OBSERVATIONS ON THE BIODIVERSITY OF SULFIDIC KARST HABITATS

ANNETTE SUMMERS ENGEL

Department of Geology and Geophysics, Louisiana State University, Baton Rouge, LA 70803 USA, aengel@geol.lsu.edu

Abstract: Recognition of the metabolic process of chemosynthesis has recently overthrown the ecological dogma that all life on earth is dependent on sunlight. In complete darkness, complex ecosystems can be sustained by the energy and nutrients provided by chemosynthetic microorganisms. Many of these chemosynthetically-based ecosystems result from microbial manipulation of energy-rich sulfur compounds that can be found in high concentrations in groundwater. Subsurface environments in general can be highly stressful habitats (i.e., darkness, limited food, etc.), but in the case of sulfidic groundwater habitats, organisms must also tolerate and adapt to different stresses (e.g., toxic levels of gases or lethally low oxygen concentrations). Nevertheless, these habitats, and specifically cave and karst aquifers, have a richly diverse fauna. This review focuses on the biodiversity (as the number and types of species) of sulfur-based cave and karst aquifer systems. The relationships among ecosystem productivity, biodiversity, and habitat and ecosystem stresses are explored. The relatively high numbers of species and complex trophic levels could be attributed to the rich and plentiful, chemosynthetically-produced food source that has permitted organisms to survive in and to adapt to harsh habitat conditions. The geologic age and the hydrological and geochemical stability of the cave and karst aquifer systems may have also influenced the types of ecosystems observed. However, similar to non-sulfidic karst systems, more descriptions of the functional roles of karst aquifer microbes and macroscopic organisms are needed. As subterranean ecosystems are becoming increasingly more impacted by environmental and anthropogenic pressures, this review and the questions raised within it will lead to an improved understanding of the vulnerability, management, and sustainability challenges facing these unique ecosystems.

INTRODUCTION

Caves represent discontinuous continental subsurface habitats that are characterized by complete darkness, nearly constant air and water temperatures, relative humidity near saturation, and generally a poor supply of nutrients. Excluding climatic fluctuations that could bring thermally- or chemically-contrasting air or water into a cave's interior, the physical arrangements and constraints of most subterranean habitats have remained relatively unchanged for thousands, if not millions, of years (e.g., Gale, 1992). For most people who have sat in the sunless silence of a cave, the concept that life could flourish in such conditions for even a short period of time is profound. Indeed, colonizing the subsurface requires specific adaptations to the stresses of living in darkness and to the extreme environmental conditions encountered, such as nutrient and energy limitations, the possibility of experiencing oxygen deprivation, high-water pressures due to living at deep aquifer depths, or geochemically variable solutions. Recently, studies have focused on the metabolic and evolutionary mechanisms that address the survival of subsurface- or cave-adapted faunas (e.g., Jones et al., 1992; Howarth, 1993; Hervant et al., 1999a; Porter and Crandall, 2003; Hervant and Malard, 2005; Hüppop, 2005;

Lefébure et al., 2006). Due to such specialized adaptations, many species of obligate subsurface troglobites (living in terrestrial habitats) or stygobites (living in aquatic habitats) have high degrees of endemism (Barr, 1967; Culver et al., 2003).

The paucity of a continuous nutrient supply is one of the critical extreme conditions affecting subsurface-adapted fauna, as most are quite dependent on the flux of nutrients and energy from the surface, specifically from photosynthetically-produced organic matter. Often, this material comes in the form of wind-blown, meteoric-, and stream-derived detritus (e.g., particulate matter like leaves or woody debris, or as dissolved organic carbon), or from bat and other animal guano (Barr, 1967; Culver, 1976; Brown et al., 1994; Poulson and Lavoie, 2000; Gibert and Deharveng, 2002; Simon et al., 2003; Hüppop, 2005). Consequently, organisms reliant on the transport of easily-degraded organic matter may experience prolonged periods of starvation. Numerous studies have shown that increased feeding efficiency, lower metabolic rates, slower growth rates, and reduced fecundity are linked to nutritional stress (e.g., Hervant et al., 1999b; Hüppop, 1985, 2005). However, a growing body of evidence reveals that some subsurface and cave ecosystems do not rely exclusively on surface-derived

organic matter (e.g., Stevens, 1997; Krumholz, 2000; Amend and Teske, 2005).

