewater inputs retained on average 7 and 5 kg ha⁻¹ year⁻¹, respectively, of NO₃-N and SRP. Instream processing of NO₃-N (12 to 46 % of the total load) and SRP (10 to 81 % of total load) removed large quantities of NO₃-N and SRP (129 - 906 kg NO₃-N day⁻¹ and 56 - 779 kg SRP day⁻¹) during all months. Using an average river width of 50 m, the 200 km reach of the MRG downstream of the urban wastewater inputs sequesters on average ~ 26 µg NO₃-N min⁻¹ m⁻² and ~ 18 µg SRP min⁻¹ m⁻². Estimates indicate that ~ 132 and 89 metric tons of NO₃-N and SRP, respectively, are retained annually by the agricultural irrigation network, and ~ 132 and 96 metric tons of NO₃-N and SRP are retained annually by in-stream processes.

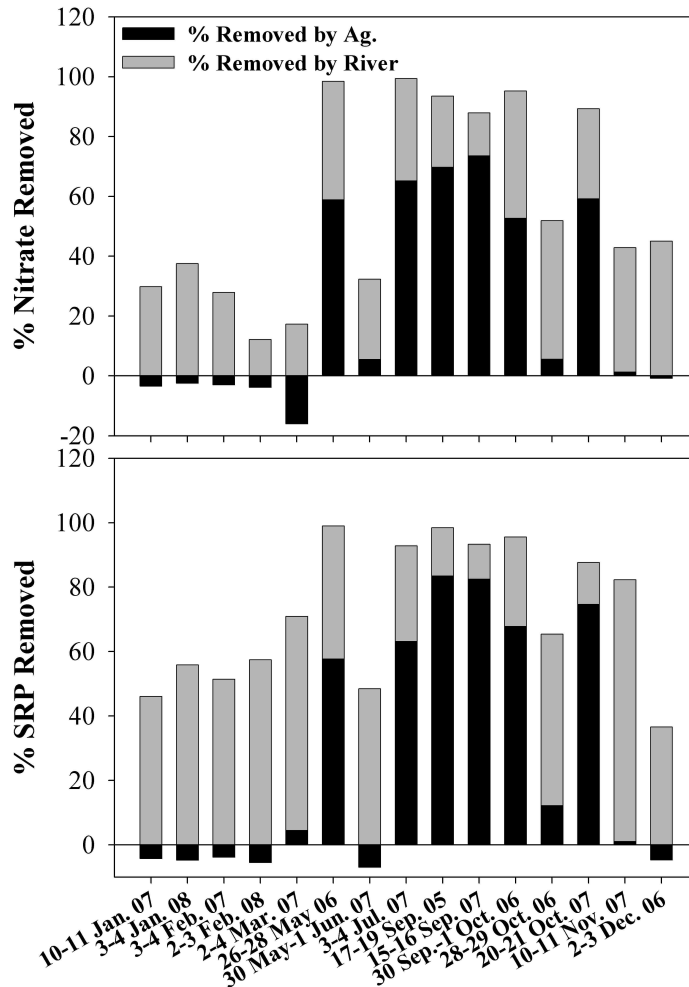


Figure 4. Percentage of MRG NO₃ and SRP loads removed by instream processing and the agricultural system. Sampling events are ordered by calendar month, not chronologically. Values above 0% show removal while values below are inputs. In-channel removal is shown in gray with agricultural system removal or addition in black.

Results from data collected in the March 2007 sampling event, which took place during the initial spring flush of the irrigation network, were dramatically different from those collected during the other months. The agricultural system contributed a substantial amount of NO₃-N to the MRG during this month, effectively offsetting the nutrients removed by instream processing (Fig. 4). During the September 2006 and April 2007 sampling events precipitation inputs resulted in increased flow for the downstream

portion of the MRG reach. Nitrate retention during these events was significantly lower than retention during other irrigation months (Table 3).

Water Loss Associated with Agricultural Irrigation

Average water loss in the MRG during months in which the irrigation system was running was 63 ± 27 % of the total volume of water entering the MRG. The northern, central, and southern sub-reaches lost on average 25 ± 17 , 23 ± 16 , and 15 ± 12 % of the total incoming water, respectively. Average losses of water entering individual sub-reaches were 25 ± 17 %, 33 ± 21 %, and 36 ± 26 % for the north, central and southern sub-reaches, respectively.

Discussion

Nutrient Sources and Sinks in the MRG

River systems receive nutrient loading from upstream sources, point source inputs such as wastewater and industrial discharge, and diffuse inputs such as atmospheric deposition, surface runoff, and nutrient enriched groundwater recharge (Carpenter et al. 1998). The primary sink for nutrients in stream ecosystems is in-channel processing by autotrophic and heterotrophic organisms (Reddy et al. 1999, Seitzinger et al. 2002). Longitudinal sampling in the MRG identified the primary sources of nutrients to this river reach as point source inputs from wastewater treatment plants and loading from upstream sources. The largest nutrient sinks were in-channel retention and sequestration in the extensive irrigation network. This analysis of nutrient sources and sinks focused exclusively on NO_3 and SRP, which are the dominant inorganic nutrient forms in streams and rivers. These nutrient species are highly responsive to anthropogenic impacts while

organic and total dissolved forms are much less responsive to human influences on watersheds (Caraco 1995, Caraco and Cole 1999, 2001, Oelsner et al. 2007).

Wastewater inputs to the MRG increased instream nutrient loads by an order of magnitude. The dominance of wastewater effluent as a nutrient source confirms research conducted by Caraco and Cole (1999), who investigated sources and retention of NO_3 in 35 rivers worldwide and found point sources are the most significant contributors of nutrients in xeric catchments. Dominance of point sources is due to high rates of within-catchment retention of potential non-point source inputs due to low rates of runoff and the limited application of fertilizers in xeric watersheds (Caraco and Cole 1999). These findings are supported by previous investigations of the nutrient dynamics in the MRG, which found wastewater inputs were the major source for N and P to the river (Passell et al. 2005, Oelsner et al. 2007) and identified agriculture as a potential sink of N (Oelsner et al. 2007).

Loading of nutrients from upstream sources contributed to nutrient concentrations and loads in the MRG. However, these inputs represented a small percentage of the total nutrient loading, highlighting the relatively unimpacted nature of the URG and MRG prior to entering the urbanized portion of the MRG watershed. The presence of Cochiti Reservoir at the upstream end of the MRG reach may serve to further retain nutrient inputs from the URG, as impoundments have been shown to decrease nutrient exports in a variety of river ecosystems (Baxter 1977, Hillbricht-Ilkowska 1999) including the MRG (Oelsner et al. 2007). An analysis of URG nutrient data from 1977 to 2002 confirms the low upstream nutrient values reported here (Passell et al. 2005).

The role agricultural irrigation return drains played in the nutrient dynamics of the MRG varied by sub-reach and season. Drains from the northern sub-reach, which are supplied with low nutrient river water diverted from the MRG upstream of the urban inputs, consistently contributed small amounts of NO_3 and SRP to the MRG. Data from return drains from the two sub-reaches downstream of the urban inputs to the MRG indicate this portion of the agricultural system acts as a substantial nutrient sink during the irrigation season. Average NO_3 and SRP irrigation return concentrations and loads in the central and southern sub-reaches were consistently lower than water diverted out of the river to the irrigation system. Decreased nutrient concentrations in return drains are in contrast to an increase in the concentration of conservative solutes such as bromide and chloride (data not shown). These results suggest that evaporative and transpirative processes concentrate solutes in the irrigation network, while other processes remove non-conservative solutes such as nutrients. Non-irrigation month concentration and load data for drains from all three sub-reaches revealed minimal loading to the MRG, indicating the shallow alluvial groundwater responsible for this flow contains low nutrient concentrations. This result is supported by an investigation of recently recharged groundwater in the United States (Nolan et al. 2002) that found low NO_3 concentrations in the shallow groundwater associated with the Albuquerque reach of the MRG.

Low nutrient loading from northern sub-reach drains and high nutrient retention in the drains of central and southern sub-reaches are of interest because agricultural drainage ditches in mesic catchments have been shown to be a conduit for nutrient transport to river ecosystems. For example, a study of NO_3 export from irrigation drains in Maryland found annual export rates of $1.7 - 24.9 \text{ kg NO}_3\text{-N ha}^{-1}$ (Schmidt et al. 2007). These

results suggest that flood irrigation, the dominant form of irrigation in the MRG, does not result in the movement of large quantities of inorganic nutrients from fields to the river ecosystem. Furthermore, these results indicate that some components of the MRG irrigation system are responsible for retaining added nutrients at a rate of 7 and 5 kg ha⁻¹ yr⁻¹ for NO₃-N and SRP, respectively, making this system unusual as compared to irrigation drainage systems from mesic watersheds.

In-channel retention of nutrients in the MRG was relatively constant throughout the year with average retention values of ~ 30 and 45% of NO₃ and SRP inputs and retention rates of 26 µg NO₃-N min⁻¹ m⁻² and 18 µg SRP min⁻¹ m⁻². These retention values are comparable to those found in other large river reaches. A study that used a longitudinal sampling design similar to the one used in this study found NO₃ retention at low discharge for a 645 km reach of the Ohio River was on average 39% of inputs (Bukaveckas et al. 2005). Two studies that used a modeling approach to estimate instream retention of NO₃ and SRP in European rivers draining into the North Sea (Behrendt 1996) and the Baltic Sea (Behrendt and Bachor 1998) found average NO₃ retention was 42% of inputs and average SRP retention was 50% of inputs. These in-channel N retention rates from the MRG are also similar to predictions of a simple model of nitrogen removal developed by Seitzinger et al. (2002) that is based on the total length of stream reaches within a watershed. When applied to the MRG, this model predicts 32 % of the nitrogen would be removed in the 200 km reach below the major wastewater input. Areal nutrient uptake rates in the MRG are also comparable to rates in other systems. A review of ~ 150 nutrient spiraling experiments in streams and rivers found the interquartile range for NO₃-N and SRP retention was 5 – 66 and 6 – 35 µg min⁻¹ m⁻²,

respectively, with median values of 15 and 14 $\mu\text{g min}^{-1} \text{m}^{-2}$, respectively (Ensign and Doyle 2006). In general, these findings show instream processing rates in the MRG that are comparable to those found in rivers draining more mesic watersheds.

The range of combined in-channel and agricultural retention of NO_3 and SRP inputs to the MRG was 9-99% and 37-99%, respectively. The considerable monthly variation in retention spans a range similar to that found for a variety of mesic catchments in Europe, where in-stream retention ranged from 9 - 80%, and 4 - 88% of inputs for nitrogen and phosphorus, respectively. These values represented total retention in the entire river network (Behrendt, 1996; Behrendt and Bachor, 1998). The MRG mean total retention values of $62 \pm 32\%$ and $73 \pm 22\%$ for $\text{NO}_3\text{-N}$ and SRP, respectively, were higher than those found for these European rivers that retained on average 42 and 50% of $\text{NO}_3\text{-N}$ and SRP inputs. The greater total mean retention of nutrients in the MRG is driven by higher retention rates during summer months when total retention exceeds 95% of inputs.

This finding of significant total retention of nutrients in the MRG is generally in agreement with a study that investigated N dynamics in the MRG during five summer samplings (Oelsner et al. 2007). At low volumes of river discharge, NO_3 concentrations below major wastewater inputs declined substantially, presumably due to both dilution from low nutrient irrigation return flows and instream processing (Oelsner et al. 2007). However, Oelsner et al. (2007) found that NO_3 concentrations elevated by wastewater inputs remained high at sites up to 200 km downstream during sampling events conducted at high river discharge. These results are similar to results from my September 2006 and April 2007 sampling events during which precipitation inputs resulted in

increased flow for the downstream portion of the MRG reach. Total NO₃ retention was dramatically lower during these events, indicating that storm flow may significantly increase NO₃ transport and decrease retention in the MRG. Additional research focused on storm flow events is necessary to fully describe nutrient retention in the MRG.

Potential Mechanisms for the Agricultural Nutrient Sink in the MRG

The strong evidence for the role of the MRG agricultural system in retaining nutrients was surprising since agricultural inputs have been identified as the single largest contributor to the eutrophication of aquatic ecosystems both in the United States and globally (Carpenter et al. 1998, Bennett et al. 2001, Tilman et al. 2001, Foley et al. 2005). Factors that likely contribute to nutrient retention in the MRG agricultural system include, 1) the limited fertilization of dominant crop types, 2) retention during flood irrigation, and 3) the distribution of large portions of MRG discharge into the extensive irrigation network.

Primary crops grown in the MRG floodplain require minimal fertilization. Alfalfa, pasture, and fallow fields represent ~39, 27, and 22% of the agricultural land use in the MRG, respectively. Fertilizer application schedules for the MRG indicate minimal N application for alfalfa seedlings (22 kg hectare⁻¹) and no N additions for established stands (Glover and Baker 1990), as alfalfa plants establish symbiotic relationships with the nitrogen fixing bacteria *Rhizobium*. Alfalfa production does require significant phosphorus fertilization with a suggested application rate of 135 kg P₂O₅ hectare⁻¹ in the MRG (Glover and Baker 1990), which is in excess of requirements for corn production (Schlegel and Havlin 1995). Minimal data exist for fertilization rates of pasture land and

fallow fields; however, these agricultural land uses generally are not associated with fertilizer application.

