# DISCUSSION: PERSISTENT COLIFORM CONTAMINATION IN LECHUGUILLA CAVE POOLS

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Hunter *et al.* (2005) recently reported, based on crude presumptive tests, that *Escherichia coli* is present within many of the drinking pools of Lechuguilla Cave, indicating fecal contamination of these pools by explorers. We believe that these results are misrepresented and do not accurately reflect the presence of fecal contamination within these pools.

The bacterium *E. coli* is a common resident of the intestinal system of most mammals, but is generally not known to persist for more than about 2–3 weeks in the environment (Neidhardt *et al*. 1996). *E. coli* is also easily identified by its ability to grow in the absence of oxygen (referred to as fermentation) on lactose with the production of acid and gas. The source of *E. coli* in the mammalian intestinal tract, its easy identification and rapid loss from the environment allows this bacterium to be used in the rapid identification of fecal contamination incidents *(*Edberg *et al*. 2000). Although the concept of using *E. coli* as an indirect indicator of water quality and health risk is sound, it is complicated in practice. This is due to the presence of other enteric bacteria such as *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics (Holt *et al.* 2000). As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is therefore not a taxonomic classication, but rather a working definition to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas. Consequently, while the presence of coliforms in the environment may be indicative of fecal contamination, it is by no means a confirmation of the presence of *E. coli* or direct evidence of fecal contamination itself. Rather, these tests are considered presumptive and must be followed by additional experiments to either conclusively confirm or rule out the presence of fecal *E. coli* strains (USEPA, 2001).

In their study, Hunter *et al.* used a number of presumptive tests to determine whether the lakes in Lechuguilla Cave were contaminated with fecal coliforms. These tests included the LaMotte [sic] Company TC-5 coliform indicator test, the most probable number (MPN) test and the use of mENDO indicator plates. The TC-5 coliform test is used to identify lactose fermentor species with the production of acid and gas (LaMotte Company, personal communication, 2005). The most probable number (MPN) test contains the detergent lauryl sulfate, which excludes the growth of gram-positive bacteria and false positives by members of the *Lactobacilli, Propionibacteria, Serratia and Streptococci*. Finally, the mENDO agar test is slightly more selective for coliforms, with the addition of deoxycholate to limit the growth of *Proteus* species. Nonetheless, members of the genus *Aeromonas,* which are routinely identified in karstic waters, display an identical growth pattern to *E. coli* on mENDO plates (D. Lye, EPA, personal communication, 2005; Legnani *et al*., 1998). Each of these tests is presumptive: in order to conclusively identify fecal contamination within this water, the presence of thermotolerant *E. coli* must be identified by growth at 44.5°C (Edberg *et al*. 2000; Neidhardt *et al*. 1996). Indeed, standard regulations by the Environmental Protection Agency (EPA) and the American Public Health Association require presumptive tests to be confirmed by the mTEC test for thermotolerant *E. coli* before any statement regarding fecal contamination can be made (USEPA, 2001).

We therefore believe that the results presented by Hunter *et al.* (1995), while indicative of lactose-fermenting bacterial species within the pools of Lechuguilla Cave, do not represent conclusive evidence of fecal contamination; for example, in a recent study on bacterial species isolated from Carlsbad Cavern, Barton and collaborators were able to demonstrate that 23% of isolated species demonstrated sufficient lactose fermentation to produce a false positive coliform test (Barton, unpublished results, 2005). It is interesting to note that the MPN tests carried out by Boston failed to identify coliforms within many of these pools during 1999 (Hunter *et al.* Table 1, we assume 'ND' corresponds to microbiological convention of 'none-detected', although this is not claried in the paper), which may reflect the more selective nature of this test (Hunter *et al.*, 2004).

