ICS 2009

15TH INTERNATIONAL CONGRESS OF SPELEOLOGY

Proceedings, Volume 1

Symposia Part 1

OUR SPONSORS

National Cave and Karst Research Institute

NATIONAL PARK SERVICE

Volume 1, Symposia, Part 1

Proceedings

15th International Congress of Speleology

Kerrville, Texas
United States of America
July 19–26, 2009

Editor:
William B. White

Cover design:
Beth Fratesi

Layout and design:
Greyhound Press

Library of Congress Control Number 2009930608
ISBN 978-1-879961-33-3

PROCEEDINGS

15TH INTERNATIONAL CONGRESS OF SPELEOLOGY

VOLUME 1

SYMPOSIA, PART 1

Kerrville, Texas

United States of America

July 19–26, 2009

Produced by the organizing Committee of the

15th International Congress of Speleology

Published by the International Union of Speleology

Printed in the United States of America.

2005–2009 BUREAU OF THE INTERNATIONAL UNION OF SPELEOLOGY

President:	Andrew Eavis
General Secretary	Dr. Fadi Nader
Vice Presidents	Dr. Pavel Bosák Dr. Alexander Klimchouk
Adjunct Secretaries	Carlos Benedetto Roman Hapka Dr. Stein-Erik Lauritzen Efrain Mercado Dr. Andrej Mihevc Dr. George Veni Dr. Paul Williams Dr. Kyung Sik Woo

PRODUCTION OF THE PROCEEDINGS

Science Committee	Dr. Louise D. Hose (USA) – Chairman Dr Joaquin Arroyo-Cabrales (Mexico) – Paleontology Dr. Penelope Boston (USA) – Life and Planetary Sciences Dr. James Brady (USA) – Archaeology Dr. Linda El-Dash (Brazil) – Stewardship/Social Sciences Dr. Alexander Klimchouk (Ukraine) – Earth Science/Exploration Dr. Donald McFarlane (UK and USA) – Paleontology/Earth Science Dr. Oana Moldovan (Romania) – Life Sciences Dr. José Palaciós-Vargas (Mexico) – Life Sciences Dr. James Pisarowicz (USA) – Exploration/Social Sciences Dr. Wilfried Rosendahl (Germany) Archaeology Dr. Patricia Seiser (USA) – Stewardship/Social Sciences Dr. Rickard Toomey (USA) – Paleontology/Stewardship Abel Vale (Puerto Rico) – Stewardship/Social Sciences Dr. George Veni (USA) – Earth Science/Archaeology Dr. Paul Williams (New Zealand) – Earth Sciences Dr. Kyung-Sik Woo (Korea) – Earth Sciences
Proceedings Editor	Dr. William B. White (USA)
Production Manager	G. Thomas Rea (USA)

DISCOVERING NEW DIVERSITY IN HAWAIIAN LAVA TUBE MICROBIAL MATS

MATTHEW G. GARCIA[1], MONICA MOYA[1], MICHAEL N. SPILDE[2], FRED D. STONE[3] , DIANA E. NORTHUP[1]
[1]Department of Biology, MSC03 2020, University of New Mexico, Albuquerque, NM 87131
[2]Institute of Meteoritics, MSC03 2050, University of New Mexico, Albuquerque, NM 87131
[3]University of Hawai'i at Hilo, Hilo, HI 96720 and Bishop Museum, Honolulu, HI

Bacterial mats cover walls and ceilings of lava tubes around the world, yet little is known about their composition and role in the ecosystem or what controls their diversity. To address these issues, we ask: (1) What bacterial species are found in the mats? (2) Does diversity vary with respect to the different ages of lava flow? (3) Does species composition differ between differently colored mats? and, (4) What is the amount of organic carbon present in the drip water entering the cave system that can fuel heterotrophic growth?

