PERSISTENT COLIFORM CONTAMINATION IN LECHUGUILLA CAVE POOLS
Response: Barton and Pace Discussion

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Barton and Pace (2005) criticized the results of Hunter *et al.* (2004) in terms of (1) the accuracy of our results suggesting the presence of fecal contamination, and in particular, *E. coli*, in pools in Lechuguilla Cave and (2) the assertion that *E. coli* can persist in the environment. In this response we clarify certain methodologies, further address the issue of persistence of *E. coli* in the environment, and present additional tests that confirm the presence of *E. coli* in previous samples from 2004.

The persistence of *E. coli* and other coliforms in natural systems is a complex issue (e.g., Szewzyk *et al*., 2000; Byrd & Colwell, 1993). Since the Hunter *et al*. (2004) manuscript was accepted for publication in the *Journal of Cave and Karst Studies*, we have delved further into the literature of coliform persistence in other environments and conducted additional experiments to try to confirm or rule out our previous results. We discuss those here and recap our previous efforts.

We too have been surprised by the apparent persistence of organisms normally requiring high organic nutrient levels in an oligotrophic cave environment. Thus, we have kept an eye on the Red Lake area for a number of years. While some of our tests have been presumptive, they have consistently shown the presence of coliforms and in some instances *E. coli* over the course of six years from 1999 through our most recent samples taken in December 2004. The persistence of high nutrient-requiring coliforms in a low nutrient cave environment over relatively long periods of time can be explained by three possibilities: 1) a surface source of coliform input is reaching the Red Lake area through fracture percolation, 2) reinfection through human visitation in spite of official closure of the area, or 3) the coliforms are actually persisting in the environment and not dying off as one might initially expect. We believe that to make the case for the first possibility would be difficult. If the cave underlies heavy human or mammal population areas then it might be plausible, but the low annual volume of surface precipitation input (especially severe over the past seven years of drought) and the low mammal and other vertebrate density common to the desert environment above Lechuguilla Cave conspire to make this an implausible scenario. The second possibility of illicit visits is beyond our ability to determine and is a matter for NPS personnel. We like to believe that this is not happening. The third possibility appears to us as the most plausible; that is, that our collective ignorance in the microbiological community about the real lives of organisms in nature means we have a drastically incomplete picture of them. They may well be capable of things that our imaginations have not yet grasped.

To summarize our prior observations, presumptive *E. coli* was detected in Red Lake during the January 1999 trip by Boston much to her surprise (Boston, 1999). Additionally, we assayed for the presumptive presence of *E. coli* and other coliforms in the soils of Huapache Camp near Red Lake (1994–1995), and on the trails leading to Red Lake during a 2000 trip (Northup *et al.*, 1997; Northup *et al.*, 2000). The confirmed *E. coli* contamination present in the soils and small pools adjacent to Red Lake is indicative of fecal contamination (DOH, 2004). These tests were originally conducted because of an outbreak of digestive tract illness in a caving party that had drunk water from a siphon hose and spigot at the Red Lake pool.

In the paper under discussion here, drinking water pools were first tested using the positive/negative TC-5 coliform indicator kits (Hunter *et al.*, 2004). Pools that tested positive were then re-tested for total coliforms using U.S. Geological Survey water sampling protocols in the National Field Manual for the Collection of Water-Quality Data (Webb *et al.*, 1998). Total coliforms were quantified using the membrane filtration technique, an accepted and approved technique by hydrologists and microbiologists for total coliform sampling (Webb *et al.*, 1998). *E. coli* was not tested in drinking water resources during the 2000 or 2001 trips (Hunter *et al.*, 2004, Table 2).

In December of 2004, samples were collected at Red Lake once again. We used clinical Chromagar™ of various types and a series of dilution plates to get a better handle on any presence of coliforms, especially *E. coli* (Alonso *et al*., 1996). Chromagar™ media utilize the principle of species-indicative enzymes. Cleavage of various substrates and reaction with specific dyes yields diagnostically colored colonies. For example, the enzyme ßgalactosidase releases a dye that produces pink

colonies in the presence of *E. coli*, whereas another chromogen is targeted towards ßglucosidase releasing a blue dye that indicates *Enterococcus* species. Such chromogenic media are revolutionizing the screening and detection in water quality and the public health field. The Becton Dickinson Company even claims that no further tests are required for a very high confidence detection rate of many organisms including *E. coli* and enterococci and minimal false positives. However, we did follow up on all putative *E. coli* tests with the spot indole test (Miller & Wright, 1982) and a gram stain. Chromagar™ *E. coli* yielded positives on three of seven samples. Chromagar™ EEC yielded positive *E. coli* tests on the same three samples, and positive for other coliforms on six of the seven samples. Chromagar™ Orientation, which differentiates between coliforms and other typical urinary tract and scar pathogens, yielded four positives for *E. coli*, five positives for *Enterococcus*, and one positive for *Pseudomonas.* Chromagar™ O157 fortunately yielded no positives for this enterohaemorrhagic strain of *E. coli* (Bettelheim, 1998). These were presence/absence tests, not intended to quantify the number of bacteria present in the water column and surfaces. All samples were also grown on EMB (Eosin-Methylene Blue) Agar. The same four samples that had yielded *E. coli* positives on various Chromagar media also developed the characteristic metallic green sheen byproduct of glucose metabolism that interacts with the medium dyes and indicates *E. coli* on many of the colonies. The repeated confirmation of *E. coli* and other coliform presence by a number of different presumptive tests has increased our confidence that we are really seeing them in the Red Lake samples. Based on these results, we have collected sample colonies from these tests for later DNA analysis that is not yet completed.