Following the identification of lactose-fermenting species within the cave pools, Hunter *et al.* proposed that microbial biofilms forming on tubing support the growth and persistence of *E. coli* species. Such a hypothesis, and the data presented in Figure 6, would imply that *E. coli* was selectively enriched by the presence of this biofilm. Given the nature of biofilm structure and formation, this enrichment would suggest that *E. coli* demonstrated either a grazing or predatory nature (Hall-Stoodley *et al*. 2004). This behavior would be the first description of any such activity by this highly characterized organism (Neidhardt *et al.* 1996).

To support their biofilm hypothesis, Hunter *et al.* presented a bacterial growth curve that they propose demonstrates an enrichment of *E. coli* growth in the presence of biofilm material (Hunter *et al.* Figure 6). The investigators clearly state that

these tubes were set up with a "loop-full" of *E. coli* starter culture, rather than a defined number of bacterial colony forming units. While the investigators control against the numbers of organisms at Day 0, there is no accounting for how the variability in the total number of cells added may affect the growth rate. It is known that cell density, access to nutrients and quorum sensing have significant effects on the growth rate of *E. coli*, but the investigators did not control against this variability (Neidhardt *et al.* 1996; Sperandio *et al*. 2001). There is also no indication that the experiment was performed in triplicate (with no error or standard deviation bars on Figure 6) to control against the inherent variability in growth assays. Finally, the experiment was only carried out for six days in a medium that was shown to support the growth of *E. coli,* preventing any conclusions from being drawn regarding persistence. This makes it impossible to conclude from the data presented in Figure 6 whether the biofilm material is directly responsible for *E. coli* growth and long-term persistence.

It is the transient nature of *E. coli* in the environment that makes it such an ideal indicator organism of fecal contamination (Edberg *et al*. 2000; Neidhardt *et al.* 1996; Sperandio *et al*. 2001). The only exception to this rule is in highly organic-rich tropical soils and effluent pools associated with animal farming *(Carrillo et al. 1985; Rahn et al*. 1997). Numerous research groups have attempted to identify conditions that would promote *E. coli* long-term survival in low-nutrient conditions without success, while other investigators have suggested that *E. coli* may survive extended starvation by entering the viable but non-culturable state (Bogosian *et al*. 1996; Carrillo *et al.* 1985). However, to date it has not been possible to demonstrate the persistence of this organism within the environment or entry into the VBNC state (Bogosian *et al*. 1998; Bogosian *et al*. 1996). In conclusion, we believe that the work presented by Hunter *et al.* does not provide sufficient evidence to conclude that there is fecal contamination within the pools of Lechuguilla Cave, or that this paper demonstrates a dramatic shift in our understanding of the natural history and ecology of *E. coli*.

The work carried out by Hunter *et al.* should be commended for its goal toward understanding the impact that human activity has on pristine cave environments. Their demonstration that certain tubing is inappropriate for long-term storage within the cave and should be replaced by non-biogenic formulations is an important step toward minimizing impact during exploration. Nonetheless, with new microbial species and phylotypes being identified on a regular basis in cave environments, we should be careful when using tests that have been developed for chemically defined surface environments to analyze microbial communities within caves (Barns & Nierzwicki-Bauer 1997; Barton *et al*. 2004; Chelius & Moore 2004; Kuznetsov *et al*. 1979; Northup *et al*. 2003; Pedersen 2000; Sarbu *et al*. 1994).

Finally, while it is important to monitor and limit human impact in cave environments, our understanding of the structure and potentially unique microbial habitats that these caves represent is still solely dependent on the initial exploration and description by speleologists; there remains a delicate balance between the appropriate techniques to safely map and explore cave environments and the needs of minimal impact to conserve them. As microbiologists, with a much deeper understanding of the intricacies of microbial growth and metabolic activity, it is essential that we take a great deal of care when providing material to cavers and land managers who may not be able to objectively critique microbiological data. Such care is especially important, given that resultant management decisions could have profound impacts on our abilities as microbiologists to identify and understand microbially-important cave environments in the future.

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