Flood irrigation is the dominant method of irrigation used in the MRG. Water not lost to evapotranspiration following flood irrigation percolates through the soil column without aid from tile drains or subsurface drainage pipes, seeps into low lying drainage ditches, and eventually flows back to the river. As irrigation water follows this pathway there are numerous potential sinks for transported wastewater-derived nutrients as well as fertilizers applied to the crops. Sorption to soil particles is important for removing nutrients such as SRP from agricultural leachate as irrigation water percolates through the soil column (Sinaj et al. 2002). This mechanism is responsible for low phosphorus exports from agricultural areas drained via subsurface drainage networks (Skaggs et al. 1994, Herzon and Helenius 2008). This retention pathway could be responsible for the low irrigation return drain SRP values reported in this study, in spite of the intensive phosphorus fertilization used in MRG alfalfa cultivation. Nitrate also may be removed during this process as flood irrigation and subsequent percolation of irrigation water likely create conditions similar to those in natural floodplains, which have been demonstrated to rapidly remove NO_3 from flood water via denitrification (Forshay and Stanley 2005).

The extensive network of irrigation ditches and drains also may act as a sink for nutrients in the MRG. Irrigation ditches contain high levels of organic material, leading to strong redox gradients and intense biogeochemical cycling in ditch sediments (Needelman et al. 2007). Water percolating from irrigated fields into conveyance channels passes through these biologically and chemically active sediments as the water

seeps into return ditches. Nutrients that enter ditches also may be removed during downstream transport, as high rates of nutrient uptake have been documented in a variety of ditch systems (Kroger et al. 2007, Hall et al. 2009, Olli et al. 2009) through processes including sorption of SRP to sediments (Sharpley et al. 2007) and microbial uptake of SRP and NO_3 (Sharpley et al. 2007, Strock et al. 2007). Additionally, the low elevation of the drainage ditches promotes the flow of water from the mainstem of the MRG, through the shallow alluvial aquifer underlying the riparian zone, and into the ditch system. As water follows this flow path nutrients are removed by riparian vegetation (Tibbets and Molles 2005) and microbial uptake.

A final factor that likely contributes to the retention of nutrients in the MRG agricultural irrigation system is the increased residence times for water within the irrigation system as compared to water that remains in the main channel of the river. The irrigation network effectively transforms a large river into a network of small streams that are spread over the floodplain. This process of distribution, collection and return increases the travel time of water moving through the MRG, a factor which is strongly and positively correlated with nutrient retention in river ecosystems (Howarth et al. 1996, Seitzinger et al. 2002). Increased travel time facilitates a significant amount of interaction between transported nutrients and soil particles, crops, and the benthic zones of numerous small ditches, all of which lead to nutrient uptake.

These cumulative processes lead to substantive amounts of nutrient uptake from nutrient-enriched irrigation waters. The distributary hydrology typified by the highly modified MRG makes catchment scale modeling of nutrient export extremely challenging. Measurements of nitrate retention in irrigation ditches could not be scaled up

to predict instream NO_3 concentrations using a model based on the accumulation of water and solutes through a topographically driven hydrologic network that had been developed for a North American cross biome comparison (Helton et al. *In Review*).

Comparison of Nutrient Retention in the MRG to Other River Ecosystems

High rates of NO_3 and SRP retention in the MRG (up to 99% of inputs during months where significant portions of flow are diverted for irrigation) are much greater than those observed in mesic watersheds (Behrendt 1996, Behrendt and Bachor 1998, Caraco and Cole 1999, 2001, Bukaveckas et al. 2005). Rates are similar, however, to NO_3 retention rates observed in other aridland rivers such as the Nile, Orange, Murray-Darling and Zambezi rivers (Caraco and Cole 1999, 2001) (Fig. 5).

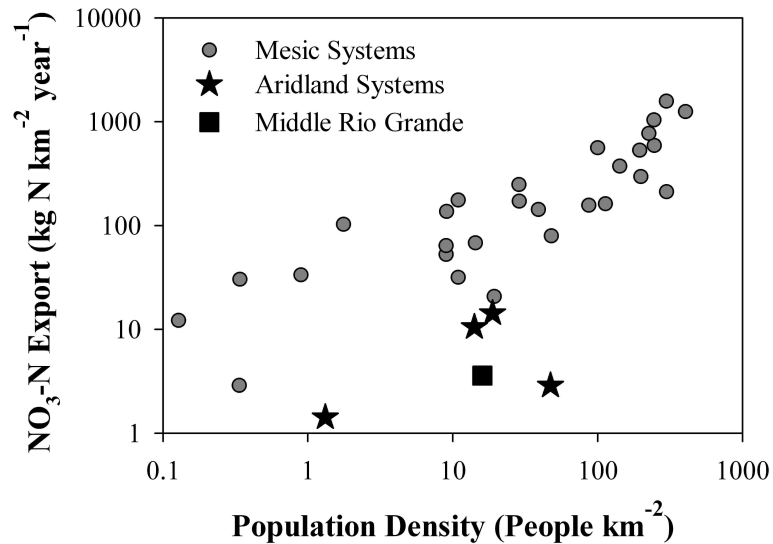


Figure 5. Export of nitrate-N ($\text{kg N km}^{-2} \text{ year}^{-1}$) from a study of 33 rivers from around the world versus watershed population density (people km^{-2}) (Modified with permission from Caraco and Cole, 2001).

Two mechanisms proposed to explain high retention rates in these other aridland systems were high within watershed retention due to low precipitation runoff and high instream

retention (Caraco and Cole 1999, 2001). We used the available literature to evaluate whether the water removal and potential nutrient sinks discussed as potentially important for nutrient removal in the MRG might also be responsible for nutrient retention in the Murray-Darling, Nile and Orange river systems. Germane literature was not found for the Zambezi River.

Extensive agricultural development has occurred adjacent to each of these three aridland rivers. The Murray-Darling Basin (MDB) is the largest and most productive agricultural region in Australia (Banens and Davis 1998) producing over 40% of the nation's gross value of agricultural production (Smith and Maheshwari 2002). Similarly, 90% of the Nile Delta is under cultivation and is one of the most agriculturally productive areas in Egypt (Sultan et al. 1999). The arid portion of the lower Orange River flows through the Kalahari and Namib deserts in South Africa and supports ~ 71,000 hectares of irrigated agricultural production (Department of Water Affairs and Forestry 2004). Additionally, agricultural cultivation along each of these rivers uses a substantial portion of river flow. Agriculture in the MDB uses ~ 70% of all of the water used in Australia (Smith and Maheshwari 2002), cultivation in the Nile Delta uses ~ 80% of all of the water used in Egypt (Wahaab and Badawy 2004), and crops along the Orange utilize ~65% of the total water removed from the Orange river (Earle et al. 2005, Lange et al. 2007). The irrigated portion of each of these systems contains an extensive network of ditches and drains that supplies water to fields and drains excess water back into the river, preventing soils from salinizing. The MDB contains over 6,000 km of irrigation drains, which have been shown to be potential sinks for nutrients (Bowmer et al. 1994). After the closure of the Aswan High Dam, over 13,000 km of irrigation drains were constructed in

the Nile delta (Nixon 2003). An extensive network of irrigation ditches has been constructed along the Orange River to connect the numerous impoundments to irrigated farmland (Gillitt et al. 2005). Although a range of irrigation practices is used in each of the three systems, some form of flood irrigation is common in each system (Clemmens et al. 1999, Bethune 2004, Gillitt et al. 2005, Wood and Finger 2006).

Two case studies from western United States indicate similar retention mechanisms may be important in river systems geographically related to the MRG. A mass balance analysis of wastewater N in the Central Arizona – Phoenix area quantified the intensive reuse of wastewater for agricultural and municipal irrigation, aquifer recharge, and nuclear power generation, resulting in a 78% reduction in wastewater N inputs to the Gila River (Lauver and Baker 2000). An investigation of longitudinal N levels in the South Platte River in eastern Colorado found N retention ranged from 53 to 100% of inputs due to denitrification (50%) and diversion of water from the river for municipal and agricultural use (35%) (Sjodin et al. 1997). The combination of instream and anthropogenic nutrient sinks results in higher nutrient retention in aridland ecosystems when compared to more mesic environments (Fig. 5) via mechanisms illustrated in the conceptual model presented in Figure 6.

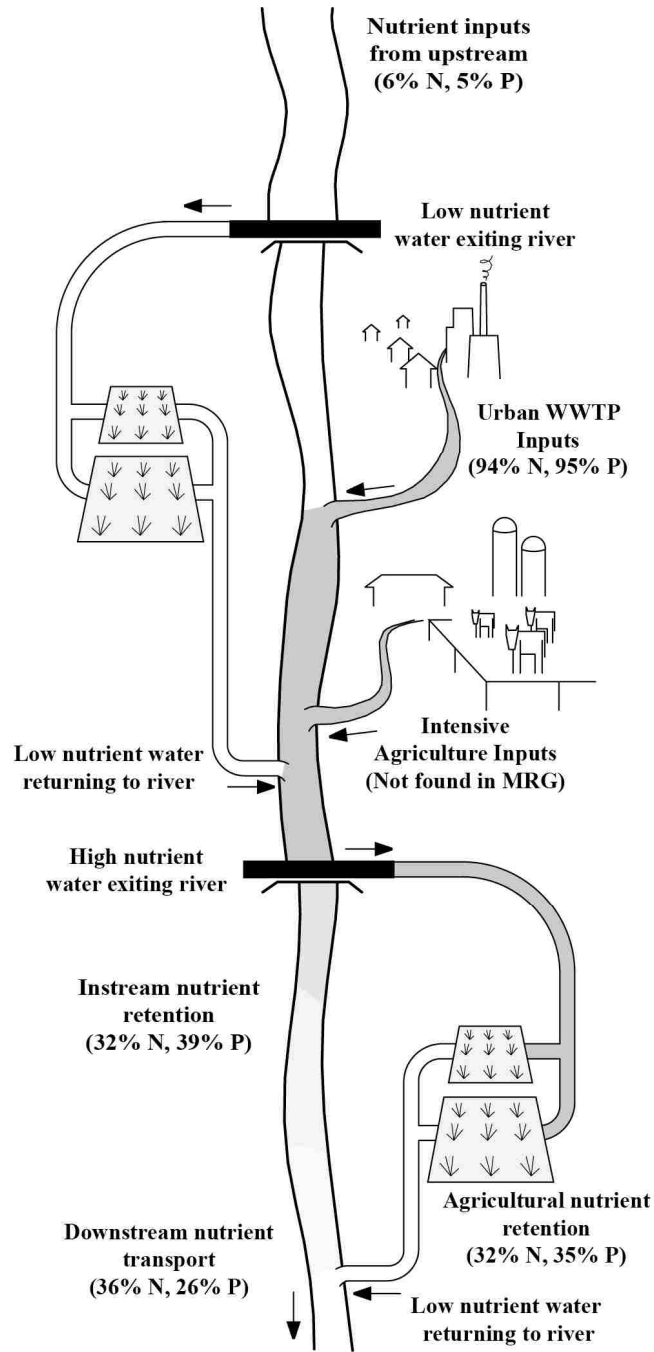


Figure 6. A conceptual model of nutrient retention in aridland ecosystems where sources of nutrients include upstream inputs and wastewater and agricultural effluent and significant portions of river flow are removed for use in agriculture irrigation. The annual percentages are given for the total NO_3 (N) and SRP (P) loads added to the system by upstream and urban inputs, and the percentage of the loads either retained by in-channel or agricultural processes or transported downstream out of the MRG.

Conclusions

The primary contributions of nutrients to the MRG come from wastewater treatment plants. Two mechanisms responsible for removing these inputs from the MRG are instream processing and the retention of nutrients within the agricultural irrigation system. Percentages of nutrient loads retained by instream processing in the MRG are comparable to those found in mesic river systems. Additional removal by the agricultural system during the irrigation season results in greater than 90% total removal of the NO_3 and SRP added to the MRG. Similar water use patterns in other aridland river basins suggest agricultural irrigation may play a major role in explaining high nutrient retention in these ecosystems. Although these results demonstrate that agriculture is providing an important ecosystem service of water purification for downstream water users and ecosystems, the cost for this service is evapotranspirative loss of a significant portion of the MRG discharge during the irrigation season.

Acknowledgements

I acknowledge Abdul-Mehdi Ali, Evan Bussanich, John Craig, Nick Engquist, Becky Engquist, and Casey Seaman for assisting with field work, laboratory analysis, and data management. Funding for this project was provided by the U.S. Army Corps of Engineers and the U.S. Fish and Wildlife Service. Support was provided to David Van Horn through the Alvin R. and Caroline G. Grove Doctoral Scholarship. The U.S. National Science Foundation provided support to Lydia Zeglin through the Integrative Graduate Education and Research Traineeship (IGERT) Freshwater Sciences Interdisciplinary Doctoral Program (DGE 9972810) and the Graduate Research Fellowships Program (GRFP). Support for Clifford Dahm came through the Experimental

Program to Stimulate Competitive Research (EPSCoR) (EPS 0814449), and to Clifford Dahm, David Van Horn, and Lydia Zeglin through the Sevilleta Long-Term Ecological Research (LTER) Project (DEB-0620482). This is publication number (XXX) from the Sevilleta LTER Project.

Literature Cited

- Alexander, R. B., R. A. Smith, and G. E. Schwarz. 2000. Effect of stream channel size on the delivery of nitrogen to the Gulf of Mexico. *Nature* **403**:758-761.
- Banens, R. J. and J. R. Davis. 1998. Comprehensive approaches to eutrophication management: The Australian example. *Water Science and Technology* **37**:217-225.
- Baxter, R. M. 1977. Environmental effects of dams and impoundments. *Annual Review of Ecology and Systematics* **8**:255-283.
- Behrendt, H. 1996. Inventories of point and diffuse sources and estimated nutrient loads - A comparison for different river basins in Central Europe. *Water Science and Technology* **33**:99-107.
- Behrendt, H. and A. Bachor. 1998. Point and diffuse load of nutrients to the Baltic Sea by river basins of north east Germany (Mecklenburg-Vorpommern). *Water Science and Technology* **38**:147-155.
- Behrendt, H. and D. Opitz. 2000. Retention of nutrients in river systems: Dependence on specific runoff and hydraulic load. *Hydrobiologia* **410**:111-122.
- Bennett, E. M., S. R. Carpenter, and N. F. Caraco. 2001. Human impact on erodible phosphorus and eutrophication: A global perspective. *Bioscience* **51**:227-234.