When testing drinking water, total coliforms are used to determine water treatment adequacy and distribution system integrity (EPA, 2005). The absence of total coliforms minimizes the likelihood that fecal pathogens are present (EPA, 2005). Thus, total coliforms are used to determine the vulnerability of a system to fecal contamination (EPA, 2005). Drinking-water resources sampled during the 2001 trip (noted in Table 2, Hunter *et al*., 2004) are positive for total coliforms and are therefore vulnerable to fecal contamination (EPA, 2005).

The Total Coliform Rule (published 29 June 1989/effective 31 December 1990) set both EPA Maximum Contaminant Level Goals (MCLGs) for health and Maximum Contaminant Levels (MCLs) as legal limits for total coliform levels in drinking water (EPA, 2005). The EPA MCL for coliform bacteria in drinking water is zero (or no) total coliform per 100 ml of water (EPA, 2005). There have been waterborne-disease outbreaks in which researchers have found very low levels of coliforms, suggesting that any level indicates some health risk (EPA, 2005). Given the positive indication of total coliforms using two separate tests (TC-5 Total Coliform, Membrane Filtration) on three different occasions (Hunter *et al.*, 2004, Table 2), park service management recommendations were made to limit access to the water resources in question if the coliforms persisted. Further management recommendations included the following: 1) Quantify the number of coliforms in all pools routinely, 2) identify the major sources of coliforms (i.e. surface infiltration, dirty boots, hands, etc.), 3) measure total and dissolved organic carbon present in pools with/without biofilms, 4) identify dominant species present within siphoning hose biofilms using molecular methods (Hunter, 2001).

The MPN tests carried out by Boston only identified coliforms within Red Lake pool during 1999 (Hunter *et al.*, 2004, Tables 1 and 2). As noted in (Hunter *et al.*, 2004, Tables 1 and 2), "ND" stands for the convention of Not Determined (Hunter *et al.*, 2004).

Regarding the biofilm experiment: The same loop size was used for each "loop-full" of *E. coli* starter culture. Any variability in original numbers of *E. coli* organisms added would have also been reflected in the control (as described in the methods for the coliform growth preference experiment in Hunter *et al*., 2004). Any variance in initial quantity of *E. coli* cells added was also taken into account with the triplicate vials and triplicate plating for each of those vials using 1:100,00 serial dilutions, the results of which were averaged to produce the line graph in (Hunter *et al.*, 2004, Fig. 5 and 6). Colony count data from the *E. coli/Hyphomicrobium*/biofilm experiment were analyzed using the analysis of variance (ANOVA) procedure in the SAS software release 6.12 (SAS Institute, Cary, NC, USA). Signficant differences among treatments were detected ($P = 0.0001$). This experiment emphasized *E. coli* growth preference in medium containing biofilm over growth in medium containing cultured *Hyphomicrobium*-like organisms.

Persistence, as noted in the article title, references total coliform contamination. For purposes of this study, *E. coli* was not specifically tested for during the 2000 and 2001 sampling trips. *E. coli* was used in the lab "coliform growth preference" experiment, however, to determine if *E. coli* had a preference towards biofilm that would potentially be of interest if the positive total coliform results of this study were followed up by more specific *E. coli* field tests in the future. Regardless, the soils and Red Lake water tested prior to the 2000 and 2001 sampling were positive for *E. coli.* Additionally, total coliforms were repeatedly tested and positively identified during that two-year period.

Total coliforms represent a health risk, and if they are still present, then additional testing identifying the source should be done. Precautions should still be taken by those using the above mentioned water resources.

The matter of persistence in the environment of human-associated bacteria including *E. coli* and other coliforms is of both academic and practical management interest. In a seminal paper, Byrd and Colwell (1993) reported *E. coli* persistence over long periods of time (more than three years) in a starved state in artificial seawater while retaining both culturability and the viability of their indigenous plasmids. In another study,

Barcina *et al*. (1997) concluded that besides nutrient scarcity, the most negative factors on survival of allochthonous bacteria (i.e. those introduced from elsewhere) in aquatic surface systems were temperature, osmotic stress, visible light, and grazing by protozoa. We note that caves are thermally quite stable, moist, and dark places without either visible light or particularly ultraviolet radiation sources. We have seen no evidence of significant protist activity in any samples from pool waters (Hobbs, unpublished data), wall rock, or other materials in Lechuguilla Cave. A temperature study of *E. coli* cells starved for carbon and/or nitrogen showed that their temperature optima departed from the usual 37°C (~ human body temperature) and survivability of starved cells was greater at 20°C (Nelson *et al*., 1996). This metabolic downshifting indicates that such cells radically adjust their so-called "normal" behavior to meet environmental exigencies.