Rock samples were collected from microbial mats in six different lava tubes found on the Big Island of Hawai`i. Sampled mats ranged in color and included yellow, white, pink, blue-green, and an organic butterscotch ooze. Samples were aseptically collected from each cave, and DNA was extracted and then purified. The 16S rRNA gene was amplified using polymerase chain reaction (PCR) (~1365 bp), cloned, and then later sequenced. From this, closest relatives were found using the Ribosomal Database Project II and BLAST databases, and a parsimony phylogenetic tree was constructed using PAUP. *Actinobacteria* occurred in five of the six lava tubes sampled and were found to dominate where they occurred. *Acidobacteria and Proteobacteria* occurred in all six lava tubes, but which proteobacterial subdivisions were present varied. *Nitrospirae* occurred in four of the six lava tubes sampled. Other closest relatives were found to be *Cyanobacteria, Firmicutes OP11, Chloroflexi, Verrucomicrobia, Gemmatimonadetes, Planctomycetes, Bacteroidetes/Chlorobi* Group, and *Deinococcus-Thermus.*

The different types of morphology we find in these bacterial mats were visualized with a JEOL 5800 scanning electron microscope (SEM) equipped with an energy dispersive x-ray analyzer (EDX). These analyses showed diverse morphologies including some that are similar to *Actinobacteria.* Samples of drip water were collected in the caves that had constant drips from the ceiling for dissolved organic carbon (DOC) analysis using a Shimadzu TOC-5050A Total Organic Carbon Analyzer. DOC values ranged from 4.85 mg/L to 36.95 mg/L; level of DOC did not correspond to level of rainfall or vegetation.

Our studies show a great deal of novel diversity and several of the closest relatives of our sequences come from other cave studies. Overall, the diversity in our Hawaiian lava tube samples spans thirteen phyla of *Bacteria*, revealing a very diverse community in these striking mats.

1. Introduction

Worldwide lava tubes exhibit stunning microbial mats that cover the walls and ceilings with remarkable colors, and patterns. Even though these mats are commonly found in lava tubes, very little is known about them and they have received even less attention than microorganisms in limestone caves (Northup and Welbourn, 1997; Northup et al., 2008). Culture-dependent techniques have been the only methods applied in studying these mats, nicknamed "lava wall slime." Howarth (1981) suggested that slimes are important sites of nutrient recycling (e.g. nitrogen). Ashmole et al. (1992) found slimes present in humid caves in the Canary and Azores Islands, but never in dry caves. Studies done in lava tubes in Washington, USA, have found slime consisting of different species of bacteria, including actinomycetes in the genus *Streptomyces* (Staley and Crawford, 1975). Researchers have since assumed that microbial mats in lava tubes are primarily composed of actinomycetes. Certain types of actinomycetes are medicinally and culturally significant because they excrete antibiotic products to repel invaders (Lazzarini et al., 2000). Antibiotic properties of many bacterial species make them interesting to the medicinal industry, providing a rationale for studying these microbial mats in lava tubes.

Our preliminary studies of a white and a yellow microbial mat in one lava tube in New Mexico, USA, revealed a more diverse microbial community than expected, which included *Actinobacteria, Chloroflexi, Verrucomicrobia, Acidobacteria,* and *Betaproteobacteria*. The greater diversity led us to expand our studies of lava tube microbial mats to investigate the species composition of microbial mats of different colors and from different locations. We are studying mats from lava tubes on the Big Island of Hawai`i by applying culture-independent phylogenetic techniques to uncover the diversity in these mats. Organic carbon present in drip water, composition of rock, and precipitation are some of the abiotic factors being taken into account so that we may further understand what controls the diversity of these microbial communities.