- Bethune, M. 2004. Towards effective control of deep drainage under border-check irrigated pasture in the Murray-Darling Basin: A review. *Australian Journal of Agricultural Research* **55**:485-494.
- Bowmer, K. H., M. Bales, and J. Roberts. 1994. Potential use of irrigation drains as wetlands. *Water Science and Technology* **29**:151-158.
- Bukaveckas, P. A., D. L. Guelda, J. Jack, R. Koch, T. Sellers, and J. Shostell. 2005. Effects of point source loadings, sub-basin inputs and longitudinal variation in material retention on C, N and P delivery from the Ohio River Basin. *Ecosystems* **8**:825-840.
- Caraco, N. F. 1995. Influence of human populations on P transfers to aquatic systems: A regional scale study using large rivers. Pages 235-244 *in* H. Tiessen, editor. *Phosphorus in the Global Environment*. John Wiley & Sons, Chichester.
- Caraco, N. F. and J. J. Cole. 1999. Human impact on nitrate export: An analysis using major world rivers. *Ambio* **28**:167-170.
- Caraco, N. F. and J. J. Cole. 2001. Human influence on nitrogen export: A comparison of mesic and xeric catchments. *Marine and Freshwater Research* **52**:119-125.
- Carpenter, S. R., N. F. Caraco, D. L. Correll, R. W. Howarth, A. N. Sharpley, and V. H. Smith. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications* **8**:559-568.
- Chesterikoff, A., B. Garban, G. Billen, and M. Poulin. 1992. Inorganic nitrogen dynamics in the River Seine downstream from Paris (France). *Biogeochemistry* **17**:147-164.

- Clemmens, A. J., Z. El-Haddad, and T. S. Strelkoff. 1999. Assessing the potential for modern surface irrigation in Egypt. *Transactions of the American Society of Agricultural Engineers* **42**:995-1008.
- Dahm, C. N., J. R. Cleverly, J. E. A. Coonrod, J. R. Thibault, D. E. McDonnell, and D. F. Gilroy. 2002. Evapotranspiration at the land/water interface in a semi-arid drainage basin. *Freshwater Biology* **47**:831-843.
- Department of Water Affairs and Forestry, South Africa. 2004. Internal strategic perspective: Lower Orange water management area. Department of Water Affairs and Forestry, Pretoria, South Africa.
- Earle, A., D. Malzbender, A. Turton, and E. Manzungu. 2005. A preliminary basin profile of the Orange/Senqu River. African Water Issues Research Unit, University of Pretoria, Cape Town.
- Ensign, S. H. and M. W. Doyle. 2006. Nutrient spiraling in streams and river networks. *Journal of Geophysical Research* **111** doi:10.1029/2005JG000114.
- Foley, J. A., R. DeFries, G. P. Asner, C. Barford, G. Bonan, S. R. Carpenter, F. S. Chapin, M. T. Coe, G. C. Daily, H. K. Gibbs, J. H. Helkowski, T. Holloway, E. A. Howard, C. J. Kucharik, C. Monfreda, J. A. Patz, I. C. Prentice, N. Ramankutty, and P. K. Snyder. 2005. Global consequences of land use. *Science* **309**:570-574.
- Forshay, K. J. and E. H. Stanley. 2005. Rapid nitrate loss and denitrification in a temperate river floodplain. *Biogeochemistry* **75**:43-64.
- Gibson, C. A. and J. L. Meyer. 2007. Nutrient uptake in a large urban river. *Journal of the American Water Resources Association* **43**:576-587.

- Gillitt, C. G., W. L. Nieuwoudt, and G. R. Backeberg. 2005. Water markets in the lower Orange River catchment of South Africa. *Agrekon* **44**:363-382.
- Glover, C. R. and R. D. Baker. 1990. Fertilizer guide for New Mexico A-128, New Mexico State Cooperative Extension, Las Cruces, NM.
- Haggard, B. E., E. H. Stanley, and D. E. Storm. 2005. Nutrient retention in a point-source-enriched stream. *Journal of the North American Benthological Society* **24**:29-47.
- Haggard, B. E., D. E. Storm, and E. H. Stanley. 2001. Effect of a point source input on stream nutrient retention. *Journal of the American Water Resources Association* **37**:1291-1299.
- Hall, R. O., J. L. Tank, D. J. Sobota, P. J. Mulholland, J. M. O'Brien, W. K. Dodds, J. R. Webster, H. M. Valett, G. C. Poole, B. J. Peterson, J. L. Meyer, W. H. McDowell, S. L. Johnson, S. K. Hamilton, N. B. Grimm, S. V. Gregory, C. N. Dahm, L. W. Cooper, L. R. Ashkenas, S. M. Thomas, R. W. Sheibley, J. D. Potter, B. R. Niederlehner, L. T. Johnson, A. M. Helton, C. M. Crenshaw, A. J. Burgin, M. J. Bernot, J. J. Beaulieu, and C. P. Arango. 2009. Nitrate removal in stream ecosystems measured by N-15 addition experiments: Total uptake. *Limnology and Oceanography* **54**:653-665.
- Hansen, H. P. and F. Koroleff. 1983. Determination of nutrients. Pages 159-226 in K. Grasshoff, K. Kremling, and M. Ehrhardt, editors. *Methods of Seawater Analysis*. Weinheim: Verlag Chemie.
- Helton, A. M., G. C. Poole, J. L. Meyer, W. M. Wollheim, B. J. Peterson, P. J. Mulholland, J. A. Stanford, C. Arango, L. R. Ashkenas, L. W. Cooper, W. K.

- Dodds, S. V. Gregory, R. O. Hall, S. K. Hamilton, S. L. Johnson, W. H. McDowell, J. D. Potter, J. L. Tank, S. M. Thomas, H. M. Valett, J. R. Webster, and L. H. Zeglin. *In Review*. Thinking outside the channel: Nitrogen cycling in networked river ecosystems. *Frontiers in Ecology and the Environment*.
- Herzon, I. and J. Helenius. 2008. Agricultural drainage ditches, their biological importance and functioning. *Biological Conservation* **141**:1171-1183.
- Hillbricht-Ilkowska, A. 1999. Shallow lakes in lowland river systems: Role in transport and transformations of nutrients and in biological diversity. *Marine and Freshwater Research* **408**:349-358.
- Howarth, R. W., G. Billen, D. Swaney, A. Townsend, N. Jaworski, K. Lajtha, J. A. Downing, R. Elmgren, N. Caraco, T. Jordan, F. Berendse, J. Freney, V. Kudeyarov, P. Murdoch, and Z. L. Zhu. 1996. Regional nitrogen budgets and riverine N&P fluxes for the drainages to the North Atlantic Ocean: Natural and human influences. *Biogeochemistry* **35**:75-139.
- Kroger, R., M. M. Holland, M. T. Moore, and C. M. Cooper. 2007. Hydrological variability and agricultural drainage ditch inorganic nitrogen reduction capacity. *Journal of Environmental Quality* **36**:1646-1652.
- Lange, G.-M., E. Mungatana, and R. Hassan. 2007. Water accounting for the Orange River Basin: An economic perspective on managing a transboundary resource. *Ecological Economics* **61**:660-670.
- Lauver, L. and L. A. Baker. 2000. Mass balance for wastewater nitrogen in the central Arizona-Phoenix ecosystem. *Water Research* **34**:2754-2760.

- Marti, E., J. Aumatell, L. Gode, M. Poch, and F. Sabater. 2004. Nutrient retention efficiency in streams receiving inputs from wastewater treatment plants. *Journal of Environmental Quality* **33**:285-293.
- Mulholland, P. J., A. M. Helton, G. C. Poole, R. O. Hall, S. K. Hamilton, B. J. Peterson, J. L. Tank, L. R. Ashkenas, L. W. Cooper, C. N. Dahm, W. K. Dodds, S. E. G. Findlay, S. V. Gregory, N. B. Grimm, S. L. Johnson, W. H. McDowell, J. L. Meyer, H. M. Valett, J. R. Webster, C. P. Arango, J. J. Beaulieu, M. J. Bernot, A. J. Burgin, C. L. Crenshaw, L. T. Johnson, B. R. Niederlehner, J. M. O'Brien, J. D. Potter, R. W. Sheibley, D. J. Sobota, and S. M. Thomas. 2008. Stream denitrification across biomes and its response to anthropogenic nitrate loading. *Nature* **452**:202-205.
- Needelman, B. A., D. E. Ruppert, and R. E. Vaughan. 2007. The role of ditch soil formation and redox biogeochemistry in mitigating nutrient and pollutant losses from agriculture. *Journal of Soil and Water Conservation* **62**:207-215.
- Nixon, S. W. 2003. Replacing the Nile: Are anthropogenic nutrients providing the fertility once brought to the Mediterranean by a great river? *Ambio* **32**:30-39.
- Nolan, B. T., K. J. Hitt, and B. C. Ruddy. 2002. Probability of nitrate contamination of recently recharged groundwaters in the conterminous United States. *Environmental Science and Technology* **36**:2138-2145.
- Oelsner, G. P., P. D. Brooks, and J. F. Hogan. 2007. Nitrogen sources and sinks within the Middle Rio Grande, New Mexico. *Journal of the American Water Resources Association* **43**:850-863.

- Olli, G., A. Darracq, and G. Destouni. 2009. Field study of phosphorus transport and retention in drainage reaches. *Journal of Hydrology* **365**:46-55.
- Passell, H. D., C. N. Dahm, and E. J. Bedrick. 2004. Hydrological and geochemical trends and patterns in the Upper Rio Grande, 1975 to 1999. *Journal of the American Water Resources Association* **40**:111-127.
- Passell, H. D., C. N. Dahm, and E. J. Bedrick. 2005. Nutrient and organic carbon trends and patterns in the upper Rio Grande, 1975-1999. *Science of the Total Environment* **345**:239-260.
- Peterson, B. J., W. M. Wollheim, P. J. Mulholland, J. R. Webster, J. L. Meyer, J. L. Tank, E. Marti, W. B. Bowden, H. M. Valett, A. E. Hershey, W. H. McDowell, W. K. Dodds, S. K. Hamilton, S. Gregory, and D. D. Morrall. 2001. Control of nitrogen export from watersheds by headwater streams. *Science* **292**:86-90.
- Rabalais, N. N. 2002. Nitrogen in aquatic ecosystems. *Ambio* **31**:102-112.
- Reddy, K. R., R. H. Kadlec, E. Flaig, and P. M. Gale. 1999. Phosphorus retention in streams and wetlands: A review. *Critical Reviews in Environmental Science and Technology* **29**:83-146.
- Schlegel, A. J. and J. L. Havlin. 1995. Corn response to long-term nitrogen and phosphorus fertilization. *Journal of Production Agriculture* **8**:181-185.
- Schmidt, J. P., C. J. Dell, P. A. Vadas, and A. L. Allen. 2007. Nitrogen export from coastal plain field ditches. *Journal of Soil and Water Conservation* **62**:235-243.
- Seitzinger, S. P., R. V. Styles, E. W. Boyer, R. B. Alexander, G. Billen, R. W. Howarth, B. Mayer, and N. V. Breemen. 2002. Nitrogen retention in rivers: Model

- development and application to watersheds in the northeastern U.S.A.
Biogeochemistry **57/58**:199-237.
- Sharpley, A. N., T. Krogstad, P. J. A. Kleinman, B. Haggard, F. Shigaki, and L. S. Saporito. 2007. Managing natural processes in drainage ditches for nonpoint source phosphorus control. *Journal of Soil and Water Conservation* **62**:197-206.
- Sinaj, S., C. Stamm, G. S. Toor, L. M. Condron, T. Hendry, H. J. Di, K. C. Cameron, and E. Frossard. 2002. Phosphorus exchangeability and leaching losses from two grassland soils. *Journal of Environmental Quality* **31**:319-330.
- Sjodin, A. L., W. M. Lewis, and J. F. Saunders. 1997. Denitrification as a component of the nitrogen budget for a large plains river. *Biogeochemistry* **39**:327-342.
- Skaggs, R. W., M. A. Breve, and J. W. Gilliam. 1994. Hydrologic and water-quality impacts of agricultural drainage. *Critical Reviews in Environmental Science and Technology* **24**:1-32.
- Smith, A. and B. L. Maheshwari. 2002. Options for alternative irrigation water supplies in the Murray-Darling Basin, Australia: A case study of the Shepparton Irrigation Region. *Agricultural Water Management* **56**:41-55.
- Strock, J. S., C. J. Dell, and J. P. Schmidt. 2007. Managing natural processes in drainage ditches for nonpoint source nitrogen control. *Journal of Soil and Water Conservation* **62**:188-196.
- Sultan, M., M. Fiske, T. Stein, M. Gamal, Y. A. Hady, H. El Araby, A. Madani, S. Mehane, and R. Becker. 1999. Monitoring the urbanization of the Nile Delta, Egypt. *Ambio* **28**:628-631.

