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Impacts of Alterations of Organic Inputs on the Bacterial Community within the sediments of Wind Cave, South Dakota, USA

Marisa K. Chelius[1,4], Guy Beresford[1], Howard Horton[2], Meghan Quirk[2], Greg Selby[1], Rodney T. Simpson[1], Rodney Horrocks[3], and John C. Moore[1,2]

Abstract:

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Wind Cave (WICA) in the Black Hills of South Dakota, like many mostly dry caves in temperate regions is an energy-starved system. The biotic communities that reside in these systems are low in diversity and simple in structure, and sensitive to changes in external inputs of organic matter. Caves open to tourist traffic offer an opportunity to study the impacts of organic matter amendments in the form of human and rodent hair and dander, clothing lint, material from rodent activity (nesting materials and feces), and algal growth in and around artificial lighting. This study reports on the impacts of carbon amendments from humans and rodents on the bacterial and archaeal communities within the sediments of WICA from annual surveys and from a manipulative study that added lint ('L'; cellulose plus rodent dander and rodent hair), rodent feces ('F'), and a combination of both ('LF'). The survey confirmed that bacterial biomass was higher in regions of the cave with the highest rates of lint (hair and natural clothing fibers) input. The manipulative study found that organic amendments in the forms of lint (L) and rodent feces (F) altered the WICA bacterial community structure in both abundance and diversity, with the combined lint and feces (LF) amendment having the most significant response. The high similarity of the LF and L communities suggests that the cave bacterial community is more carbon than nitrogen limited. The implication of cave development to management practices is immediate and practical. Even small amounts of lint and organic matter foreign to cave bacteria significantly compromise the integrity of the endemic community resulting in the replacement of undescribed species by assemblages with at best, unknown impacts to natural cave features.

Keywords: Wind Cave, caves, bacterial communities, diversity, geomicrobiology, detritus

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INTRODUCTION

The amount of bioavailable energy from organic sources derived from primary production and detritus are critical to the structuring of ecological communities (Elton, 1927; Hutchinson, 1959; Moore et al., 2004). Theoretical models and empirical comparisons of natural gradients in productivity reveal clear lower limits in the levels needed to support producers and consumers (Pimm & Lawton, 1977; Oksanen et al., 1981; Moore et al., 1993; Rosenzweig, 1995). At extremely low levels of input, energetically efficient biota like microbes may form highly diverse consortia that contribute to the formation and degradation of organic substrates (Schink & Stams, 2006; Barton et al, 2007). These energy poor communities may be sensitive to small increases in existing or to introductions in new exotic organic inputs that could potentially invite additional species, supplanting those that are present. Many caves in temperate regions occupy the lower end of the range of carbon inputs seen globally and the communities that reside in them are generally energy starved. While *in situ* energy production from chemolithoautotrophic microbes and autochthonous internal cycling of organic matter occurs within caves (Northup & Lavoie, 2001; Sarbu et al., 1996), in their natural state, most cave communities are supported by organic inputs that originate from outside or allochthonous sources.

Under natural conditions, the mix of external and internal energy sources entering at low rates creates an interesting milieu from which basic ecological principles can be studied that relate energy inputs to diversity and community structure. Previous work in Wind Cave demonstrated that the invertebrate

1. Natural Resource Ecology Laboratory, Colorado State University, Ft. Collins, CO 80524 USA

2. School of Biological Sciences, University of Northern Colorado, Greeley, CO 80639 USA

3. Wind Cave National Park, RR 1 Box 190, Hot Springs, SD 57747 USA

4. Sitka Sound Science Center, PO Box 1373, Sitka, AK 99835, current address

community is largely detritus-based, dependent upon allochthonous inputs of organic carbon (Peck, 1959; Moore et al., 1996; Jesser, 1998; Moore & de Ruiter, 2000). Additional work has shown that the microbial component in a relatively undisturbed, remote region is also detritus dependent (Chelius & Moore, 2004). The phylogenetic affiliations of most of the bacterial DNA sequences retrieved from a remote portion of the cave excluded known chemolithoautotrophs common to systems with abundant sources of reduced chemical energy and carbon dioxide.

Wind Cave, like many large caves, has been modified to entertain upwards of hundreds of thousands of visitors every year. These activities may interfere with the natural state of the cave environment and alter population densities of the resident biota and species composition of the community. The expansion of natural entrances, the building of trails, and the installation of artificial lighting to accommodate tours can significantly alter the energy budgets and climate of the cave. Moreover, the tourists deposit exotic forms of organic matter as clothing lint, hair and dander. These inputs may induce shifts in the community composition of cave microbial communities from their natural configurations to ones that are based on non-endemics and alter the natural chemical and physical regime of the cave (Watson et al., 1997; Ikner et al., 2007). Informed cave management practices can minimize the impacts of exotic substrates but a more intractable consequence of cave development is the significant deposition of dust fall or external chemical inputs. Dust fall (hereafter referred to as lint) in Wind Cave is composed of fine mineral dust, plant matter such as pollen, and organic and textile debris (58% organic and 42% synthetic) shed from humans (Jablonski, 1992; Michie, 1999). Finer lint material may be advected to inner cave areas causing pervasive damage. In Wind Cave, lint input rates are the highest along tour routes and fall markedly beyond these routes. Wind Cave biota are clearly responsive to lint since diversity trends of arthropods and nematodes along tour routes parallel both lint input rates and human activity (Moore et al., 1996; Jesser, 1998; Horton, 2005).