2. Methods

At each site, entrance elevation, GPS coordinates, cave temperature and humidity were recorded using an IMC Digital Thermometer probe. Age of the lava flow and average area rainfall were researched and recorded for later comparison. Small samples of wall rock covered with bacterial mats were collected from the six Hawaiian Island caves under a National Park Service collecting permit or permission of land owners. Samples were covered with sucrose lysis buffer to preserve the DNA and transported to the lab where they were stored in a -80° C freezer until DNA extraction. Yellow, white, and pink microbial mats were sampled, along with a blue-green mineral deposit from six different caves across the Big Island of Hawai`i. On the west side of the island or the "dry" side, we sampled three caves. In the northwestern region samples were collected from Beall's lava tube, a privately owned cave. In the southwestern portion of the island we sampled from Maelstrom and Kula Kai lava tubes, part of the Kipuka Kanohina Cave Preserve (KKCP) founded by the Cave Conservancy of Hawai`i. Both lava tubes in the KKPC are found in the same flow of lava. For the eastern side, the "wet" side of the island we sampled three caves as well. In the central part on the eastern side we sampled Kaumana and Epperson's lava tube. Kaumana lava tube is in Hawai'i Volcanoes National Park and Epperson's lava tube is privately owned. The last site is Bird Park lava tube that is in the southeast part of the island.

DNA was extracted and purified using the MoBio PowerSoil™ DNA Isolation Kit using the manufacturer's protocol (MoBio, Carlsbad, CA). Extracted DNA was amplified with universal bacterial primers 46 forward (5'-GCYTAAYACATGCAAGTCG-3') and 1409 reverse (5'-GTGACGGGCRGTGTGTRCAA-3')(Vesbach, personal communication). Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, Maryland) and were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sent to Washington University Genome Sequencing Facility for sequencing of 96 clones per sample with primers M13F and M13R. Once received, sequences were edited Sequencher 4.8. (Gene Codes, Ann Arbor, Michigan). To check the orientation of our sequences and to convert from antisense to sense OrientationChecker (www.cardiff.ac.uk/biosi/research/biosoft/) was used. Chimeras were detected using the Mallard software (http://www.bioinformatics-toolkit.org/Mallard/). Rarefaction curves were generated using DOTUR (http://schloss.micro.umass.edu/software/dotur.html) to ascertain whether sequencing had detected a comprehensive set of community members (Schloss and Handelsman 2005). Sequences were then classified at the phylum level using RDP classifier (Maidak et al., 2001). Sequences were analyzed using BLAST to identify closest relatives (NCBI; Altschul et al., 1997). Initial alignment was completed with Greengenes (greengenes.lbl.gov/) and manually corrected using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), guided by 16S primary and secondary structure considerations. Parsimony analysis was performed using PAUP (version 4.0b10, distributed by Sinauer; http://paup.csit.fsu.edu/) and bootstrap analyses were conducted on 1000 re-sampled datasets.

Samples of the lava tube wall rock covered with microbial colonies were examined on a JEOL 5800 scanning electron microscope (SEM) equipped with an Oxford (Link) Isis energy dispersive x-ray analyzer (EDX). Rock samples with adherent bacterial colonies were mounted directly on an SEM sample stub while in the cave and then coated by evaporation with Au-Pd in the lab prior to imaging.

3. Results and Discussion

Overall, sequences from the eight lava tubes sampled from Hawai`i fall into thirteen phyla: all divisions of the *Proteobacteria* (except zeta), *Actinobacteria, Acidobacteria, Chloroflexi, Cyanobacteria, Nitrospirae, Verrucomicrobia, Gemmatimonadetes, Planctomycetes, Bacteroidetes/Chlorobi* Group, *Deinococcus-Thermus, OP11,* and *Firmicutes.* Because of space constraints, we will highlight only two microbial mat communities: The yellow microbial mat sample is from Kula Kai lava tube and a blue/green microbial sample is from Maelstrom lava tube. These communities are the focus of this discussion, yellow because it is one of the most dominant colors found in these Hawaiian lava tubes and blue/green because of its rarity.