- Tibbets, T. M. and M. C. Molles. 2005. C : N : P stoichiometry of dominant riparian trees and arthropods along the Middle Rio Grande. *Freshwater Biology* **50**:1882-1894.
- Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W. H. Schlesinger, D. Simberloff, and D. Swackhamer. 2001. Forecasting agriculturally driven global environmental change. *Science* **292**:281-284.
- Wahaab, R. A. and M. I. Badawy. 2004. Water quality assessment of the river Nile system: An overview. *Biomedical and Environmental Sciences* **17**:87-100.
- Webster, J. R. and B. C. Patten. 1979. Effects of watershed perturbation on stream potassium and calcium dynamics. *Ecological Monographs* **49**:51-72.
- Wollheim, W. M., C. J. Vorosmarty, B. J. Peterson, S. P. Seitzinger, and C. S. Hopkins. 2006. Relationship between river size and nutrient removal. *Geophysical Research Letters* **33**:L06410.
- Wood, M. L. and L. Finger. 2006. Influence of irrigation method on water use and production of perennial pastures in northern Victoria. *Australian Journal of Experimental Agriculture* **46**:1605-1614.

Chapter 4: GUT MICROBIAL COMMUNITY COMPOSITION AND VARIABILITY IN THREE PLANORBID SNAIL SPECIES

D. J. Van Horn¹, J. R. Hall¹, E. S. Loker², K. R. Mitchell¹, G. M. Mkoji³, C. M. Adema²,
& C. D. Takacs-Vesbach^{1*}

¹Department of Biology, University of New Mexico, 167 Castetter Hall MSC03 2020, 1
University of New Mexico, Albuquerque, NM 87131-0001

²Center for Evolutionary and Theoretical Immunology, Department of Biology,
University of New Mexico, 167 Castetter Hall MSC03 2020, 1 University of New
Mexico, Albuquerque, NM 87131-0001

³Kenya Medical Research Institute (KEMRI), Nairobi, Kenya

Abstract

Little is known about the microbial gut flora of planorbid snails in spite of the importance of some members of this family of gastropod mollusks in the transmission of schistosomiasis, a parasitic disease which affects ~ 200 million humans worldwide. This study used culture independent methods to describe the community composition and the variability of gut microbes within and among three species of planorbid snails, *Helisoma duryi* (North American species), *Bulinus africanus* (African species), and *Biomphalaria pfeifferi* (African species). Three hundred and fourteen unique bacterial operational taxonomic units (OTUs, DNA sequences with <98% similarity) were found in the guts of the three snail species. This diversity was distributed across 23 bacterial phyla with the largest number of OTUs found in the *Proteobacteria* and *Bacteroidetes* groups. A small percentage of bacterial clones from every snail species were related to opportunistic pathogens that infect a range of hosts including snails and humans. Measures of β

diversity revealed minimal divergence among the gut microbial communities both within and among the three planorbid species, with samples differing primarily in the abundance of sequences within bacterial lineages and not in the presence or absence of lineages.

These results suggest the presence of highly diverse and relatively similar gut microbial communities in the three snail species in spite of varying levels of phylogenetic and geographic separation, and highlight the need for additional study to determine the roles gut microbes play in the physiology of these important intermediate hosts for digenetic trematodes of medical and veterinary significance.

Introduction

Microbes outnumber the cells of their eukaryotic host organisms by a factor of ten (Savage 1977, Turnbaugh et al. 2007). The realization that microbes are not just pathogens but are important symbiotic partners for eukaryotes has resulted in new paradigms and research programs. Recently the term microbiome was coined, which refers to the genomes of all the microbes associated with an organism (Turnbaugh et al. 2007), and a major effort to sequence the human microbiome (Turnbaugh et al. 2007) has begun in recognition that a complete understanding of any organism includes knowledge of its microbiome. Additionally, recent theories of evolution consider organisms along with their associated microorganisms as discrete units of selection (Zilber-Rosenberg and Rosenberg 2008).

The gut has long been recognized as one of the most diverse and important sites of microbe/host interactions and as a result, gut microbiota have been investigated in a variety of organisms ranging from earthworms (Karsten and Drake 1995) to humans (Collins and Gibson 1999). These investigations have revealed an initial microbial

colonization of the host gut that begins during or after birth or hatching. This colonization is followed by succession of the microbial community as microbes capable of inhabiting physical and chemical niches in the gut environment establish viable populations. The sources of these microbes include the general host environment, food sources, and excrement of other members of same species (Dillon and Dillon 2004). Communities of these populations stabilize, forming an indigenous or autochthonous gut microbial community for an individual (Savage 1977). The factors that affect the habitability of host guts for microbes include: diet, gut structure, the nature of the gut lining, and the physical and chemical conditions inside the gut (Harris 1993).

A stabilized indigenous gut microbial community interacts with host organisms in a variety of ways. A primary mutualistic function of gut microbes is to assist the host organism in obtaining nutrients from food sources. This can take several forms including: an improved ability to survive on suboptimal diets, improved digestion efficiency, and acquisition of digestive enzymes and vitamins (Dillon and Dillon 2004). Gut microbes also assist their hosts by metabolizing and detoxifying secondary compounds found in food sources (Bhat et al. 1998, Dillon and Dillon 2004). The extent to which gut microbes provide nutritional aid depends on the food source of the host and the residence time and microenvironments in the gut. If food sources are rich in lignin or other indigestible compounds, as in termites and ruminates, complex gut structures and long residence times provide microbes with conditions necessary to process these recalcitrant materials.

Autochthonous gut flora also protect their hosts from invasion by pathogenic exogenous microbes, a service termed colonization resistance (Rolfe 1997b, Dillon and

Dillon 2004). Indirect and direct antagonism are used by indigenous bacteria to prevent settlement by foreign microbes. Indirect mechanisms include modification of bile salts, induction of immunological processes and stimulation of peristalsis. Direct antagonism includes depletion of essential substrates, creation of restrictive physiologic environments, and production of antibiotic-like substances (Rolfe 1997a).

The structure and functions of gut microbial communities discussed above have been elucidated primarily for mammals and insects, specifically humans, ruminates and termites. Currently little is known about the composition and function of gut flora in mollusks in general. There is very little information specifically about gut microbes from the gastropod family *Planorbidae* (*Planorbidae*, *Pulmonata*, *Gastropoda*, *Mollusca*), a group of snails that is of particular interest because it includes the genera *Biomphalaria* and *Bulinus* that serve as intermediate hosts of digenetic trematodes of the genus *Schistosoma* (Morgan et al. 2002). According to the World Health Organization, these parasites infect ~200 million people worldwide (Gryseels et al. 2006). These and other planorbid snails also support the larval development of many other digeneans that are pathogenic as adults in vertebrates of veterinary significance, or that are of concern to conservation biologists.

The existing information about snail gut microbes is focused on terrestrial phytophagous and saprophagous species that feed on lignin rich food sources. These studies have found a range of pH, oxygen and hydrogen levels and distinct microenvironments in helcid snails feeding on terrestrial plants and detritus. High oxygen uptake rates near the gut epithelium may be related to bacterial degradation of plant compounds such as lignin and phenols (Charrier and Brune 2003a). These findings

indicate a specialized gut community may be found in helicid snails due to their reliance on recalcitrant food sources and their specialized gut morphology.

Planorbid snails, which live primarily in freshwater environments and feed primarily on detritus, decaying macrophytes, and algae, may have significantly different gut flora than the terrestrial snails discussed above due to the phylogenetic distance between these organisms, differences in the environments they inhabit, and the composition and lability of their food sources. The few studies that have investigated the microbial community in planorbid snails used whole organism tissue samples and culture dependent methods to characterize the microbiota (Ducklow et al. 1979, Ducklow et al. 1981). These data provided limited information about gut microbes due to the inability of culture dependent methods to recover more than a small fraction of the entire microbial community (Amann et al. 1995) and because whole organism samples were used.

The goals of this study were to: 1) gain a more complete understanding of planorbid snails by investigating the microbiome from the guts of three species of *Planorbidae* (*Biomphalaria pfeifferi*, *Bulinus africanus*, and *Helisoma duryi*), 2) compare intestinal bacterial communities between individual snails, and 3) compare gut microbe community similarities and differences between species.

Materials and Methods

Collection and Identification of Snails

Adult specimens from snail species representing three genera of *Planorbidae* were obtained from natural aquatic environments. *Helisoma duryi* were collected from Shady Lakes, a recreational fishing and aquatic plant nursery facility in Albuquerque, New Mexico, USA (35° 12.99'N, 106° 35.91'W). *Biomphalaria pfeifferi* and *Bul. africanus*

snails were collected in the Tala/Kangundo area of Kenya, from the seasonal stream Kakulutuine (1° 12.54'S, 37° 20.38'E) and near the Kisukioni Dam (1° 13.50'S, 37° 16.90'E), respectively.

Preliminary field identification of the snails was verified by analyzing the 28S rRNA gene. Briefly, DNA was extracted from whole tissue samples using a CTAB extraction method (modified from Winnepenninckx et al. 1993). Extracted DNA was amplified using PCR forward 5'-GTAACGGCGAGTGAAG-3' and reverse 5'-GTACAATCTGAGGAACCAG-3' primers constructed to yield amplicons 773 bp in length. Using AmpliTaq Gold (Applied Biosystems Inc.), the PCR thermal cycling (ABI GeneAmp 2700, Applied Biosystems Inc.) consisted of 10 min 95°C, 30 cycles of 1 min 95°C, 30 s 52°C, 1 min 72°C, and 7 min 72°C. The amplified 28S rRNA gene was sequenced directly on an ABI 3100 Capillary DNA Sequencer (Applied Biosystems Inc.). Sequences were analyzed using CodonCode Aligner and NCBI BLAST (McGinnis and Madden 2004) searches to verify the species identification.

Bacterial Gut Flora 16S rRNA Gene Amplification and Sequencing

Snails were fasted for at least 24 hours prior to dissection to empty their guts of partially digested food (*Biomphalaria glabrata* snails have been shown to have food transit times of ~ 3 hours, Florschütz and Becker 1999). This ensured that any bacterial 16S rRNA genes isolated were from autochthonous bacteria closely associated with the gut and not associated with a transient food source. Fasted snails were stored at -70°C until processing. The intestine, the portion of the digestive tract distal to the stomach to the anus, was obtained by thawing each snail, wiping its shell with ethanol, and removing the digestive tract using sterile instruments and techniques. The intestine was dissected

and processed from three *Biom. Pfeifferi* and *H. duryi* specimens (samples Bio1 – Bio3, and Hel1 – Hel3 respectively), and two *Bul. africanus* specimens (samples Bul1 and Bul2). After dissection, the intestinal tissues were homogenized using sterile pestles and DNA was extracted using the CTAB extraction technique described above. Bacterial 16S rRNA gene sequences were amplified using the bacteria-specific forward primer 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1492R 5'-GTTTACCTTGTTACGACTT-3' in 50 µl reactions containing 5 µl 10X buffer (Promega Buffer B w/ 1.5 mM MgCl₂), a 12.5 mM concentration of each dNTP (BioLine USA Inc.), 20 pmol each of 8F and 1492R primers, 2.5 U Taq polymerase (Promega US), and approximately 50 ng of DNA. The PCR thermal cycling (ABI GeneAmp 2700, Applied Biosystems Inc.) consisted of 30 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. The amplified 16S rRNA genes were spin-purified using a DNA Purification Kit (MoBio Laboratories) and cloned using a TOPO TA Cloning Kit (Invitrogen). Clones were sequenced using 8F primers, and a representative clone from each major phylogenetic clade was fully sequenced using bacteria-specific, internal primers with the BigDye terminator cycle sequencing kit (PE Applied Biosystems).

Statistical Analysis

Bacterial intestinal flora 16S rRNA gene sequence data was checked for quality using CodonCode Aligner (CodonCode Corporation). High quality sequences greater than 500 bp were exported to Greengenes (<http://greengenes.lbl.gov>) for alignment (NAST Alignment Tool, DeSantis et al. 2006a) to identify the most closely related 16S rRNA gene sequences previously characterized from cultured and uncultured bacteria (DeSantis et al. 2006b). A distance matrix of the aligned sequences was generated in ARB (Ludwig

et al. 2004). This matrix was analyzed in DOTUR (Schloss and Handelsman 2005) (<http://schloss.micro.umass.edu/software/dotur.html>) to divide sequences into operational taxonomic units (OTUs) within each snail species using a 98% DNA sequence similarity cutoff, and to generate rarefaction curves for each individual snail using both a 98% and 95% DNA sequence similarity cutoff. The general distribution of bacterial phyla in the three snail species was assessed by adding a representative from each identified OTU to a backbone phylogenetic tree of 6634 bacterial 16S rRNA gene sequences (Hugenholtz 2002). Each OTU representative was then assigned to the phylum to which it grouped in the backbone tree. A representative from each OTU was submitted to a NCBI BLAST search (McGinnis and Madden 2004). The bacterial sequences representing the ten closest matches for each sequence were recorded, along with the environments from which these bacteria were isolated. All clones with BLAST hits to potential pathogens were included in an initial phylogenetic analysis of pathogens, and redundant sequences were later removed.

Sequence data for the unique OTUs defined in DOTUR was used in Arlequin (Schneider et al. 2000) to perform analysis of molecular variance (AMOVA). AMOVA estimates variance components and F-statistic (F_{ST}) analogs defined here as Φ -statistics (Φ_{ST}) (Excoffier et al. 1992). These parameters describe the correlation of molecular diversity at different levels of hierarchical subdivision (Excoffier et al. 1992). Arlequin also generated traditional fixation index (F_{ST}) values for pairwise comparisons between all samples. The F_{ST} parameter is a measure of population differentiation based on variance in genetic diversity within and among populations. Pairwise comparisons among

organisms generate F_{ST} values ranging from 0 to 1, with 0 indicating no genetic differentiation. Values greater than 0.15 indicate significant divergence (Wright 1978).