In this paper, we compare the bacterial assemblages between remote and developed sites of Wind Cave to better understand the impact of tourist traffic on cave biota. We also explored the response of a remote microbial community to controlled organic amendments that mimic organic matter inputs associated with tourist traffic and the concomitant expanded access to rodents. This work also raises questions related to the relationship between the sources and quality of organic inputs on microbial community structure. Since previous work has shown that the culturable fraction of bacteria in Wind Cave is not representative of the larger microbial community (Chelius & Moore, 2004), these investigations rely on molecular biological methodologies to capture a meaningful proportion of the microbial community including those not responsive to standard cultivation practices.

MATERIALS AND METHODS

Site Location

Wind Cave, South Dakota (WICA) is one of the world's oldest, longest and most complex caves. It originates from the Madison Limestone Formation where the first passageways developed approximately 320 million years ago, receiving the major portion of its development 10 to 40 million years ago along gypsum deposits and paleokarst zones (Palmer and Palmer 2000). Cave walls consisting of limestone and dolomite bedrock have undergone weathering processes, exposing one of the most extensive displays of a honeycomb pattern of calcite lamina known as 'boxwork.' The cave is a network maze with extensive passageways beneath approximately 1.8 square kilometers of land. The current survey exceeds 166 km, however a recent estimate based on passage density indicates that the cave length could range from 400-1,760 km, (Horrocks & Szukalski, 2002) that would make it the fourth longest cave in the world.

The first recorded biotic survey of WICA was published by Peck (1959) focusing primarily on sediment dwelling arthropods (e.g., insects, Collembola, and mites). Moore et al. (1996) provided a more extensive survey to include estimates of microbial, protozoan, and arthropod densities, and species lists using traditional sampling and culture techniques. Chelius and Moore (2004) used molecular techniques to access the diversity of bacterial and archaeal communities from sediment estimated to have been deposited approximately 300 mybp (Palmer & Palmer, 2000; Horrocks & Szukalski, 2002). The present report focuses on a survey of nine sites initiated in 1998 designed to estimate microbial and faunal community structure and a manipulative study within one of the sites initiated in 2001 aimed at studying the impacts of organic amendments on bacteria populations.

Experimental Design - Survey

In 1998, we identified nine sites along a 1000 m NW-SE transect that followed the Natural Entrance Tour Route to monitor the rate of input of lint and the structure of the microbial and invertebrate communities within the sediments. The sites were arranged in groups of three into three Regions (I-III) positioned in a perpendicular manner at the end points and mid-region of the transect. Region I included the sites (Natural Entrance, Juice Room, and Fairy Palace) at the NW-end of the transect (closest to the natural entrance), Region II included three sites in the mid-range of the transect (~300 m from NW end) off the Post Office Room (Room Draculum - Wet, Room Draculum - Dry, and Rainbow Falls), and Region III included three sites located at the SE-end of the transect farthest from the natural entrance near the elevators (Mammoth Gallery, Nudist Colony, and Buffalo Gap). Sediments from the sites were collected aseptically and analyzed (see below) to characterize their physical and chemical attributes, and sampled at 12 to 15 month intervals (three times) between August 1998 to January 2002 for total lint input

(clothing fibers and hair), and microbial (bacteria and fungi) and invertebrate biomass (protozoa, nematodes, and arthropods). We present our findings for lint and bacteria in this paper.

Sediment Assays

The chemical and physical properties of sediments collected from within each site are presented in Table 1. Five samples of between 25-50 g of sediment were collected aseptically and placed in sterile sealable plastic bags from each of the nine sites (one sediment sample within one meter of each five Petri dish within each site used to estimate lint inputs as described below) for a total of 45 samples. The samples were stored on ice for approximately 24 hours during transport and at 4 °C prior to processing. Sediment pH was measured using 2:1 water extraction (EPA, 1996a). Organic matter content was determined using the Walkley-Black method (Nelson & Summers, 1982). Nitrate-N was measured by cadmium reduction (EPA, 1996b). Phosphorus content was measured using Olsen's method (Olsen & Summers 1982). Potassium content was measured by atomic emission (Knudsen et al, 1982). Percent moisture was determined by measuring gravimetric water content.

Lint Estimation

Five open sterile Petri dishes (standard 100 mm diameter) were placed at set stations within each of the nine sites discussed above. After approximately 1 year (12-15 months) in the cave, the dishes were covered, sealed and returned to the lab for processing. We adapted a line-grid insect technique used to estimate the lengths of roots to estimate the length and mass of lint and hair (Newman, 1966; Giovannetti & Mosse, 1980). The dishes were placed over a grid with 1 cm cells. The bottom of dish and grid are viewed using a stereo-binocular dissecting microscope at a magnification of 10-30x. The total number of natural lint fibers (58% of total fiber count per Jablonski et al., (1994)) and hair (separately) that crossed a grid line are recorded for the entire area of the dish. The total lengths (meters) of lint and hair are estimated using the following formula (Newman, 1966):

$$R = \frac{\pi \times N \times A}{2 \times H}$$

where R is the length of the fiber (m), N is the number of times the fibers intersect a grid line, A is the total area of the dish (m^2), and H is the cumulative length of the transect (m). The dry weights of the natural lint were determined using the length to mass (dry) conversions of 4.5 mg m^{-1} of cotton lint with a carbon content of 24% (Jablonsky et al., 1994). Dry weights of hair were based on averages provided by our own estimates and those of Rutherford and Hawk (1907) assuming a mass conversion of 4.9 ± 0.4 mg m^{-1} and a carbon content of 44%.