In the absence of light, reactive rock surfaces and mineral-rich groundwater provide a wide assortment of potential energy sources that microbial *chemolithoautotrophs* (translated loosely as rock-eating self-feeder) can use to gain cellular energy while making organic carbon molecules from inorganic carbon (i.e. CO_2, HCO_3^-). Chemolithoautotrophs are distinguished from photosynthetic organisms based on whether the energy source is from inorganic chemicals (*litho-*) or from light (*photo-*). Conversely, *heterotrophs* use organic carbon for cellular energy and as a carbon source, and *chemoorganotrophs* use organic compounds for a carbon source and obtain cellular energy from chemical transformations. Chemolithoautotrophs are important to global biogeochemical cycles and ecosystem-level processes because they can cycle various elements simultaneously while generating considerable amounts of organic carbon and serving as the base of ecosystem food webs. Some researchers have hypothesized that subsurface chemolithoautotrophic primary productivity may surpass the activity of photosynthetic organisms on the Earth's surface (e.g., Stevens, 1997).

Prior to the 25th anniversary issue of the Bulletin of the National Speleological Society in 1966, and in the years that followed, the concept that chemosynthesis could sustain subsurface ecosystems was not commonly accepted (nor understood), as chemolithoautotrophic activity was considered insufficient to support ecosystem-level processes (e.g., Schreiber, 1929; Wolters and Schwartz, 1956; Barr, 1966, 1967; Caumartin, 1963; Poulson and White, 1969; Ginet and Decou, 1977). The discovery of chemolithoautotrophically-based ecosystems at the deep-sea hydrothermal vents in the late 1970s (e.g., Jannasch, 1985; Deming and Baross, 1993) toppled the dogma that all life on earth was dependent on sunlight. In 1986, another important breakthrough further changed perceptions of life in the continental subsurface, and of cave ecosystems in general; that discovery was the uniquely diverse chemolithoautotrophically-based ecosystem from the hydrogen sulfide-rich (*sulfidic*) groundwater associated with the Movile Cave, Romania (Sarbu, 1990; Sarbu et al., 1996).

Sulfur, as the 14th most abundant element in the Earth's crust, is biogeochemically important because proteins and other cellular components of all life are comprised of at least 0.5–1% sulfur by dry weight (Zehnder and Zinder, 1980). Nearly all organisms get their required sulfur either from consuming organic sulfur compounds or from assimilatory sulfate reduction. Sulfur exists in a variety of valence states, from the most reduced form as hydrogen sulfide (H_2S) to the most oxidized form as sulfate (SO_4^{2-}). Changes in valency are attributed to the geochemically reactive nature of the various sulfur compounds (e.g., Millero et al., 1987;

Megonigal et al., 2005), and prokaryotes (from the domains *Bacteria* and *Archaea*) can gain energy by transforming one valence state to another. Many of the transformations within the sulfur cycle are catalyzed almost exclusively by microorganisms, and biological sulfur cycling must be tightly coupled with oxidation-reduction (redox) reactions to out-compete the abiotic reactions (for a review, see Megonigal et al., 2005). The relationship between the metabolic requirements for sulfur and oxygen (O_2) causes many sulfur-dependent microbes to occupy interface, or gradient, habitats with a range of O_2 concentrations from highly-oxygenated (*aerobic*) to O_2-deprived (*anaerobic*).

Chemolithoautotrophic ecosystems have been identified from marine sediments (e.g., D'Hondt et al., 2002; Amend and Teske, 2005), continental aquifers (e.g., Stevens and McKinley, 1995; Stevens, 1997; Amend and Teske, 2005), and other caves and karst settings (e.g., Pohlman et al., 1997; Vlasceanu et al., 2000; Engel et al., 2004a). In some deep, isolated continental aquifers, chemolithoautotrophic methanogenic microbial communities are supported by the geochemical production of molecular hydrogen (H_2) (Stevens and McKinley, 1995; Amend and Teske, 2005). No higher trophic levels, including microscopic eukaryotes, have been reported to date from these microbial ecosystems; this starkly contrasts with the trophic diversity found at the deep-sea vents and from sulfidic karst systems where sulfur compounds are exploited by chemolithoautotrophs (e.g., Jannasch, 1985; Sarbu et al., 1996; Engel, 2005).