Closest relatives of clones from a yellow microbial mat in the Kula Kai Caverns resided in four phyla: *Actinobacteria,*

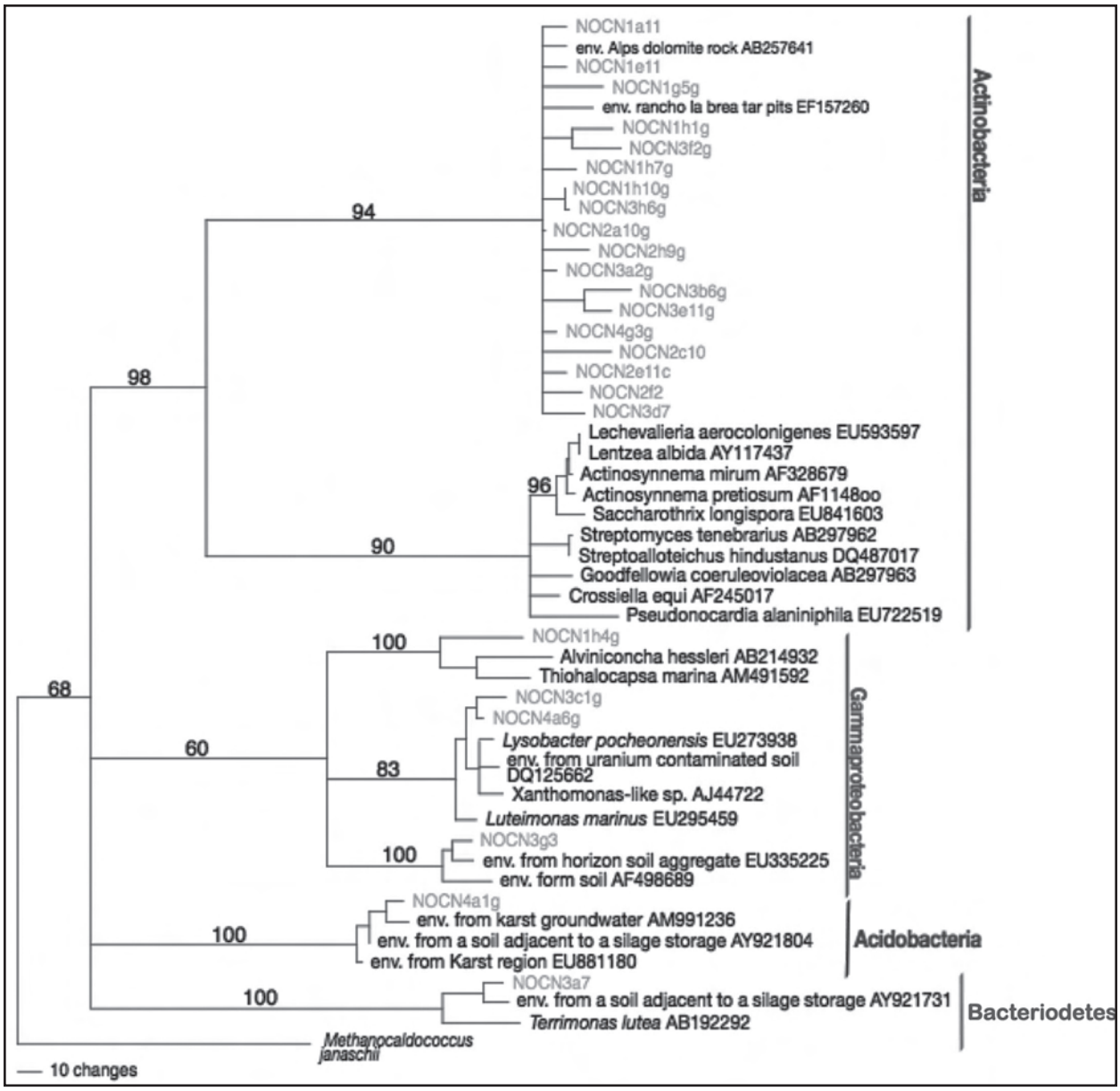

Figure 1*: Parsimony tree of bacterial clone sequences from a yellow microbial mat from Kula Kai Caverns on the big island of Hawai'i. Numbers on the branches indicate bootstrap values from 1000 replicates and indicate the degree of support for relationships in this tree topology.*