Bacterial Density and Biomass Estimation

Methods for enumerating bacteria were modified from Frey et al. (1999) and Bloem et al. (1995). The initial processing of the sediment samples (sampling protocol described under Sediment Assays) was done under a laminar flow hood to minimize aerial contamination, while the serial dilutions and staining were conducted aseptically in a sterile biohazard hood. A 5 g sediment sample was added to 45 ml of filter sterilized, autoclaved, de-ionized water and blended in a Waring blender for one minute. A 1 ml aliquot was immediately added to 9 ml of sterile de-ionized water in a test tube and capped. The test tube was then transferred to a biohazard hood for immediate pipetting of five 10 µl samples onto pre-cleaned, 10-well (6mm) microscope slides. A total of two samples were placed per slide and allowed to air dry. Samples were then stained with DTAF (5-(4,6 dichlorotriazin-2-yl) aminofluorescein) per Bloem et al. (1995), rinsed and allowed to air dry. A drop of immersion oil (type FF) was placed on each well and a cover slip was affixed to each slide. All finished samples were stored at 4° C until direct counts could be made. Bacterial direct counts were made at 1500x magnification using an Olympus confocal microscope with fluorescent capabilities for the survey, and an Olympus microscope with Nemarski optics and fluorescent capabilities for the manipulative study. Bacterial estimates were expressed as number of cells per gram of dry soil. The bacterial cell counts were converted to estimates of bacterial biomass using a conversion of 6.65 x 10^{-13} g bacteria $cell^{-1}$ (Ilic et al. 2001).

Table 1. The chemical and physical characteristics of sediments collected from within the sites used in the survey and manipulative study. Different

Region	Cave location	pH	organic matter(%)	NO_3-N(ppm)	P (ppm)	K (ppm)	Percent moisture
I	natural entrance	7.6 **a**	0.5 **a**	93 **a,b**	9.9 **a**	413 **a**	10.05 **b**
	Juice room	7.5 **a**	0.05 **b**	117 **a**	16.53 **a**	108 **b**	16.64 **a,b**
	Fairy palace	7.7 **a**	*<0.01 **b**	3 **b**	23.69 **a**	28 **c,d**	20.86 **a**
II	Room-draculum-drt	7.3 **a**	0.19 **b**	3 **b**	9.37 **a**	150 **b,c**	11.10 **a,b**
	Room draculum-wet	7.6 **a**	*<0.01 **b**	3.33 **b**	6.34 **a**	41 **b,d**	15.44 **a,b**
	Rainbow falls	7.4 **a**	*0.01 **b**	4 **b**	6.61 **a**	28 **b**	18.08 **a,b**
III	Mammoth gallery	7.5 **a**	*0.01 **b**	2.33 **b**	5.78 **a**	40 **b,d**	11.50 **a,b**
	Buffalo gap	7.3 **a**	0.065 **b**	3 **b**	10.19 **a**	21 **d**	8.66 **b**
	Nudist colony	8.0 **a**	*0.01 **b**	3.67 **b**	9.64 **a**	65 **b,c,d**	17.16 **b**

Experimental Design – Organic Matter Manipulation

The manipulative study consisted of a 2x2 full factorial randomized block design in five blocks. Five 1.21 m^2 plots, representing the blocks, were located in Room Draculum; three in an area of Room Draculum that received drips directly from the ceiling (Room Draculum – Wet), and two in an area that did not receive drips from the ceiling (Room Draculum – Dry). The plots within Room Draculum – Wet did not have drips fall directly on the plots. Each plot was sectioned into four 0.25 m^2 sub-plots separated by a 0.1 m 'no sample zone.' The sub-plots were randomly assigned one of the following treatments: Control (C), Lint (L), Feces (F), and Lint + Feces (LF) (Figure 1).

The Control (C) treatment received no augmentation of organic inputs other than those that occurred naturally. The Lint (L) treatment (1.5 g) was composed of a combination of laboratory grade cellulose powder (0.375 g; 100% Cellulose) to emulate clothing fibers and rodent hair and skin (1.125 g; 42.6% Protein) to represent dander and hair. We initially attempted to use cotton-clothing fibers in our lint to simulate deposits from tourists, but were unable to get the material to separate and spread in a uniform manner over the plots. As a compromise we substituted the clothing fibers with powdered cellulose. The Feces (F) treatment (1.5 g; 77.9% Organic Matter; 42.1% Carbon; 3.8% Nitrogen) was composed of fresh rodent feces that had been collected at monthly intervals during our surveys from along the trail within the first 100 m of Natural Entrance. The Lint + Feces (LF) treatment (1.5 g) included equal amounts of lint and feces. The rodent skins and hair were obtained from discarded laboratory mice used as controls in clinical trials. The hides were removed from mice, freeze-dried, and grated with a cheese grater. The rodent feces were collected from within the cave and stored in a cold room before being dried and grated. The lint and feces were weighed into individual vials for each treatment in the amounts described above and sterilized via gamma-irradiation (nine jolts of $2.5x10^6$ rads). A random sampling of the gamma-irradiated substrates revealed no viable culturable bacteria or fungi. The sub-plots received two pulses of the treatments described above, one in January 2001, and one in March 2002.