The phylogeny of the bacterial 16S rRNA genes from the eight snail samples was also analyzed using UniFrac (Lozupone and Knight 2005, Lozupone et al. 2006). Briefly, all sequences were added using the parsimony add option in ARB (Ludwig et al. 2004) to the large backbone phylogenetic tree of bacterial 16S rRNA gene sequences described above. This tree was imported into UniFrac to calculate the UniFrac metric, which is defined as the phylogenetic distance between sets of taxa in a tree calculated as the percentage of branch length that leads to descendants from only one of a pair of environments represented in a single phylogenetic tree (UniFrac metric) (Lozupone and Knight 2005). This UniFrac metric was then used to perform a variety of tests. The UniFrac significance test was used to determine if samples were statistically different from each other (Lozupone et al. 2006). Environment distance matrices (EDM) were calculated to measure distances between all sample pairs in a tree (Lozupone et al. 2006). These EDMs were then used to hierarchically cluster samples using an un-weighted pair group method with arithmetic mean (UPGMA) algorithm (Lozupone et al. 2006). Jackknife analysis was used to assess confidence in the nodes of the UPGMA tree (Lozupone et al. 2006). Finally, the environment distance matrices were also used to perform a principal coordinate analysis (PCoA) (Lozupone et al. 2006).

Each of the above tests was performed using both the weighted and un-weighted analysis option available in UniFrac (Lozupone et al. 2007). The un-weighted analysis provides a qualitative measure of β diversity that disregards the relative abundance of lineages and focuses on the presence or absence of bacterial lineages within a community.

This measure is useful for determining whether differences in environmental factors between sample environments are large enough to promote the growth of different microbial lineages (Lozupone et al. 2007). The weighted analysis provides a quantitative measure of β diversity that takes into account the relative abundance of lineages. This analysis is useful for investigating how transient environmental factors affect microbial community structure (Lozupone et al. 2007).

Phylogenetic Analysis

A general phylogenetic tree of the snail intestinal bacteria was created using a subset of the total 616 partial sequences found in all of the samples. These sequences were chosen by building a tree of all of the partial sequences and then collapsing the branches into a tractable number of clades. A representative sample was then chosen from each clade for complete sequencing (see above). Sequences were assembled using PHRAP in CodonCode Aligner and edited by hand. The initial forward sequence reads (with the 8F primer) were used in phylogenetic analysis for seven OTUs that did not have a fully sequenced representative due to plasmid insert loss. Phylogenetic analysis was performed in ARB (Ludwig et al. 2004). Topology was explored using neighbor joining, parsimony, and maximum likelihood analyses with various masks based on nucleotide frequency at each alignment position. The final trees were made in ARB by creating a parsimony tree with full-length sequences and adding the shorter sequences with parsimony quick-add. The full-length trees were bootstrapped before the shorter sequences were added using 1000 replicates. All trees were rooted with

Methanocaldococcus jannaschii.

Accession Numbers

The snail 28S and bacterial 16S rRNA gene sequences were deposited in the GenBank nucleotide sequence database under accession numbers: EF152570 (*Biom. africanus*), EF152571 (*Bul. pfeifferi*), FJ423081 (*H. duryi*), and FJ228740 through FJ229355 (bacteria).

Results

Snail Identification and Phylogeny

The 28S rRNA gene sequences from the three snail species confirmed the field identifications of the North American species as *H. duryi*, and the African species as *Bul. africanus*, and *Biom. pfeifferi*. *Helisoma. duryi*, and *Biom. pfeifferi* are phylogenetically close, considered to be sister genera in the *Planorbinae* sub-family while *Bul. africanus* belongs to a separate sub-family *Bulininae* (Morgan et al. 2002) (Fig. 1) .

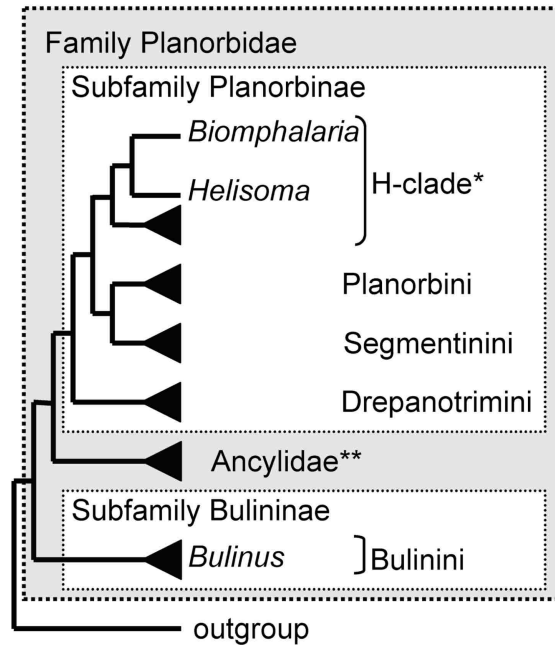


Figure 1: Simplified phylogeny of the family *Planorbidae* (*Gastropoda*, *Mollusca*), based on 28S rDNA genes and exon 2 of a cytoplasmic actin gene, using a physid snail (family *Physidae*, *Mollusca*) to represent the outgroup (Morgan et al. 2002). The term H-clade designates a tribe-level group for which no previous formal name existed (Morgan et al. 2002). Several species of ancyloid snails, previously considered as members of the related basommatophoran family *Ancylidae*, cluster within the *Planorbidae*. The relative phylogenetic position of these *Ancylidae* remains equivocal (also see Jørgensen et al. 2004; Albrecht et al. 2007).

General Distribution of Bacterial 16S rRNA Sequences and OTUs

A total of 616 high quality partial 16S rRNA gene sequences were obtained. These sequences were distributed among the snail species with 150 from the two *Bul. africanus* samples, 215 from the three *Biom. pfeifferi* samples, and 251 from the three *H. duryi* samples. A total of 314 OTUs (cut-off 98% similarity) were recovered from the entire 616 sequence data set. When the communities from the snail species were considered individually, 99 OTUs were recovered from *Bul. africanus*, 74 from *Biom. pfeifferi*, and 156 from *H. duryi*.

Rarefaction curves from the eight samples demonstrated that a significant portion of the genus level diversity (95% DNA sequence similarity) was successfully described for all samples, with the exception of the Hel1 sample (Fig. 2). Additionally, a smaller but still substantial portion of the species level diversity (98% DNA sequence similarity) was successfully described for five of the eight samples, with some evidence of under sampling for the Bio1, Bul2 and Hel1 samples (Fig. 2). While many microbial communities are too complex to be sampled completely (Hughes et al. 2001), the rarefaction curves from this study indicate that we successfully described a significant portion of the gut microbial diversity from the snails sampled.

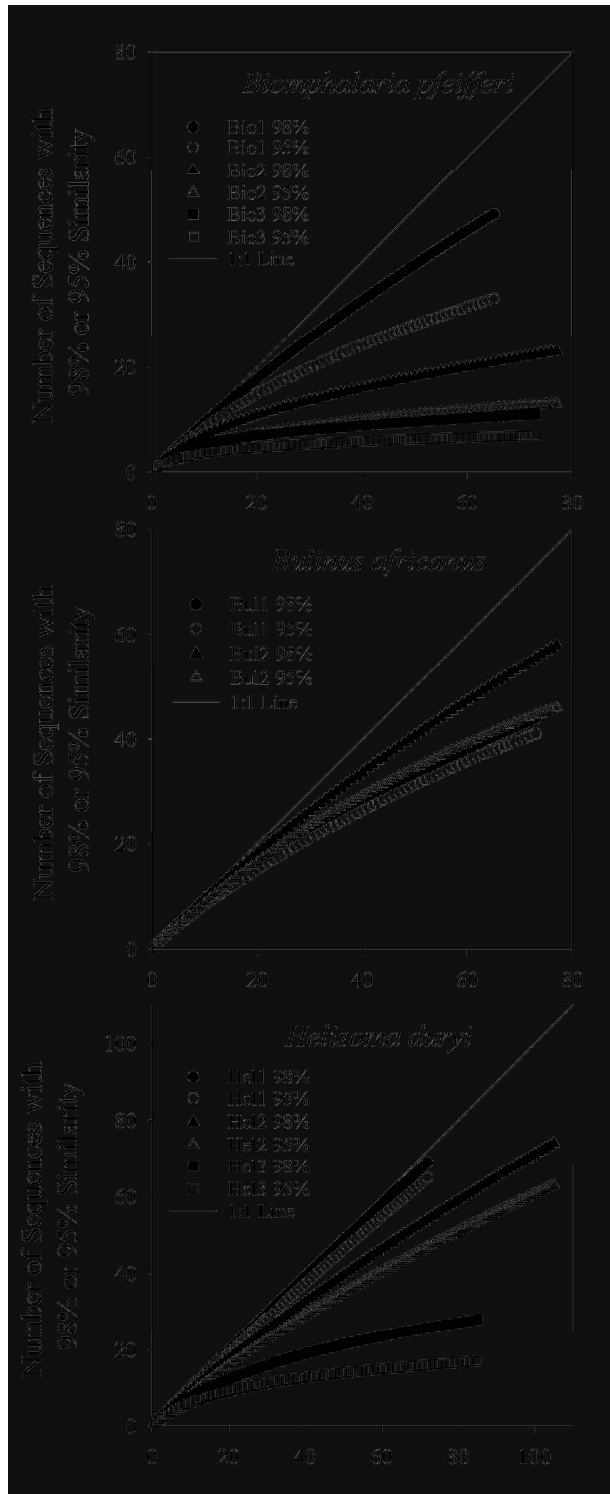


Figure 2: Rarefaction curves create in Dotur (<98% similarity cut-off) for the gut microbial communities from the eight snail samples obtained by analysis of a distance matrix created from sequence data in ARB.

The bacterial sequences from the snail species were distributed among 23 bacterial phyla (Figs. 3a and 3b).

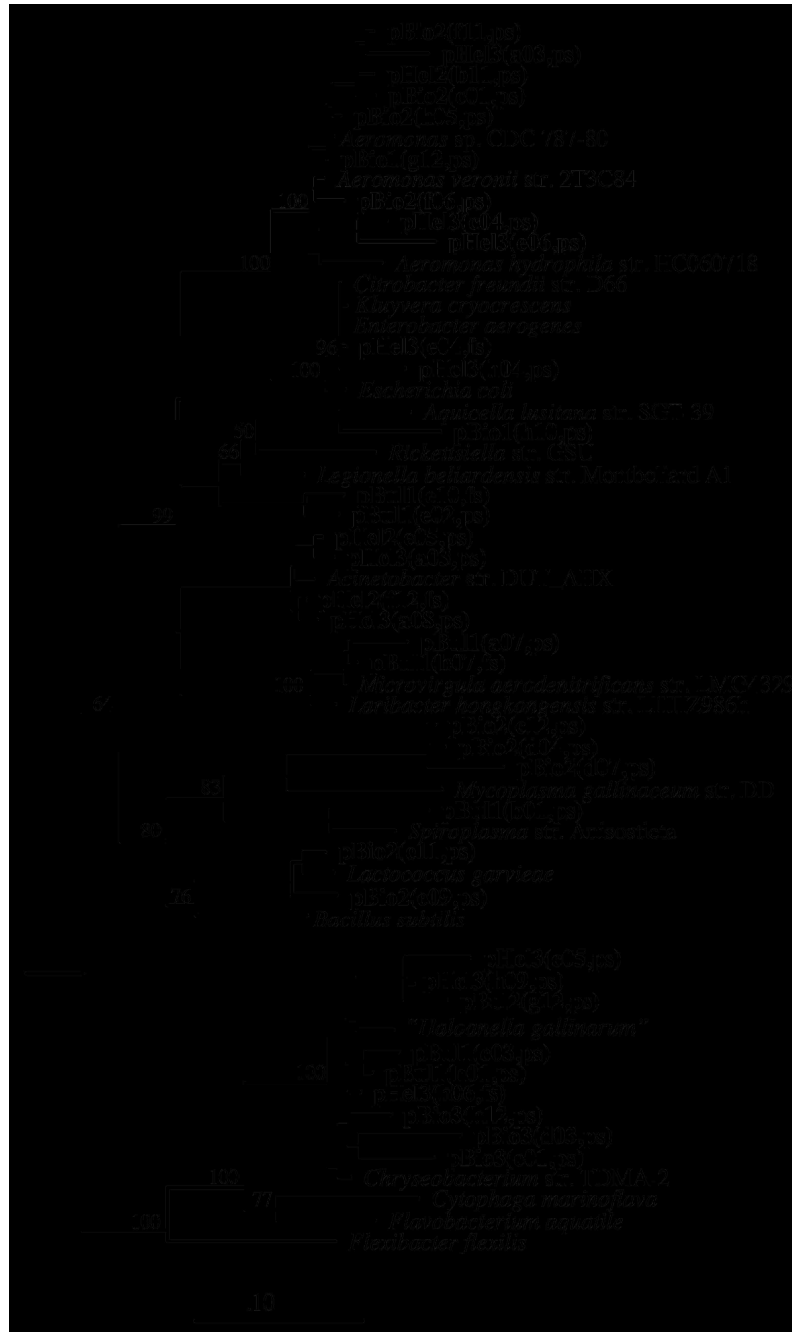


Figure 3a: Parsimony phylogram of planorbid gut bacterial 16S rRNA genes for representatives from the major bacterial clades excluding *Proteobacteria*. Percent bootstrap values of 1000 iterations are shown at the nodes. Clones from this study are highlighted with bold typeset.