Here I explore the biodiversity of sulfidic cave and karst ecosystems. The motivation for this review was to evaluate the relationships among ecosystem productivity, biodiversity (as the number and types of species), and habitat and ecosystem stresses with respect to ecosystem stability. Of the known locations for sulfidic karst (Fig. 1), there is generally a clumped distribution of systems in North America and Europe. This could relate to the abundance of (bio)speleologists on these continents, but also to the geologic and hydrostratigraphic history of the karst. It is likely that more sulfidic karst systems are distributed worldwide; as such, considerable adventures await. This review concludes with a perspective on the directions of future work.

ORIGIN OF SULFIDIC CAVE AND KARST SYSTEMS

The classic speleogenesis model invokes carbonic acid dissolution of carbonate rocks, usually at shallow depths and rarely far below the water table (e.g., Palmer, 1991). The alternative karstification process of sulfuric acid speleogenesis was initially proposed by S.J. Egemeier from work in Lower Kane Cave, Wyoming (Egemeier, 1981), where groundwater bearing dissolved sulfide discharges as springs into the cave passage. Hydrogen sulfide gas

Figure 1. Approximate locations for sulfidic caves and karst aquifers reported in the literature. Some of the sites are discussed in detail herein.
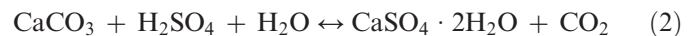
volatilizes from the groundwater to the cave atmosphere[1] and is oxidized to sulfuric acid on moist subaerial surfaces:

$$H_2S + 2O_2 \leftrightarrow H_2SO_4 \qquad (1)$$

The acid reacts with and replaces carbonate with gypsum

$(CaSO_4 \cdot 2H_2O)$,

$$CaCO_3 + H_2SO_4 + H_2O \leftrightarrow CaSO_4 \cdot 2H_2O + CO_2 \qquad (2)$$

This speleogenetic process has been suggested to explain the formational history of active cave systems globally (Hubbard et al., 1990; Sarbu et al., 1996; Galdenzi and Sarbu, 2000; Hose et al., 2000; Sarbu et al., 2000), ancient caves like Carlsbad Cavern, New Mexico (Hill, 1996; Polyak and Provincio, 2001), and some continental karst aquifers at or just below the water table (Hill, 1990, 1995; Schindel et al., 2000). Lowe and Gunn (1995) suggest that sulfuric acid may be important for all nascent subsurface carbonate porosity generation, and Palmer (1991, 1995) further speculates that sulfuric acid speleogenesis is more important for the evolution of carbonate-hosted petroleum reservoirs than it is for the origin of caves, as the process has been linked to the karstification of reservoirs, e.g., the Lisburne field in Prudhoe Bay, Alaska (Jameson, 1994; Hill, 1995).

Various biological, geologic, and hydrostratigraphic parameters generate H_2S. As all life generates small amounts of H_2S from the breakdown of sulfur-containing organic compounds (e.g., proteins), H_2S is produced during the decay and decomposition of organic matter, such as in swamps. Microbial reduction of sulfate-bearing minerals, such as gypsum, or dissolved sulfate in marine or fresh water generates H_2S (see discussion below). Microbial

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[1] **A Safety Note:** Cave explorers and researchers working in active sulfidic caves are exposed to harsh conditions, including toxic gases and the possibility of reduced oxygen levels. Hydrogen sulfide is a colorless flammable gas that can cause headaches, dizziness, nausea, and irritability with prolonged, low-level exposure. The rotten eggs odor (detectable to 0.5 ppbv in air) is not a good indicator of the atmospheric concentration; exposure dulls the sense of smell. At higher exposure levels, this desensitization can lead to coma and death. Above 20 ppmv, H_2S causes eye and mucous membrane irritation, and pulmonary edema in few cases. In some caves, concentrations exceeding 100 ppmv have been reported (e.g., Hose et al., 2000). It is recommended that cave air be monitored for H_2S and oxygen, as well as other gases (CH_4, CO) using a multigas monitor (e.g., PhD Ultra Atmospheric Monitor, Biosystems, Middleton, CT) at all times while working in active sulfidic caves. Although the concentration of H_2S may be less than both the OSHA and NIOSH short term exposure limit (STEL) of 10 ppmv for 10 min, acute irritation is possible. Level-C respiratory protection, such as a half-face air-purifying gas mask with organic/acid vapor cartridges (H_2S escape), should also be worn. Such masks are effective for SO_2, organosulfur gases, and radon, but have only short term protection against high H_2S. At high levels, a full-face mask should be used to protect the eyes and facial mucous membranes. Cartridges should be changed regularly when working in sulfidic conditions. H_2S gas negatively affects the sensitivity of oxygen sensors, and any air monitoring device should be checked periodically. Ambient air contains approx. 20.8% oxygen; under no circumstances should anyone enter a cave or passage when oxygen concentrations are <19.5% unless they have supplied oxygen available to them. According to OSHA, physical work at oxygen levels <19.5%, even with no toxic gases, is impaired due to reduced coordination, dizziness, irritability, and possibly poor circulation. At oxygen levels <10%, vomiting, mental failure, and unconsciousness occur. Concentrations <6% for 8 min can cause respiratory failure and death.