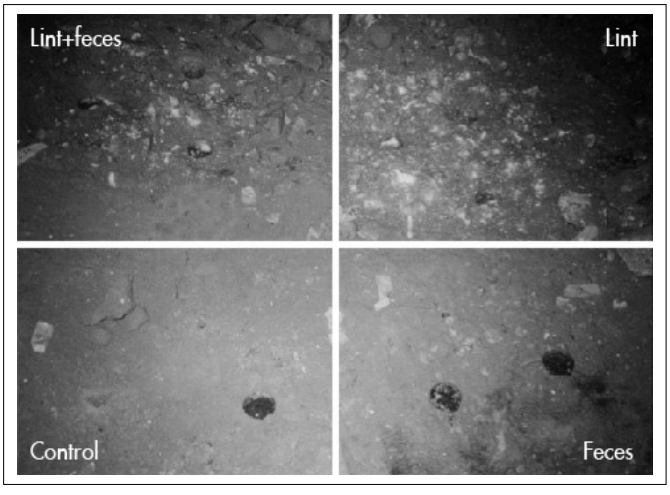

Fig. 1. Photograph of one of the five plots (1.21 m^2) used in the manipulative study. The treatments within the sub-plots (0.25 m^2) are labeled on the photograph - Control (C), Lint (L), Feces (F), and Lint + Feces (LF). The pink flagging tapes mark the corners of the 0.25 m^2 sub-plots. The black discs visible in each sub-plot are small Petri dishes (60 mm) lined with a 9:5 mixture of charcoal and plaster of Paris (Snider et al. 1969) placed to estimate the number of small arthropods (mites, Collembola and Diplura) for the broader study (results not reported here).

In July of 2002, twenty samples including 5 replicates from each of the plots treated with feces (F), lint (L), lint + feces (LF), and untreated controls (C) were aseptically collected in sterile vials and stored under ice for approximately 24 hours during transport. In the laboratory, the samples were divided into two aliquots - one to determine bacterial biomass using the technique described above for the survey and one for microbial DNA extraction and purification described below.

Microbial DNA Extraction and Purification

The samples designated for DNA and extraction and purification were stored at -80 °C prior to processing. DNA in sediment (1.0 g) was extracted and purified using the UltraClean Soil DNA Isolation Kit (MoBio, CA) with the following modification: DNA in the extraction solution was heated for 5 min at 65 °C followed by beating in a vortexer at high speed for 5 min for a total of 3 heat/beat cycles. Three of the 25 DNA extracts contained PCR inhibitors and required a scaled-up procedure of 10 g starting material with a final purification using agarose gel electrophoresis.

PCR Amplification and DNA Sequence analysis

The 16S rDNA of bacterial origin was PCR amplified for 30 cycles using primers 27f and 1492r (Lane, 1991) as previously described (Chelius & Moore, 2004; Chelius & Triplett, 2000), with the exception that the forward primer was labeled with 6-carboxyfluorescein (FAM). The 16S rDNA of archaea was amplified following the procedure of Lueders & Friedrich (2000) using primers 109f and FAM-labeled 912r. Reactions consisted of 50 µl of the following components 1X PCR buffer (Eppendorf), Taq Enhancer (Eppendorf), 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmol each primer and 2.5 U Taq polymerase (Eppendorf).

For a preliminary assessment of archaeal community diversity, clone libraries were generated and DNA was sequenced from two of the four archaeal communities (LF and L). The cloning and DNA sequence analyses were done as previously described (Chelius and Moore, 2004).

Community Profiles

(i) Restriction enzyme digestion. PCR products were digested at 37 °C with restriction enzymes *Rsa* I and *Msp* I (Promega WI). The 50 µl reaction mixture contained: 10 U restriction enzyme, 15 µl PCR product, and 1X restriction enzyme buffer (Promega, WI). Samples were digested in two steps to ensure complete

digestion, the first containing 10 U of enzyme with a 3 hr incubation followed by an additional incubation of approximately 7 hrs with another 10 U of enzyme. Digests were purified with Sephadex G-50 Superfine column to remove salts.

(ii) Capillary electrophoresis. Samples were concentrated by evaporation and dissolved in a volume of water that would result in approximately equivalent signals of fluorescence. Duration of injection time was 60 sec to enhance the signals of larger fragments since the yield of these fragments was reduced compared to smaller fragments when short injection times were used. A cutoff signal level of 50 FU was used and due to the range of the size standard (ROX 500, ABI), only fragments within 35-500 bp were scored. Fragments separated by 0.5 bp or less were scored as one peak.

(iii) Standardizing terminal restriction fragments. Normalization of peak detection threshold and peak area was done according to Kaplan et al. (2001). Briefly, the peak areas of all sample profiles were divided by the sample producing the smallest peak area and multiplied by 580, the minimum detectable peak area with Genescan software threshold of 50 units. Peak areas were then normalized for fragments larger than the new threshold by converting to a percentage of the new total area.