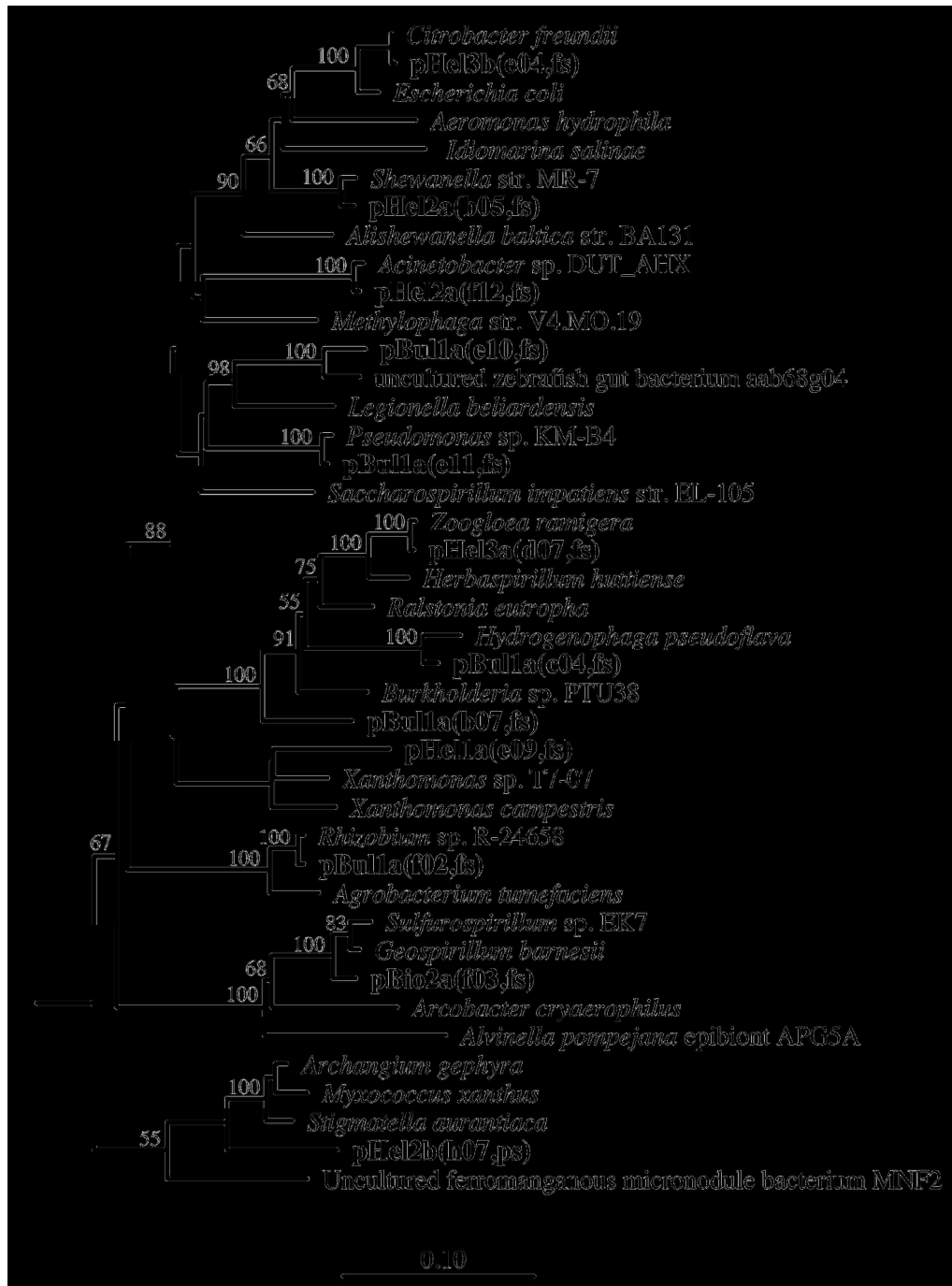


Figure 3b: Parsimony phylogram of planorbid gut bacterial 16S rRNA genes for representatives from the phylum *Proteobacteria*. Percent bootstrap values of 1000 iterations are shown at the nodes. Clones from this study are highlighted with bold typeset.

In all snail species, greater than 50% of the total OTUs were found in three or four dominant phyla (Fig. 4). The *H. duryi* samples contained the most diverse flora, with 21 phyla represented. Members of the *Acidobacteria* were most common, accounting for ~24% of the total OTUs found in the *H. duryi* samples, followed by the *Beta-* and *Gammaproteobacteria* with ~17% and 12% respectively of the total OTUs. *Bulinus africanus* samples yielded OTUs from 17 phyla, 20% of which were from *Bacteroidetes*, while *Actinobacteria*, *Deinococcus-Thermus*, and *Verrucomicrobia* represented ~16%, 10% and 10%, respectively, of the total (Fig. 4). The *Biom. pfeifferi* samples were the least diverse with 10 phyla represented. Sixty-three percent of the *Biom. pfeifferi* OTUs grouped in the *Gammaproteobacteria* with the *Bacteroidetes* containing ~15% of the total OTUs (Fig. 4).

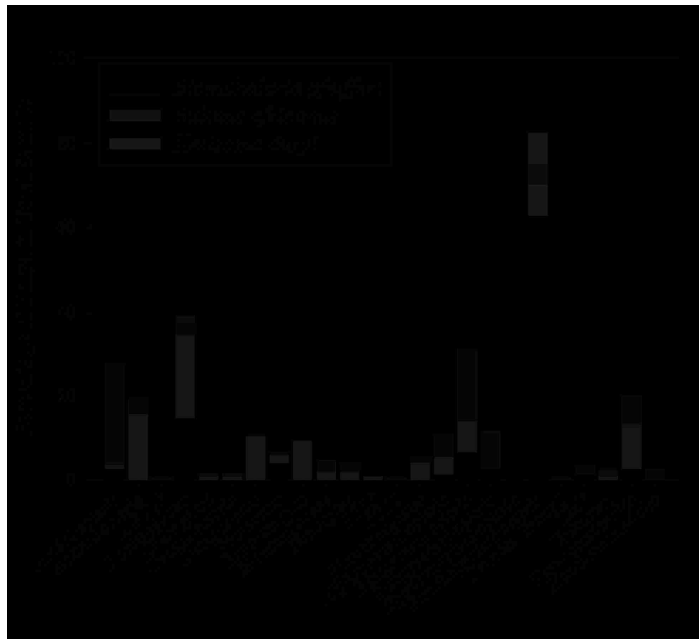


Figure 4: The general distribution of bacterial phyla in the three planorbid snail species. The data was generated by adding a representative from each identified OTU to a backbone phylogenetic tree of 6634 bacterial 16S rRNA gene sequences and assigning OTUs to the phyla they grouped with in the backbone tree.

BLAST searches of the 314 bacterial OTUs revealed that the most closely related bacterial species, based on sequence similarity, were found in a wide variety of habitats. Forty-four percent of the related sequences were found in terrestrial habitats of which the majority, 74%, were found in soil, followed by 10% in rhizospheres. Twenty-nine percent of the total related sequences were found in aquatic environments. Of these samples 46% were found in non-specific aquatic environments, 32% in sediment samples, 12% in water column samples, and 11% in marine environments. Seven percent of the related sequences were found in other gut environments with hosts including antlions, moths, fish, chickens, and humans. Six percent of related sequences were isolated from highly impacted environments such as mine tailings and polluted soil. The remaining 14% of related samples came from a variety of uncommon natural and manmade environments, such as naturally occurring tar pits, glaciers, biofilm reactors, and indoor environments. Some clones from each snail species were identified through phylogenetic analysis as close relatives of potential snail and human pathogens in the *Gamma*- and *Betaproteobacteria*, *Tenericutes*, *Firmicutes*, and *Cytophaga-Flavobacterium-Bacteroidetes* phyla. *Aeromonas* species were found in every snail species and accounted for the highest percentage of pathogen-related clones in every snail in which they were detected. Overall, potential pathogens accounted for 6.7%, 14.7%, and 29.8% of the total clones for *Bul. africanus*, *H. duryi*, and *Biom. pfeifferi* samples, respectively (Fig. 5). Interestingly, common invertebrate symbionts, such as *Rickettsia* and *Wolbachia*, were absent from all of the 16S rRNA gene clone libraries.

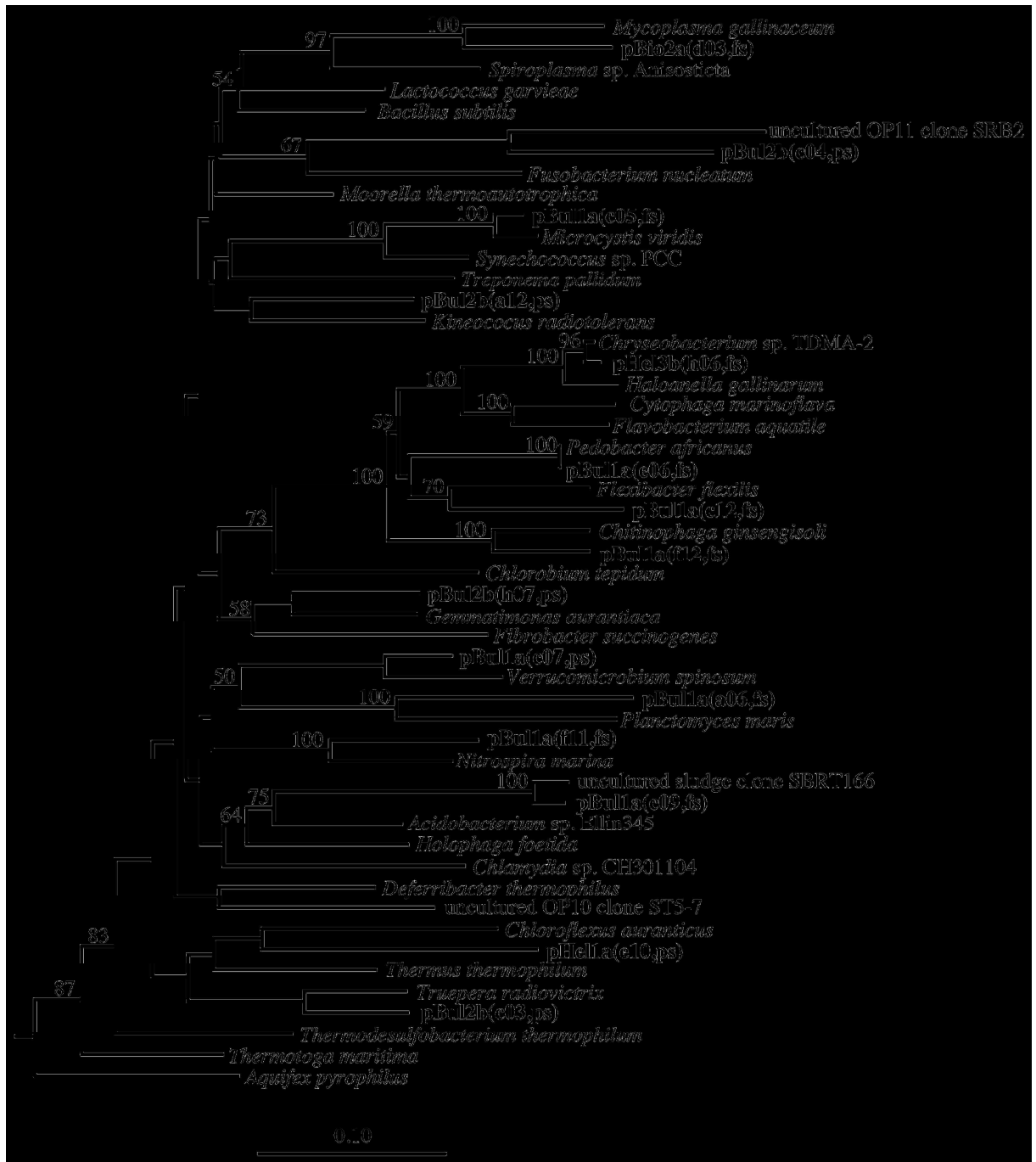


Figure 5: Parsimony phylogram of planorbid gut bacterial 16S rRNA genes for sequences identified by BLAST searches as potential pathogens. Percent bootstrap values of 1000 iterations are shown at the nodes. Clones from this study are highlighted with bold typeset.

Bacterial Community Comparison between Individual Snails and Snail Species

The AMOVA on the OTUs from each snail sample produced estimates of variance components and F -statistic analogs at three levels of hierarchical division: 1) within populations (Φ_{ST}), 2) among populations within groups (Φ_{SC}), and 3) among groups (Φ_{CT}). Ninety five percent of the total molecular variation was due to differences within the populations found in individual snails, five percent was found among populations within groups (within snail species), and no appreciable percentage of the total variation was found among groups (among snail species). The Φ_{ST} value was 0.055, the Φ_{SC} value was 0.056, and the Φ_{CT} value was -0.001. The ‘within populations’ and ‘among population within groups’ variance components and Φ_{ST} and Φ_{SC} values were statistically significant ($P < 0.001$) while the ‘among groups’ variance component and the Φ_{CT} value were not statistically significant ($P > 0.6$). The F_{ST} values for pairwise comparisons between all samples were all significant ($P < 0.001$) and relatively low, ranging from 0.007 to 0.126 (Table 1), indicating minimal to moderate divergence ($F_{ST} = 0$ indicates 100% similarity, $F_{ST} = 1$ indicates 0% similarity, $F_{ST} > 0.15$ indicates significant divergence, Wright 1978).

Table 1. Divergence of bacterial populations of individual snails, Arlequin F_{ST} values for bacterial OTUs from the eight snail samples.

	Bio1 ^a	Bio2	Bio3	Bul1	Bul2	Hel1	Hel2	Hel3
Bio1	0							
Bio2	0.056	0						
Bio3	0.091	0.126	0					
Bul1	0.034	0.070	0.104	0				
Bul2	0.016	0.052	0.086	0.030	0			
Hel1	0.010	0.047	0.082	0.025	0.007	0		
Hel2	0.020	0.056	0.090	0.034	0.016	0.011	0	
Hel3	0.048	0.084	0.117	0.062	0.044	0.039	0.048	0

^a Abbreviated species names are followed by a sample identification number.