sulfate reduction is commonly associated with petroleum reservoirs, and basinal brine solutions naturally associated with petroleum often have high concentrations of H_2S; the gas will migrate updip from reservoirs and dissolve into groundwater. Stable sulfur isotope ratio analysis has established that the source of sulfide for many cave and aquifer systems can be attributed to microbial sulfate reduction (e.g., Rye et al., 1981; Stoessell et al., 1993; Hill, 1996). When karst is proximal to volcanic terranes, volcanism gives off H_2S and other gases. Groundwater discharging as geysers, hot springs, or underwater vents will often have high dissolved sulfide content. For example, the source of H_2S and other gases was evaluated by analyzing the N_2/He and He/Ar content and He isotopes of the springs discharging into Cueva de Villa Luz (also known as Cueva de las Sardinas), Mexico (Spilde et al., 2004). The dissolved gases were found to have an upper mantle origin that could be related to El Chichón volcano ~ 50 km to the west of the cave system (Spilde et al., 2004).

MICROBIAL DIVERSITY WITHIN THE SULFUR CYCLE

Some of the earliest microbiological research regarding chemolithoautotrophic metabolism was done in the late 1880s with sulfur bacteria from sulfidic springs (e.g., Winogradsky, 1887). Much later, the microbiology of sulfidic caves was observational and predominately involved microscopy and culturing (e.g., Caumartin, 1963; Symk and Drzal, 1964; Hubbard et al., 1986, 1990; Thompson and Olson, 1988; Grubbs, 1991; Stoessell et al., 1993; Brigmon et al., 1994; Sasowsky and Palmer, 1994; Mattison et al., 1998; Ulrich et al., 1998; Humphreys, 1999; Latella et al., 1999b). Because cell morphology does not accurately determine species identity, and because most microbes in nature have not been grown in laboratory cultures, especially autotrophs (it has been estimated that $<1\%$ of known microbes are culturable; Amann et al., 1995), researchers have turned to genetic studies (culture-independent methods; Amann et al., 1990; Amann et al., 1995) involving the characterization and comparison of (predominately) 16S rRNA gene sequences and their evolutionary relationships. Recently, Barton (2006) summarized some culture-independent genetic methods that have been used to describe microbes from caves. Moreover, to understand the microbial metabolic pathways and the consequences of microbial metabolism on ecosystem function, stable and radiolabelled isotope ratio analyses of the habitat (water, rocks, air, etc.) and the microbial biomass have been done (e.g., Langecker et al., 1996; Sarbu et al., 1996; Airoldi et al., 1997; Pohlman et al., 1997; Humphreys, 1999; Porter, 1999; Vlasceanu et al., 2000; Engel et al. 2004a; Hutchens et al. 2004).

The use of genetic methods has significantly expanded our knowledge of the microbial diversity in active sulfidic cave and karst systems (Vlasceanu et al., 1997; Angert et al., 1998; Vlasceanu et al., 2000; Engel et al., 2001; Holmes et al., 2001; Brigmon et al., 2003; Engel et al., 2003a; Engel et al., 2004a; Hutchens et al., 2004; Barton and Luiszer, 2005; Herbert et al., 2005; Meisinger et al., 2005; Macalady et al., 2006). Evaluation of 16S rRNA gene sequences retrieved from microbial mats from active sulfidic karst systems reveal a diverse range of microorganisms. Available 16S rRNA gene sequences were compiled from various sources and public databases (e.g., GenBank <http://www.ncbi.nih.gov/>); this file consists of 345 partial and full-length sequences (as of May 2006) and is provided as supplemental data for future analytical work <http://geol.lsu.edu/Faculty/Engel/geomicrobiology_publications.htm>. A simple comparison of the available sequences indicates that members of the *Bacteriodetes*/*Chlorobi* and *Proteobacteria* phyla, and especially bacteria associated with the gamma and epsilonproteobacterial classes, have been identified from all of the studied, active sulfidic caves (Table 1). It is noted, however, that none of the caves have been exhaustively sampled to verify that a microbial group is truly absent from an ecosystem. Moreover, the simple retrieval of gene sequences from a particular habitat does not necessarily mean that those microbes are active in a community. Similarly, metabolic function of uncultured microorganisms is only cautiously assumed from close genetic affiliation to cultured organisms.