Interestingly, protection by biofilm has been reported for *E coli* and other pathogens. For example, Camper *et al*. (1985) have shown colonization of biofilms developed from tap water organisms by *E. coli*. Momba & colleagues (1999) have suggested that biofilms may provide significant protection for introduced pathogens in groundwater systems. We have suggested that the slimy biofilms found on introduced siphon hoses in Red Lake might be helping the survival and recoverability of viable *E. coli* and other coliforms. If this proves to be a factor, then it will have major management implications for how we should obtain water from cave pools without changing their inherent microbiology.

In conclusion, we believe that the accumulated evidence of human-associated coliforms in the Red Lake over the course of a number of years of observation warrants further monitoring. In addition, more refined attempts to determine whether we are seeing unusual persistence in these organisms or the result of subsequent reintroductions by human carriers requires additional investigations.

REFERENCES

Alonso J. L., Amoros, I., Chong, S., and Garelick, H., 1996, Quantitative determination of *Escherichia coli* in water using CHROMagar *E. coli*: Journal of Microbiological Methods, v. 25, p. 309–315.

Barcina, I., Lebaron, P., and VivesRego, J., 1997, Survival of allochthonous bacteria in aquatic systems: A biological approach: FEMS Microbiology Ecology, v. 23, p. 1–9.

Barton, H.A., and Pace, N.R., 2005, Discussion: persistent coliform contamination in Lechuguilla Cave pools: Journal of Cave and Karst Studies, v. 67, p. 55–57.

Bettelheim, K.A., 1998, Reliability of CHROMagar O157 for the detection of enterohaemorrhagic *Escherichia coli* (EHEC) O157 but not EHEC belonging to other serogroups: Journal of Applied Microbiology, v. 85, p. 425–428.

Boston, P.J., 1999, Red Lake contamination. Unpublished technical report to Carlsbad Caverns National Park, Carlsbad, New Mexico, 6 p.

Byrd, J.J., and Colwell, R.R., 1993, Long-term survival and plasmid maintenance of escherichia-coli in marine microcosms: FEMS Microbiology Ecology, v. 12, p. 9–14.

Camper, A.K., Jones, W.L., and Hayes, J.T., 1996, Effect of growth conditions and substratum composition on the persistence of coliforms in mixed-population biofilms: Applied and Environmental Microbiology, v. 62, p. 4014–4018.

DOT (Department of Health), 2004, Coliform bacteria and drinking water. Washington State Department of Health, Publication number 331–181.

EPA (Environmental Protection Agency), 2005. Total coliform rule and potential revisions and distribution system requirements. URL: http://www.epa.gov/safewater/tcr/tcr.html [accessed July 1, 2005].

Hunter, A.J., 2001, Environmental Disturbance of Oligotrophic Bacteria and Effects on Water Quality in Deep Karstic Pools. Unpublished Master's professional project report to University of New Mexico, Albuquerque, New Mexico and Carlsbad Caverns National Park, Carlsbad, New Mexico, 73 p.

Hunter, A.J., Northup, D.E., Dahm, C.N., and Boston, P.J., 2004, Persistent coliform contamination in Lechuguilla Cave pools: Journal of Cave and Karst Studies, v. 66, p. 102–110.

Miller, J.M., and Wright, J.W., 1982, Spot indole test: evaluation of four reagents: Clinical Microbiology, v. 15, p. 589–592.

Momba, M.N.B., Cloete, T.E., Venter, S.N., and Kfir, R., 1999, Examination of the behavior of *Escherichia coli* in biofilms established in laboratory-scale units receiving chlorinated and chloraminated water: Water Research, v. 33, p. 2937–2940.

Nelson, S.M., Attwell, R.W., Dawson, M.M., and Smith, C.A., 1996, The effect of temperature on viability of carbon- and nitrogen-starved *Escherischia coli*: Microbial Ecology, v. 32, p. 11–21.

Northup, D.E., Beck K.M., and Mallory L.M., 1997, Human impact on the microbial communities of Lechuguilla cave: Is protection possible during active exploration?: Journal of Cave and Karst Studies, v. 59, p. 166.

Northup, D.E., Dahm, C.N., Melim, L.H., Spilde, M.N., Crossey, L.J., Lavoie, K.H., Mallory, L.M., Boston, P.J., Cunningham, K.I., and Barns, S.M., 2000, Evidence for geomicrobiological interactions in Guadalupe caves: Journal of Cave and Karst Studies, v. 62, 80–90.

Szewzyk, U., Szewzyk, R., Manz, W., and Schleifer, K.H., 2000, Microbiological safety of drinking water: Annual Review of Microbiology, v. 54, p. 81–127

Webb, W.E., Radtke, D.B., and Iwatsubo, R.T., 1998, Surface-water sampling: Collection methods at flowing-water and still-water sites, *in* Wilde, F.D., Radtke, D.B., Gibs, J., and Iwatsubo, R.T., eds., National field manual for the collection of water-quality data TWRI Book 9: Reston, U.S. Geological Survey, p. 1–38.