Comparisons of communities

Species designations are not reliable with T-RFLP data given the lack of phylogenetic information in terminal restriction fragments. Therefore, a designation associated with a unique peak identifier is referred to as an operational taxonomic unit (OTU). OTU richness estimators and standard errors of the estimates were calculated using the species prediction and diversity estimation (SPADE) software (Chao & Shen 2003). Incidence-based Coverage Estimator (ICE) is a model based on the assumption that the detection probabilities are heterogeneous among OTUs (Chazdon et al., 1998). Chao2 uses the number of unique and duplicate OTUs to estimate the number of missing species. Both coverage estimators are used to calculate total OTU richness, including those not present in any sample and therefore give richness values that are larger than the observed richness (S_{obs}). ICE and Chao2 are calculated using incidence data only; no OTU abundance information was inferred from relative peak areas.

Compositional comparisons (and standard error estimates) were done using a Jaccard-type estimator in EstimateS 8.0 (Colwell, 2006). This abundance-based Jaccard estimator is a derivation of the classic Jaccard index of similarity using summed incidences as a proxy for relative abundance (Chao et al., 2005). The Jaccard-type estimator reduces sample size bias by including the effect of unseen shared species on similarity estimates.

Statistical Analysis

Data collected for the lint input rates, chemical analyses of sediments and population densities of sediments from the survey and the manipulative study were log-transformed [log(x+1)] to meet assumptions of normality. All data were analyzed using SAS statistics software for analysis of variance (SAS Institute, 2003). Annual lint input and bacterial biomass were analyzed using PROC GLM with room and region as main effects. For the lint and feces manipulative study, we analyzed the data using PROC GLM with lint and feces inputs as main effects. Mean separations were tested using a Bonferroni adjustment with a significance level of $p < 0.05$ unless stated otherwise.

RESULTS

Survey

Figure 2 presents estimates of annual lint input rates (mg C m^{-2} yr^{-1}) and standing bacterial biomass (mg C g^{-1} dry sediment) for the nine sites within the survey, and a summary of the sites by their designated region within the cave.

Lint inputs (minus hair) ranged from 0.17-61.85 mg C m^{-2} yr^{-1} with the highest rates occurring along the tourist path at the Natural Entrance and adjacent to the Natural Entrance and tourist path at the Juice Room (Figure 3a and 3b). Lint input rates at the remaining sites were relatively lower (<2.0 mg C m^{-2} yr^{-1}) and exhibited lower annual variation than those close to the trail. The summary of lint by region across all years revealed significantly ($p \leq 0.05$) high rates of input in Region 1, the region closest to the natural entrance, compared to Region II and Region III. Hair represented a small fraction of total lint inputs, ranging from 0-1.2 mg C m^{-2} yr^{-1} with the highest rates recorded within Region I at the Natural Entrance and the Juice Room. Given the variability and high incidences of zero counts, the summary of hair input rates by site and by region across all years found no significant differences in input rates ($p > 0.10$).

Bacterial biomass exhibited high variation within and among sites and between years (Figure 3c). Bacterial biomass ranged from 0-0.01 mg C g^{-1} dry sediment, with the highest levels of biomass occurring within the Juice Room and Fairy Palace in Region I and Mammoth Gallery in Region III. Higher levels of biomass were recorded for samples collected in 2001 and 2002 compared to 1999. The analysis revealed a significant site by year interactions ($p < 0.01$). The interaction notwithstanding, the summary of the bacteria biomass by region across all years revealed that sediments supported higher levels in Region I, the region closest to the natural entrance, compared to Region II and Region III ($p = 0.09$).

Organic Matter Manipulation Study

Bacterial densities

Bacterial populations responded to feces (F) and lint+feces (LF) amendments. The initial sampling in June 2001 (five months after treatments) revealed that levels of bacterial biomass increased significantly under the F ($p=0.0011$) and LF ($p=0.02$) amendments, but not in the L treatment ($p > 0.10$) when compared to the control (C) (Figure 3). Samples collected in February 2002 (1 year after treatment) revealed no differences between the levels of bacteria biomass