The highest F_{ST} value of any of the pairwise comparisons was between two *Biom. pfeifferi* samples ($F_{ST} = 0.126$) indicating these samples were the most different samples in the data set. The ranges of F_{ST} values between samples from the same species were 0.011 – 0.048 for the *H. duryi* samples, 0.056 – 0.126 for the *Biom. pfeifferi* samples, and 0.03 for the *Bul. africanus* samples. The ranges of F_{ST} values between samples from different species were similar to those found within species. Comparisons between *H. duryi* and *Biom. pfeifferi* samples produced F_{ST} values ranging from 0.010 – 0.117. *Helisoma duryi* and *Bul. africanus* comparison values ranged from 0.007 – 0.062 and *Biom. pfeifferi* and *Bul. africanus* samples ranged from 0.016 – 0.104.

Pairwise comparisons of the individual snail samples in UniFrac (1000 Permutations, non-normalized) indicated all samples were significantly different (Bonferroni Corrected, $P < 0.028$ for all samples) regardless of whether the analysis was weighted or un-weighted for the abundance of lineages. UPGMA clustering of the weighted data (1000 permutations, non-normalized) revealed six sample groupings that were well supported by jackknife analysis: 1) Bio1-Bio2 (97%), 2) Bio2-Bio3 (81%), 3) Bul1-Bul2 (80%), 4) Hel1-Hel2 (99%), 5) Hel3-Bio3 (100%), and 6) Hel2- Bul2 (95%) (Fig. 6a). Four of these groups represent within species groupings and two represent between species clusters, with the most strongly supported and closely related being the Hel3 and Bio3 samples. UPGMA clustering of the un-weighted data (1000 permutations, Non-Normalized) revealed five sample groupings that were well supported by jackknife analysis; within species groupings were much less clear and samples from the same snail species were less closely related than they had been in the weighted analysis (Fig. 6e). The strong jackknife support for the UPGMA clustering described above also indicates

that the sequencing effort adequately described the snail gut microbial communities (Lozupone et al. 2007). Weighted PCoA results for the eight snail samples show loose grouping of the samples from the three snail species (Fig. 6b-6d). These results also show the Hel3 sample appears to cluster with the *Biom. Pfeifferi* samples (Fig. 6b-6d), while the *Bul. africanus* samples appear the most dissimilar from the rest of the samples (Fig. 6c and 6d). Un-weighted PCoA results were similar to the weighted results. Samples from the same species, however, did not appear to cluster together as tightly, and the *Bul. africanus* samples were less clearly separated from the rest of the samples (Fig. 6f-6h).

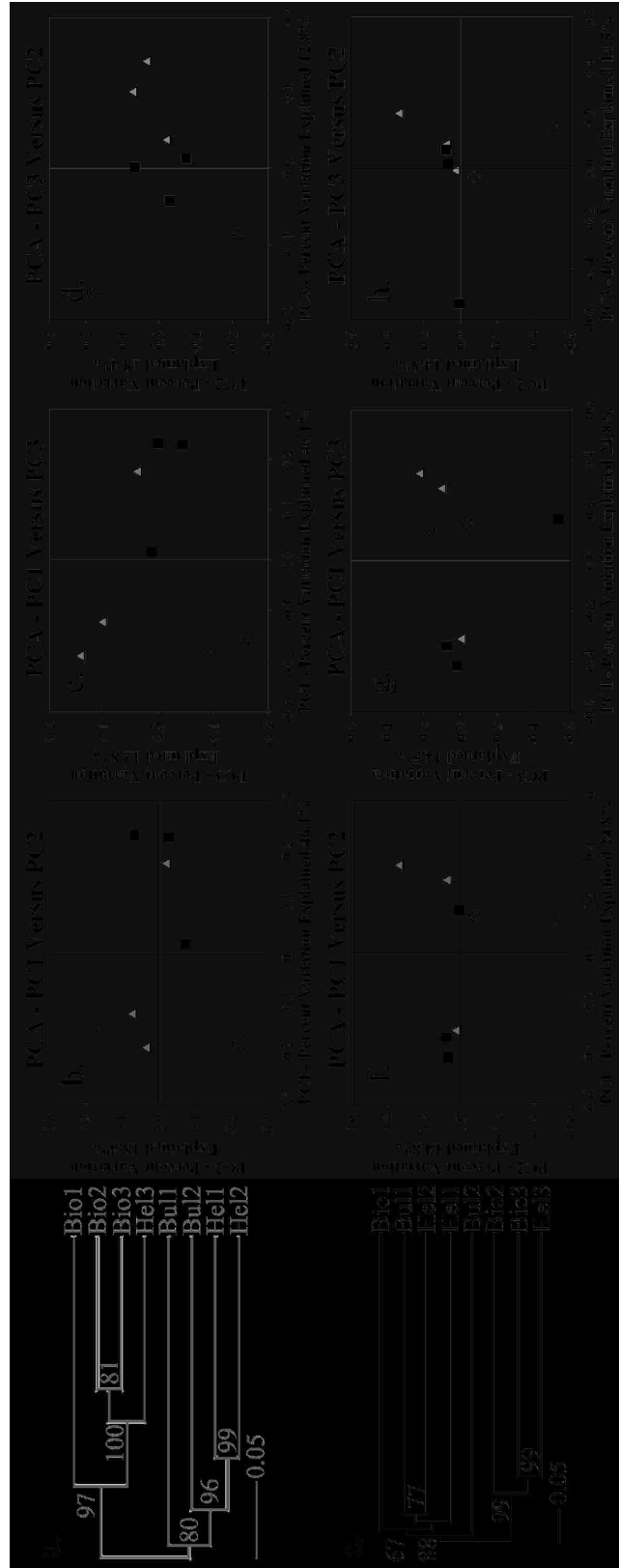


Figure 6: UPGMA dendrograms (6a, 6e) and PCoA plots (6b – 6d and 6f – 6h) for principal coordinates 1 – 3 (P1 – P3) created in UniFrac for the gut bacterial communities in eight planorbid snail samples from three species: *Biomphalaria pfeifferi* (■), *Bulinus africanus* (○), and *Helisoma duryi* (△). Figures 6a – 6d were created using the weighted option in UniFrac which provides a quantitative measure of β diversity, taking into account the relative abundance of lineages in a phylogenetic tree containing the sequence data. Figures 6e – 6h were created using the UniFrac un-weighted analysis option which provides a qualitative measure of β diversity, disregarding the relative abundance of lineages in the sample tree and focusing on the presence/absence of bacterial lineages within a community

Discussion

Gut Microbial Community Composition in Planorbid Snails

This is the first study to investigate the gut microbial community composition in three snail species from the family *Planorbidae*. The finding of more than 300 OTUs shows that the overall bacterial diversity in planorbid guts is high. Furthermore, this diversity is widely distributed across the bacterial phylogenetic tree with 23 phyla represented. In addition to the large number of OTUs found, rarefaction curves from several of the samples indicate a portion of the diversity was not sampled, providing further evidence of the diversity of these communities.

Previous studies have shown that host diet is a determining factor for the diversity and structure of gut microbial communities (Yamada et al. 2007, Ley et al. 2008). In general, host organisms that ingest recalcitrant or complex food sources rely on complex gut microbial communities to extract nutrients from these materials (Harris 1993). For example, analysis of mammal gut microbes revealed that herbivores, which ingest complex carbohydrates from plants, had the highest genus level gut microbe richness followed by omnivores and carnivores (Ley et al. 2008). Similarly, individual soil (Schmitt-Wagner et al. 2003) and wood (Yang et al. 2005) feeding termites harbor

100-200 gut microbe OTUs. This high diversity has been attributed to the complexity of microbial communities necessary to break down the recalcitrant food sources upon which these termites subsist. Previous feeding studies conducted with planorbid snails have shown that *Helisoma trivolvis* (Smith 1989) and *H. duryi* (Madsen 1992), *Biomphalaria peregrina* (Estebenet et al. 2002), *Biom. glabrata* (Cedeño-León and Thomas 1982, Thomas 1982, Thomas et al. 1985), *Biom. pfeifferi* (Madsen 1992), and *Bulinus africanus*, *Bul. truncatus* and *Bul. forskalii* (Madsen 1992) all feed primarily on detritus, followed by decaying macrophytes, diatoms and filamentous algae. Furthermore, Schmölder and Becker (1990) found *Biom. glabrata* actively and selectively fed on sand grains which aid mechanical digestion of recalcitrant materials. These mineral ingestions provide a slight growth and reproductive advantage over snails that had no mineral particles available for ingestion. Thus planorbid snails rely on recalcitrant food sources and complex diets, both of which may require a highly diverse gut microbial community for digestion, consistent with the observations in this study.

A second factor that affects the diversity and structure of gut microbial communities is host phylogeny (Yamada et al. 2007, Ley et al. 2008). While there are no available data of gut microbial diversity and structure for other planorbid snails for comparison, a few studies have investigated the microbial communities in phylogenetically more distant gastropods. A stable, potentially mutualistic gut microbial community is present in the phytophagous terrestrial pulmonate *Helix aspersa* (Charrier et al. 1998) (*Helicidae, Pulmonata*). Gut microenvironment conditions in several other phytophagous and saprophagous helicids also indicate that these snails may harbor an autochthonous gut community (Charrier and Brune 2003b). This evidence of stable

symbiotic gut microbial communities in closely related species suggests that the diverse intestinal bacterial community we observed represents an endogenous and potentially mutualistic community specific to planorbid snails.

Several studies have investigated the structure and function of gut microbes in other gastropods. A study of abalone (*Haliotis discus hannai*) investigated the structure of gut microbial communities in these marine gastropods using culture independent methods (Tanaka et al. 2004). This study, which sequenced a limited number of clones from abalone fed a variety of diets, found a total of 15 OTUs (98% identity). These clones were affiliated with five bacterial phyla (*Firmicutes*, *Fusobacteriaceae*, and *Alpha-*, *Gamma-*, and *Epsilonproteobacteria* (Tanaka et al. 2004)), four of which were represented in the planorbid snails from this study. Gut microbes have also been shown to play a role in meeting the nutritional demands of several gastropod species. Gut microbes from adult abalone, which feed on brown, red and green algae that contain complex carbohydrates resistant to digestion, have been shown to excrete enzymes that break down a variety of these compounds when grown outside the abalone digestive tract (Sawabe et al. 1995, Erasmus et al. 1997). These microbes are also responsive to changes in host diet (Tanaka et al. 2003, Tanaka et al. 2004) and form a stable and consistent community in multiple samples from the same species of abalone (Sawabe et al. 1995). Gut microbes from two other gastropod species, the sea hares *Aplysia dactylomela* and *Aplysia juliana*, are also capable of degrading plant constituents in seaweed culture medium. When antibiotics were used to depopulate the intestines of these two gastropods, the growth rates of juveniles were significantly reduced. The above studies indicate that representatives from most of the bacterial phyla found in other gastropods were present in the planorbid snails

investigated in this study. These studies also revealed that several other members of the class Gastropoda have stable gut microbial communities that are capable of processing recalcitrant food items and have been linked to host health. This is further evidence that the diverse intestinal bacterial communities recorded in this study may represent an autochthonous community specific to planorbids that is important for processing the refractory and complex diet on which these snails depend.

The nearest genetic neighbors to the snail gut bacteria found utilized many metabolic pathways and were isolated from a wide variety of habitats, but most commonly from varied terrestrial soil environments. These soil microbes are likely introduced to the planorbid digestive system through the movement of eroded soil with attached microbes into aquatic environments where they are ingested by the snails. It is likely that some of the microbes from this pool of soil bacteria are capable of filling niches in the planorbid digestive tract and become part of the autochthonous gut flora (Dillon and Dillon 2004). The second most common habitat in which the nearest neighbors to the planorbid gut microbes were found was aquatic environments, with sediment being the most common subcategory. The importance of detritus in planorbid diets (Madsen 1992) may help explain how sediment associated microbes established in the guts of these snails. Only seven percent of the bacteria related to the planorbid flora were isolated from the guts of other organisms. This modest presence of gut microbes is surprising but may be due to the relatively small number of studies that have used culture independent methods to survey the gut flora in fresh water aquatic organisms.

Some of the clones recovered had 16S rRNA gene sequences similar to pathogenic bacteria indicating that planorbid snails may be a reservoir for snail, human

and other wildlife pathogens. The closest relatives of most of these clones are gut bacteria from a variety of hosts. Phylogenetic analysis placed these clones in clades with opportunistic pathogens associated with either nosocomial or community-acquired infection in humans and/or stress-induced disease in aquatic organisms (Kirby et al. 2004, Dijkshoorn et al. 2005, Kiebre-Toe et al. 2005, Vendrell et al. 2006, Dworkin 2007, Lau et al. 2009). Though disease associated with these pathogens is rare in humans, the implication of *Acinetobacter* and *Chryseobacterium* in antibiotic resistance makes identification of environmental sources important. The closest relatives of most of these clones are gut bacteria from a variety of hosts, but all of the cultured members of these clades, except the *Tenericutes*, are either common freshwater inhabitants or parasites of freshwater amoeba (Santos et al. 2003). It is likely these bacteria were allochthonous and colonized the snails' gut through food consumption. This possibility was strengthened by the clone related to *Spiroplasma*, a phyto-pathogenic species that can be found in plant phloem (Bove 1997), and the clones related to *Lactococcus*, which had 99% similarity to strains isolated from broccoli and radishes according to NCBI matches. The only close relative known to cause disease in snails is *Aeromonas hydrophila*. Aeromonads are present in the guts of a wide range of healthy aquatic organisms, but are also associated with diseases of fish, frogs, zebra mussels (Gu and Mitchell 2002), and snails. Kiebre-Toe *et. al.* (2005) found *Aeromonas* species associated with both healthy and diseased helicid snails, though *Aeromonas hydrophila* was almost exclusively isolated from diseased snails. Given the placement and number of clones in the *Aeromonas* clade, we probably detected both normal gut flora and potentially pathogenic strains.