Gammaproteobacteria, Acidobacteria, and *Bacteroidetes* (Fig. 1). Kula Kai *Actinobacteria* clones had no close relatives and group with each other and two environmental, uncultured isolates; they did not group with the cultured relatives found in BLAST. This suggests that we may have novel species in our clone library. We found that the closest relatives to our clones were also environmental, which is common in some of our other studies. One of the most interesting relatives in this tree was *Thiohalocapsa marina*, a chemoautotroph in the *Gammaproteobacteria* that fixes CO_2. Other closest relatives in the *Gammaproteobacteria, Acidobacteria,* and *Bacteroidetes* (Fig. 1) were from uncultured soil isolates, karst soils, and groundwater respectively.

The blue/green mineral deposits from Maelstrom lava tube contained nine phyla (Fig. 2), making it one of the most diverse communities we have sampled so far. This tree did not include any sequences from the *Actinobacteria*, one of the major phyla most commonly found in lava tube microbial mats. We found that many of the closest relatives to the sequences recovered from this sample were phylotypes found in other caves, such as Altamira Cave (Spain), Frasassi Cave (Italy), the Oregon cave system (USA), and a Hawaiian (USA) lava tube. Overall, our clones relate almost

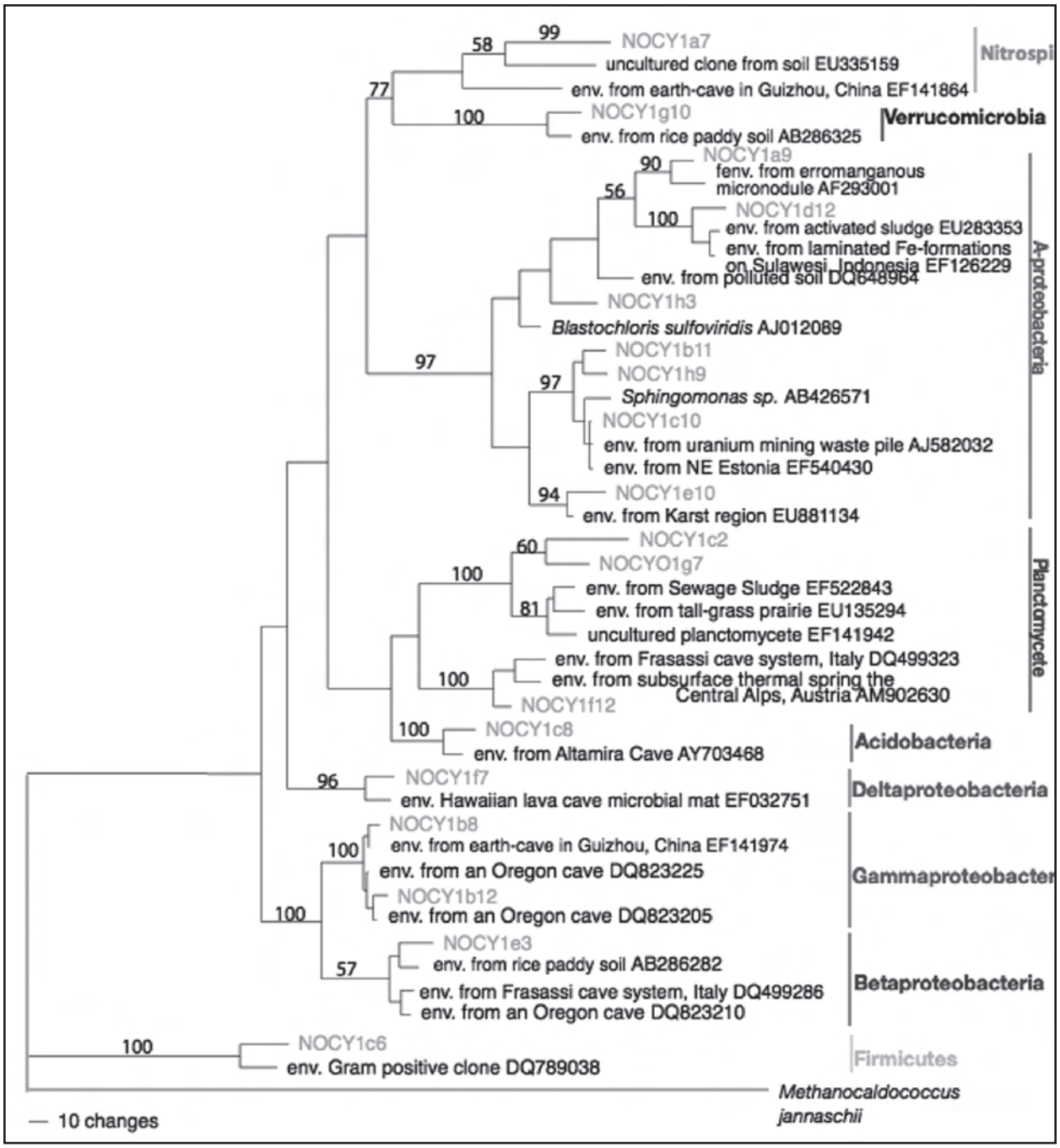