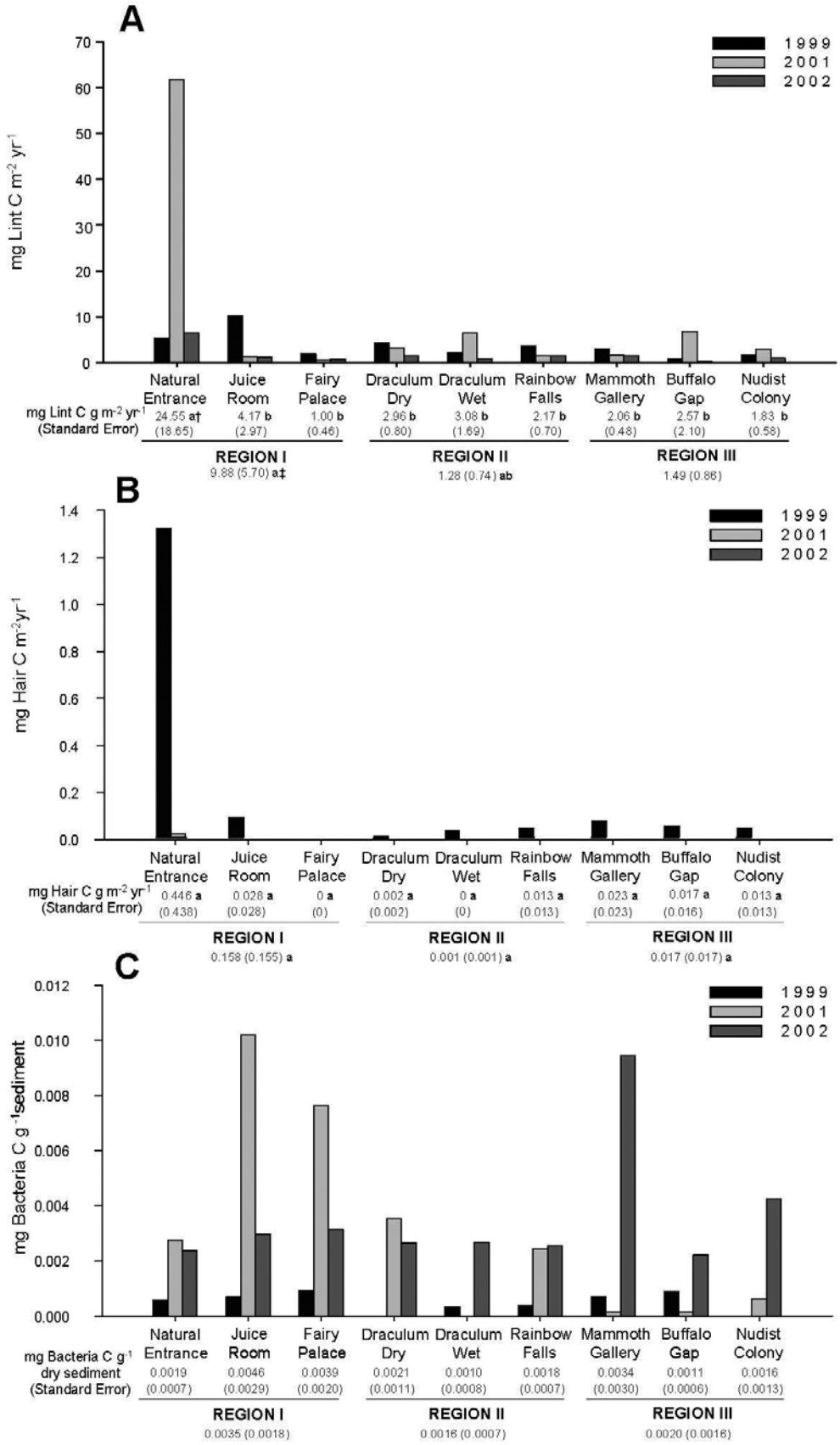

Fig. 2. A) Average annual lint input rates (mg C m^{-2} yr^{-1}) and B) hair (mg C m^{-2} yr^{-1}) by site and region for 1998-2002 (n=5 lint traps per site per year). Traps (Petri dishes) were in the field from 12-14 months. The retrieval dates of the traps were December 1999, January 2001, and January 2002. C) Average Bacterial biomass (mg C g^{-1} dry sediment) estimates by site and region from sediments collected within 1 meter of the lint traps at the time of retrieval. Values for lint, hair, and bacteria under the sites represent the three year averages with standard errors in parentheses. Values for each under the regions represent the averages of the sites within the region with the standard errors in parentheses. Different letters represent significant differences among the means for the site or region averages at the $p \leq \dagger 0.05$ and $p \leq \ddagger 0.10$ levels.

in sediments within the treatment and the control plots ($p > 0.10$). A second pulse of lint and feces were applied in March 2002. Samples from July 2002 revealed that the bacterial biomass within sediments did not differ across any of the treatments compared to the controls ($p > 0.10$).

WICA Archaea

Bacterial DNA was amplified from all of the sediment samples, however over half of the samples yielded no PCR product from archaea and therefore community comparisons were not possible. For the archaea samples that were used to generate fragment profiles, observed richness based on terminal fragments averaged only three OTUs per sample. The clone libraries generated five sequence types in the L and LF communities. Four of these sequences were highly related (98-99%) and had a terminal restriction site at the same location indicating that they would not be distinct fragments in the T-RFLP profiles. DNA sequence comparisons of clones from the two samples showed a high sequence similarity (96-98%) to WICA archaea from another remote location, Rainbow Falls (Chelius & Moore, 2004).

Community comparisons of WICA Bacteria

Trends in OTU richness of WICA bacterial communities within the sediments of the treatment and control plots were similar between estimators, ICE and Chao2 (Figure 4). Observed richness (S_{obs}) was lower than estimators since it only measures the sub-sample with no attempt to infer total richness and is therefore less reliable, especially when coverage is unknown. For both estimators the communities from the C plots were distinguishable from the communities from LF plots. Sediments amended with L and LF supported the most OTUs, but the LF community supported distinctly more OTUs than the C community. This trend was not as convincing in *Rsa* I profiles as compared to profiles generated with *Msp* I (Figure 4b). The strength of this distinction reflects restriction enzyme sensitivity to rare taxa (Engebretson & Moyer, 2003), since *Rsa* I profiles generated fewer unique peaks than *Msp* I profiles (Figure 4). Consequently, conclusions drawn by comparative analyses of WICA communities refer to *Msp* I profiles.