Gut Microbial Community Similarities between Samples and Snail Species

Additional goals of this study were to assess the gut microbial community similarities and differences between the individual snail samples and between the three snail species. The AMOVA results indicate that almost all of the molecular variance in the entire gut microbe data set collected is due to differences within individual snails and almost none is due to differences among individuals within snail species or among snail species. While this divergence is statistically significant, the low Φ_{ST} and Φ_{SC} values indicate minimal divergence within the gut microbial populations of individual snails or among populations within groups and almost no divergence between gut microbial communities from different species. This result is supported by the low F_{ST} values for all pairwise comparisons and the similar ranges of F_{ST} values for between and within species comparisons. These statistical comparisons among the samples combine to suggest planorbid snails from different sub-families and even from different continents host relatively similar gut microbial communities, although we recognize that an exhaustive sequencing effort would be needed to uncover the full diversity of the planorbid snail gut microbiome. Investigations of the gut microbial communities of other gastropods (Sawabe et al. 1995) as well as more phylogenetically distant organisms (Ley et al. 2008) have revealed relatively stable communities between individuals of the same species and similar communities between species that are closely related phylogenetically. These results suggest that planorbid snails follow this pattern, with relatively stable and similar autochthonous gut microbial communities found within species and between closely related organisms that have similar diets, even when these species are found on different continents (North America versus Africa).

The data for all of the samples were analyzed using both the weighted and un-weighted options in UniFrac. This allowed us to determine whether differences between samples or groups of samples were a result of dramatically different environmental conditions in the different host species (un-weighted), or whether the differences between host environments were small, affecting the abundance of lineages but not their presence (weighted) (Lozupone et al. 2007). The more distinct species grouping found in the PCoA plots and UPGMA dendrograms from the weighted samples as compared to the un-weighted samples suggests that the differences between species has less to do with the presence or absence of lineages and more to do with the abundance of similar OTUs within lineages. This result was expected due to the similar diets, physiology, and environments these snail species share and their close phylogenetic relationship.

Conclusions

The microbiology of the guts of planorbid snails has received very little attention to date, even though members of this family are important intermediate hosts for the transmission of human pathogens. This study described the microbial community in the guts of three planorbid species as comprised of numerous and phylogenetically diverse OTUs, and relatively similar between individuals and species. These results highlight the need for additional study to determine the roles these gut microbial communities play in host health.

Acknowledgments

I acknowledge Chris Aquino for assisting with a preliminary draft of this manuscript and Rachel Dorin for assisting with DNA extraction and amplification. A significant portion of the sequencing for this study was performed at the Genome

Sequencing Center at Washington University School of Medicine in St. Louis Missouri. Sequencing assistance and technical support were also provided by the University of New Mexico's Molecular Biology Facility which is supported by NIH Grant Number 1P20RR18754 from the Institute Development Award (IDeA) Program of the National Center for Research Resources. NIH support is acknowledged by C. M. Adema (AI052363) and E. S. Loker (AI024340).

Literature Cited

- Albrecht, C., K. Kuhn, and B. Streit. 2007. A molecular phylogeny of Planorboidea (Gastropoda, Pulmonata): Insights from enhanced taxon sampling. *Zoologica Scripta* **36**:27-39.
- Amann, R. L., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**:143-169.
- Bhat, T. K., B. Singh, and O. P. Sharma. 1998. Microbial degradation of tannins - A current perspective. *Biodegradation* **9**:343-357.
- Bove, J. M. 1997. Spiroplasmas: Infectious agents of plants, arthropods and vertebrates. *Wiener Klinische Wochenschrift* **109**:604-612.
- Cedeño-León, A. and J. D. Thomas. 1982. Competition between *Biomphalaria glabrata* (Say) and *Marisa cornuarietis* (L): Feeding Niches. *Journal of Applied Ecology* **19**:707-721.
- Charrier, M. and A. Brune. 2003a. The gut microenvironment of helcid snails (Gastropoda : Pulmonata): in-situ profiles of pH, oxygen, and hydrogen

- determined by microsensors. Canadian Journal of Zoology-Revue Canadienne de Zoologie **81**:928-935.
- Charrier, M. and A. Brune. 2003b. The gut microenvironment of helcid snails (Gastropoda: Pulmonata): In-situ profiles of pH, oxygen, and hydrogen determined by microsensors. Canadian Journal of Zoology/Revue Canadienne de Zoologie **81**:928-935.
- Charrier, M., Y. Combet-Blanc, and B. Ollivier. 1998. Bacterial flora in the gut of *Helix aspersa* (Gastropoda Pulmonata): Evidence for a permanent population with a dominant homolactic intestinal bacterium, *Enterococcus casseliflavus*. Canadian Journal of Microbiology/Revue Canadienne de Microbiologie **44**:20-27.
- Collins, M. D. and G. R. Gibson. 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. American Journal of Clinical Nutrition **69**:1052S-1057S.
- DeSantis, T. Z., P. Hugenholtz, K. Keller, E. L. Brodie, N. Larsen, Y. M. Piceno, R. Phan, and G. L. Andersen. 2006a. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Research **34**:W394-W399.
- DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006b. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology **72**:5069-5072.
- Dijkshoorn, L., E. van Aken, L. Shunburne, T. J. van der Reijden, A. T. Bernards, A. Nemeč, and K. J. Towner. 2005. Prevalence of *Acinetobacter baumannii* and

- other *Acinetobacter* spp. in faecal samples from non-hospitalised individuals. *Clinical Microbiology and Infection* **11**:329-332.
- Dillon, R. J. and V. M. Dillon. 2004. The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology* **49**:71-92.
- Ducklow, H. W., P. J. Boyle, P. W. Mangel, C. Strong, and R. Mitchell. 1979. Bacterial-flora of the schistosome vector snail *Biomphalaria glabrata* *Applied and Environmental Microbiology* **38**:667-672.
- Ducklow, H. W., K. Clausen, and R. Mitchell. 1981. Ecology of bacterial communities in the schistosomiasis vector snail *Biomphalaria glabrata* *Microbial Ecology* **7**:253-274.
- Dworkin, M., S. Falkow, E. Rosenberg, K. Schleifer, E. Stackebrandt, editor. 2007. The Genus *Enterobacter*. 3 edition. Springer New York.
- Erasmus, J. H., P. A. Cook, and V. E. Coyne. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture* **155**:377-386.
- Estebenet, A. L., N. J. Cazzaniga, and N. V. Pizani. 2002. The natural diet of the Argentinean endemic snail *Chilina parchappii* (Basommatophora: Chiliniidae) and two other coexisting pulmonate gastropods. *Veliger* **45**:71-78.
- Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**:479-491.
- Florschütz, A. and W. Becker. 1999. Gastrointestinal transit and digestive rhythm in *Biomphalaria glabrata* (Say). *Journal of Molluscan Studies* **65**:163-170.

- Gryseels, B., K. Polman, J. Clerinx, and L. Kestens. 2006. Human schistosomiasis. *Lancet* **368**:1106-1118.
- Gu, J. D. and R. Mitchell. 2002. Indigenous microflora and opportunistic pathogens of the freshwater zebra mussel, *Dreissena polymorpha*. *Hydrobiologia* **474**:81-90.
- Harris, J. M. 1993. The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microbial Ecology* **25**:195-231.
- Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. *Genome Biology* **3**:1-8.
- Hughes, J. B., J. J. Hellmann, T. H. Ricketts, and B. J. M. Bohannan. 2001. Counting the uncountable: Statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology* **67**:4399-4406.
- Jørgensen, A., T. K. Kristensen, and J. R. Stothard. 2004. An investigation of the "Ancyloplanorbidae" (Gastropoda, Pulmonata, Hygrophila): Preliminary evidence from DNA sequence data. *Molecular Phylogenetics and Evolution* **32**:778-787.
- Karsten, G. R. and H. L. Drake. 1995. Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. *Applied and Environmental Microbiology* **61**:1039-1044.
- Kiebre-Toe, M. B., A. Lacheretz, L. Villard, Y. Richard, and A. Kodjo. 2005. Pulsed-field gel electrophoresis profiles of aeromonads isolated from healthy and diseased *Helix aspersa* from French snail farms. *Canadian Journal of Microbiology* **51**:817-820.
- Kirby, J. T., H. S. Sader, T. R. Walsh, and R. N. Jones. 2004. Antimicrobial susceptibility and epidemiology of a worldwide collection of *Chryseobacterium* spp: report

- from the SENTRY Antimicrobial Surveillance Program (1997-2001). *Journal of Clinical Microbiology* **42**:445-448.
- Lau, S. K., L. C. Lee, R. Y. Fan, J. L. Teng, C. W. Tse, P. C. Woo, and K. Y. Yuen. 2009. Isolation of *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis, from Chinese tiger frog. *International Journal of Food Microbiology* **129**:78-82.
- Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon. 2008. Evolution of mammals and their gut microbes. *Science* **320**:1647-1651.
- Lozupone, C., M. Hamady, and R. Knight. 2006. UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7**:371-384.
- Lozupone, C. and R. Knight. 2005. UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**:8228-8235.
- Lozupone, C. A., M. Hamady, S. T. Kelley, and R. Knight. 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology* **73**:1576-1585.
- Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A.

- Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer. 2004. ARB: A software environment for sequence data. *Nucleic Acids Research* **32**:1363-1371.
- Madsen, H. 1992. Food selection by freshwater snails in the Gezira irrigation canals, Sudan. *Hydrobiologia* **228**:203-217.
- McGinnis, S. and T. L. Madden. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Research* **32**:W20-W25.
- Morgan, J. A. T., R. J. DeJong, Y. H. Jung, K. Khallaayoune, S. Kock, G. M. Mkoji, and E. S. Loker. 2002. A phylogeny of planorbid snails, with implications for the evolution of *Schistosoma* parasites. *Molecular Phylogenetics and Evolution* **25**:477-488.
- Rolfe, R. D. 1997. Colonization Resistance. Pages 501-536 in R. I. Mackie, B. A. White, and R. E. Isaacson, editors. *Gastrointestinal Microbiology: Volume 2 Gastrointestinal Microbes and Host Interactions*. Chapman and Hall, New York.
- Santos, P., I. Pinhal, F. A. Rainey, N. Empadinhas, J. Costa, B. Fields, R. Benson, A. Verissimo, and M. S. Da Costa. 2003. Gamma-proteobacteria *Aquicella lusitana* gen. nov., sp. nov., and *Aquicella siphonis* sp. nov. infect protozoa and require activated charcoal for growth in laboratory media. *Applied and Environmental Microbiology* **69**:6533-6540.
- Savage, D. C. 1977. Microbial ecology of gastrointestinal tract. *Annual Review of Microbiology* **31**:107-133.
- Sawabe, T., Y. Oda, Y. Shiomi, and Y. Ezura. 1995. Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microbial Ecology* **30**:193-202.

- Schloss, P. D. and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology* **71**:1501-1506.
- Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). *Applied and Environmental Microbiology* **69**:6007-6017.
- Schmölder, U. and W. Becker. 1990. Uptake of mineral particles by *Biomphalaria glabrata* (Say) (Pulmonata, Mollusca) and its importance for growth rate and egg production. *Internationale Revue der Gesamten Hydrobiologie* **75**:95-101.
- Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin. Page A software for population genetics data analysis. Genetics and Biometry Lab, Department of Anthropology, University of Geneva.
- Smith, D. A. 1989. Tests of feeding selectivity in *Helisoma trivolvis* (Gastropoda: Pulmonata). *Transactions of the American Microscopical Society* **108**:394-402.
- Tanaka, R., M. Ootsubo, T. Sawabe, Y. Ezura, and K. Tajima. 2004. Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture* **241**:453-463.
- Tanaka, R., I. Sugimura, T. Sawabe, M. Yoshimizu, and Y. Ezura. 2003. Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science* **69**:951-958.
- Thomas, J. D. 1982. Chemical ecology of the snail hosts of schistosomiasis: Snail-snail and snail-plant interactions. *Malacologia* **22**:81-91.

- Thomas, J. D., D. I. Nwanko, and P. R. Sterry. 1985. The feeding strategies of juvenile and adult *Biomphalaria glabrata* (Say) under simulated natural conditions and their relevance to ecological theory and snail control. Proceedings of the Royal Society of London, Series B: Biological Sciences **226**:177-209.
- Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon. 2007. The human microbiome project. Nature **449**:804-810.
- Vendrell, D., J. L. Balcazar, I. Ruiz-Zarzuela, I. de Blas, O. Girones, and J. L. Muzquiz. 2006. *Lactococcus garvieae* in fish: A review. Comparative Immunology, Microbiology and Infectious Diseases **29**:177-198.
- Winnepenninckx, B., T. Backeljau, and R. Dewachter. 1993. Extraction of high molecular weight DNA from mollusks. Trends in Genetics **9**:407-407.
- Wright, S. 1978. Variability Within and Among Natural Populations. The University of Chicago Press, Chicago.
- Yamada, A., T. Inoue, S. Noda, Y. Hongoh, and M. Ohkuma. 2007. Evolutionary trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites. Molecular Ecology **16**:3768-3777.
- Yang, H., D. Schmitt-Wagner, U. Stingl, and A. Brune. 2005. Niche heterogeneity determines bacterial community structure in the termite gut (*Reticulitermes santonensis*). Environmental Microbiology **7**:916-932.
- Zilber-Rosenberg, I. and E. Rosenberg. 2008. Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. FEMS Microbiology Reviews **32**:723-735.