***Figure 2**: Parsimony tree of bacterial clone sequences from blue-green deposits in Maelstrom lava tube on the big island of Hawai`i. Numbers on the branches indicate bootstrap values from 1000 replicates and indicate the degree of support for relationships within this tree topology.*

exclusively to environmental clones in soil, thermal springs, karst regions, and other cave systems.

Scanning electron microscopy (SEM) of Hawaiian lava tube microbial mat samples revealed an array of microbial morphologies. Fig. 3 shows an SEM photomicrograph of the blue/green mineral deposit with some striking features and a variety of morphologies. The SEM revealed a web of filamentous-like structures across the sample, and clearly showed the presence of bacteria. At greater magnification, Figure 4 shows the morphology of the long filamentous structures that resemble reticulated filaments. We have found these reticulated filaments in other carbonate cave samples around the world (Melim et al., 2008). The EDX analysis of the blue/green mineral deposit suggested that this mineral is chrysocolla, a copper mineral.

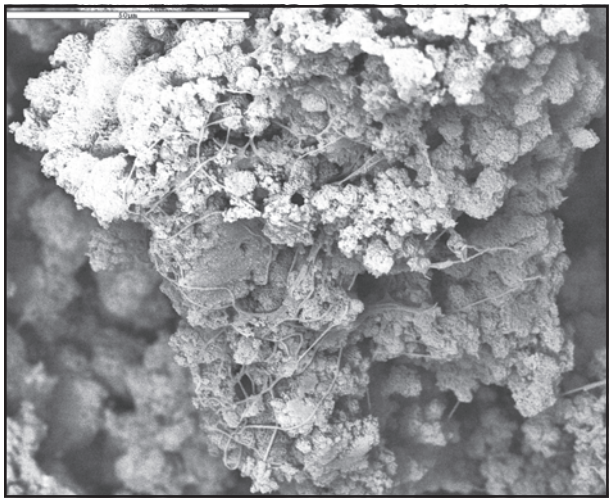

Figure 3*: Scanning electron micrograph of blue-green mineral deposits from Maelstrom lava tube. Scale bar is 50 microns.*

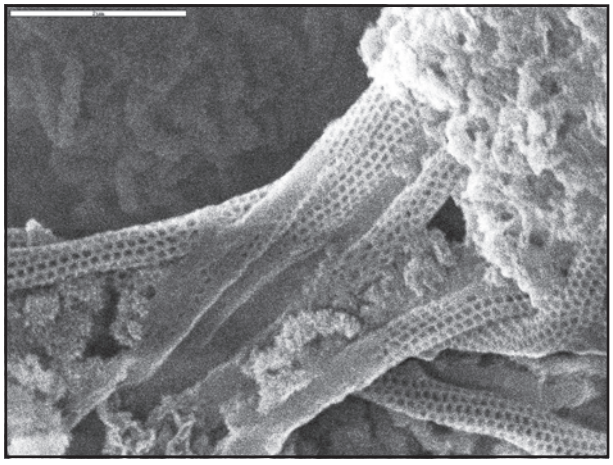

Figure 4*: Close-up of filamentous structures in Figure 3 of blue-green mineral deposits from Maelstrom lava tube. Scale bar is 2 microns.*