Pairwise compositional similarities support OTU richness findings in that the LF and L amendments shifted the community similarly and that this shift resulted in the fewest shared OTUs with the control community (Figure 5). All comparisons involving communities receiving individual amendments were equally dissimilar, however the LF community shared more OTUs with L community than the F community, suggesting that the richness of the LF community is dominated by the species within L community.

Because high doses of lethal gamma irradiation may not have destroyed all measurable DNA in the organic matter amendments, we subjected the individual L and F amendments to the same analyses as the

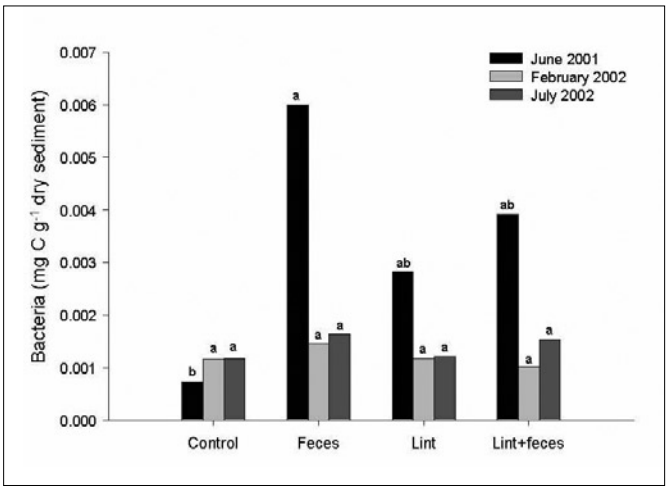

Fig. 3. Bacterial densities (g C g^{-1} dry sediment) collected from Control (C), Lint (L), Feces (F), and Lint + Feces (LF) plots in June 2001, February 2002, and July 2002. Treatments were applied in January 2001 and March of 2002. Different letters represent significant differences among treatments within a sampling date at the $p < 0.05$ level.

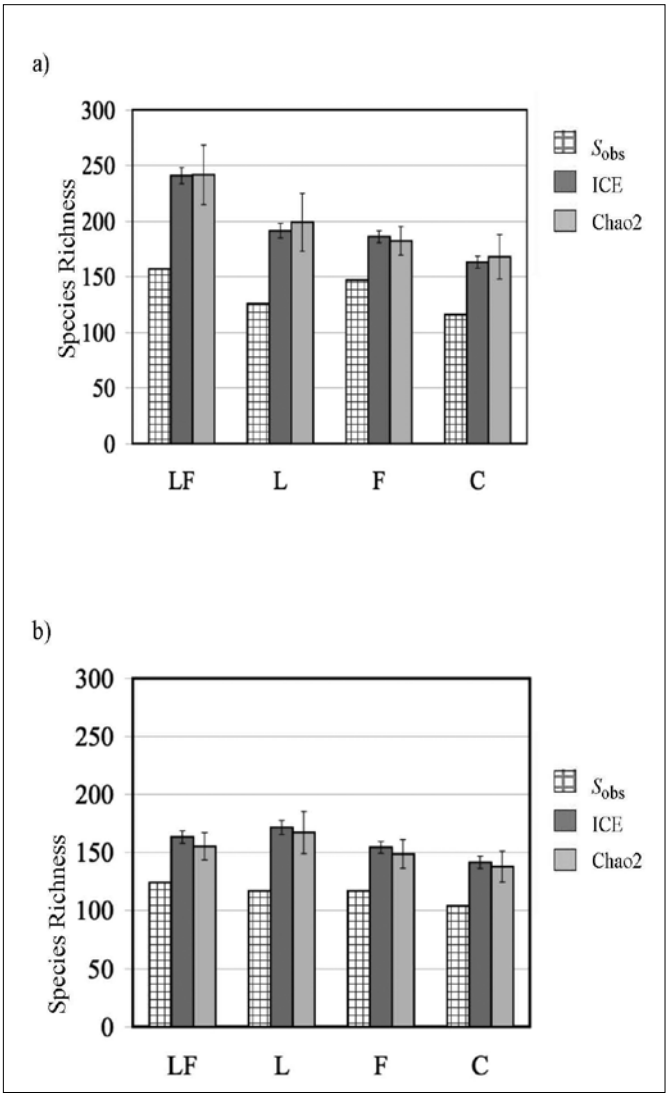

Fig. 4. Observed subsample species richness (Sobs) and operational taxonomic unit (OTU) richness (ICE and Chao2) of communities from amended WICA sediments. 1a, b are profiles generated with restriction enzymes, Msp I and Rsa I respectively. ICE and Chao2, are non-parametric estimators of total OTU richness. The standard errors of these estimates are shown.

sediments from the treated communities. No DNA was detectable in the L amendment. The feces amendment yielded some DNA but significantly fewer fragments than the sediments and revealed a pattern that was distinct from all sampled communities. This result indicates that the nucleic acids from the amendment itself did not contribute significantly to the resulting community profiles.

DISCUSSION

Previous studies from caves have demonstrated that the biodiversity and biomass of the microbial communities within caves are generally low compared to surface habitats (Sket, 1999; Moore & de Ruiter, 2000; Barton et al., 2007). The putative reasons for this pattern include the low rate of inputs and low diversity of the *in situ* and outside organic substrates. Our results are consistent with and inform both explanations, providing insights into the natural history and phylogeny of sub-surface assemblages of bacteria and Archaea, and the ecology of microbial communities in low energy environments.

The results also provided important insights into the role of the rates of inputs and the diversity of inputs on microbial community structure. From a purely natural historical perspective the low rates of production and biodiversity of caves provides an optimal situation for describing a meaningful proportion of the microbial community. This observation is supported by previous work on the microbial diversity of WICA (Chelius & Moore, 2004) and the relatively low OTU richness in Room Draculum sediments as measured by T-RFLP.

This work supports previous findings of low sequence diversity of Archaea in WICA (Chelius and Moore 2004). The majority of all WICA sequences represent a phylogenetically related cluster and lack sufficient sequence diversity for T-RFLP profiling. As a group, the Archaea that we retrieved in this study is largely equivalent to sequence types retrieved from other subsurface environments (Hansel et al., 2008; Shimizu et al., 2006), suggestive of a unique subsurface clade worthy of further investigation.

From an ecological perspective, the survey and manipulative study reported here indicate that organic amendments altered the WICA bacterial community in both richness and structure. The results on bacterial biomass derived from direct counts reported here, coupled with previous reports based on cultural bacteria (Jesser, 1998; Moore et al., 1996; Moore et al., 2006; Horton, 2005) reveal that bacterial biomass was significantly higher in regions that received higher rates of inputs and within sediments that had higher levels of organic matter and nitrogen. Microbial abundance was unresponsive to an additional pulse of organic matter. The reasons for this are unclear, but may result from an accumulation of unfavorable metabolic products after the initial amendment that inhibited further microbial growth. The high degree of variation within and among sites may reflect the heterogeneous spatial patterning in moisture and the deposition of sediments. Nonetheless, our findings are consistent with observations from other low production systems wherein microbial biomass responded to organic amendments. More generally, our findings are also consistent with ecological theory on the importance of productivity as a determinant of trophic structure, particularly at the lower end of the global productivity gradient (Hutchinson, 1959; Pimm & Lawton, 1977; Oksanen et al., 1981; Moore et al., 1993).

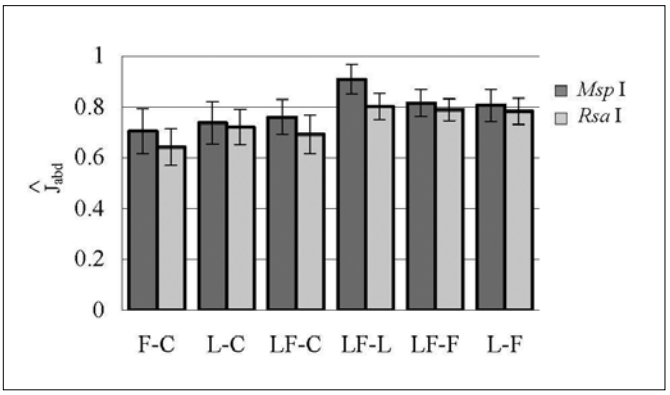

Fig 5. Pairwise-compositional similarities using an abundance-based Jaccard estimator of communities from amended WICA sediments. The standard error of this estimate is shown. Similarities are from profiles generated using restriction enzyme Msp I. Treatment labels are as follows: Control (C), Lint (L), Feces (F) and Lint+Feces (LF).

The manipulative study indicated that the microbial community is both carbon and nitrogen limited, and that it responded to increases in the amounts and diversity of organic substrates. The most significant response was with combined lint and feces (LF) amendment resulting in increased richness of the resident community and shifts to the community composition. The high similarity of the LF and L communities suggests that the lint-only community was the larger component of LF. The carbon amendments are on a C/N gradient with lint having the higher ratio compared to feces. The carbon substrates also differ in moisture content and compositional diversity. Lint is lower in moisture and nitrogen, and diversity of organic carbon compounds, factor(s) that appear to be more favorable to the WICA community. This finding indicates that the cave community is adapted to an existence on the low end of the nutrient and energy spectrum, but even so, is favorably responsive (increased OTU richness) to nutrient amendment.

How these results play against current hypotheses regarding syntrophy and microbial consortia in low carbon environments would require further, more detailed studies (Schink & Stams, 2006; Barton et al., 2007). One possibility is that our observed increase in bacterial diversity reflects a mutualistic response among bacteria as different species provide critical enzymes in the breakdown of the more complex materials (Barton et al., 2007). An alternative explanation is that the more complex substrates offer a greater diversity of resource from which the bacterial community can draw. A separate issue might include the introduction of exotic bacteria through the substrates themselves, high doses of lethal gamma-irradiation and post-irradiation sterility trials revealing no viable culturable forms notwithstanding. In either case our results unequivocally illustrate that the diversity of organic

substrates and more generally productivity and the diversity of production entering a community can alter the indigenous community structure.

Cumulatively, the data reveal that microbial biomass and diversity is responsive to the levels of organic substrate input and diversity. These inputs result in an increase in population densities and a shift in composition of the resident bacterial community. Although this conclusion is intuitively appealing, few studies have attempted to demonstrate it *in situ*, likely due in part to the difficulty in tracking the responses of microbial populations in complex communities. Further work in this cave environment will inform not only cave management practices, including a consideration of mitigation practices that control the input of external organic matter, but also conceptual models of the dynamics and contribution of microbial assemblages to ecosystem functioning.

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