A qualitative examination of elevation, precipitation above the lava tube, age of the lava flow, and amount of organic carbon entering the cave in drip water (Table 2), did not reveal any significant trends as to what factors may affect microbial diversity in the mats. The greatest number of phyla were found in the mixed yellow/white mat samples from Bealls Cave and in the blue /green deposits from Maelstrom with 12 and 9 phyla respectively.

4. Conclusions

Our studies in Hawai`i to date reveal diversity that spans 13 phyla of *Bacteria*. The *Actinobacteria* are present in all samples that we have seen to date, except the blue/green mineral deposits, and substantial overlap is observed within the *Proteobacteria* with most of our samples. From our preliminary data we observe no trend between the amount of precipitation and amount of organic carbon present in these cave systems. Also, there is no apparent trend between elevation or age of the lava flow and number of phyla with our current data, although there is a slight trend for older lava tubes to have more phyla present. DOTUR rarefaction curves indicate that more sequencing is needed for current clone libraries and analysis of additional samples will provide more insight into what controls species diversity in these microbial mats. Our preliminary results do indicate that the lava tube microbial mats are rich in diversity and contain a variety of microbial morphologies.

Acknowledgements

We would like to thank Dr. P. Boston, D. Medville, H. Medville, and D. Coons for assistance with field work and sampling. For permits and access to the lava tubes we thank the Cave Conservancy of Hawai`i, Hawaii Volcanoes National Park, and the landowners. J. Craig provided lab assistance for organic carbon analysis of the water samples. We thank Dr. B. Gannon and the UNO students for their comments on the manuscript. We thank K. Ingham for providing photographs of our sample sites. This work would not have been possible without the funding provided by the Cave Conservancy of the Virginias, Dr. J. Cook for the support of the UNO program, and the New Mexico Alliance for Minority Participation Program.

References

Altschul S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman, (1997) Gapped BLAST and PSI-BlAST: a new generation of protein database search programs. *Nucleic Acid Research* **25**, 3389–3402.

Ashmole, N.P., P. Orom, M.J. Ashmole, and J.L. Martin. (1992) Primary faunal succession in volcanic terrain: Lava and cave studies on the Canary Islands. *Biological Journal of the Linnean Society* **46**, 207–234.

Howarth, F.G. (1981) Community structure and niche differentiation in Hawaiian lava tubes. *Island Ecosystems: Biological Organization in Selected Hawaiian Communities* **15**, 318–336.

Lazzarini, A., L. Cavaletti, G. Toppo, and F. Marinelli. (2000) Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **78**, 99–405.

Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T.Parker Jr, P.R. Saxman, R.J. Farris, et al. (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* 29, 173–174.

Melim, L.A., D.E. Northup, M.N. Spilde, B.Jones, P.J. Boston, and R.J. Bixby. (2008) Reticulated Filaments in Cave Pool Speleothems: Microbe or Mineral? *Journal of Cave and Karst Studies* **70,** 135–141.

Northup, D.E., C.A. C onnolly, A. Trent, V.M. Peck, M.N. Spilde, W.C Welbourn, and D.O. Natvig. (2008) The Nature of bacterial communities in Four Windows Cave, El Malpais National Monument, New Mexico, USA. *AMCS Bulletin* **19**, 119–125.

Northup, D.E. and W.C. Welbourn. (1997) Life in the twilight zone: Lava tube ecology. *New Mexico Bureau of Mines & Mineral Resources Bulletin* **156,** 69–82.

Schloss, D.P. 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.

Staley, J.T., and R. Crawford, (1975) The biologist's chamber: lava tube slime. *Cascade Caver* **14**, 20–21.