To place the microorganisms that have been identified from sulfidic caves and karst systems into the context of the sulfur cycle, an overview of the metabolic diversity of organisms follows. It is not my intention to exhaustively cover each sulfur cycle transformation pathway here and the reader is guided to excellent recent reviews for more information (e.g., Amend et al., 2004; Brimblecombe, 2005; Canfield et al., 2005; Megonigal et al., 2005). Figure 2 illustrates the sulfur cycle in the context of other elemental cycles, including the carbon, nitrogen, and oxygen cycles.

SULFUR OXIDATION

Despite the fact that high concentrations of reduced sulfur compounds, like H_2S gas or elemental sulfur (S^0), are toxic to most organisms (e.g., Somero et al. 1989; Megonigal et al., 2005), these compounds serve as electron donors for microbial metabolism, such as in H_2S oxidation. O_2 is the electron acceptor in this reaction:

$$H_2S + 2O_2 \leftrightarrow SO_4^{2-} + 2H^+ \qquad (3)$$
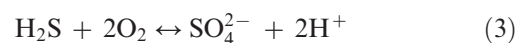
For the purposes of this review, any microbe capable of oxidizing any reduced sulfur compound will be generally referred to as a sulfur-oxidizer. For a vast majority of the sulfur-oxidizing microbes, sulfate is the end product (e.g., Canfield et al. 2005). For others, intermediate products may form, like sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$) (Equation 4), tetrathionate ($S_4O_6^{2-}$), and S^0 as intra- or

Table 1. Major affiliations for microbial communities found in sulfidic cave or karst systems.

Major Taxonomic Affiliation	Movile Cave (Romania)[a]	Frasassi Caves (Italy)[b]	Lower Kane Cave (Wyoming)[c]	Parker's Cave (Kentucky)[d]	Big Sulphur Cave (Kentucky)[e]	Cesspool Cave (Virginia)[f]	Glenwood Springs (Colorado)[g]
Bacteria							
Acidobacteria	•	•	•				
Actinobacteria			•				•
Bacteroidetes/Chlorobi	•	•	•	•	•	•	•
Chloroflexi		•	•				•
Deferribacteres		•					
Fibrobacter			•				
Firmicutes/ low G+C	•	•					•
Flexistipes							•
Nitrospirae	•		•				•
Proteobacteria			•				
Alphaproteobacteria	•		•				
Betaproteobacteria	•	•	•		•	•	
Deltaproteobacteria	•	•	•			•	•
Gammaproteobacteria	•	•	•	•	•	•	•
Epsilonproteobacteria	•	•	•	•	•	•	•
Planctomycetes	•		•		•		
Spirochaetes			•				
Termite Gut 1		•					
Verrucomicrobium	•	•	•				
Candidate Divisions	•	•	•	•			•
Archaea							
Euryarchaeota							
Thermoplasmata							•
Methanomicrobia	•	•	•				
Fungi	•				•		

[a] 16S rRNA gene sequences from Vlasceanu et al. (1997), Vlasceanu (1999), Hutchens et al. (2004), and Engel and Porter (unpublished data).
[b] 16S rRNA gene sequences from Vlasecanu et al. (2000) and Macalady et al. (2006).
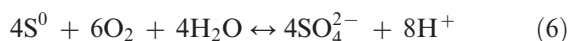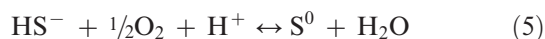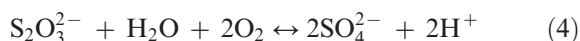[c] 16S rRNA gene sequences from Engel et al. (2003a), Engel et al. (2004a), and Meisinger et al. (2005).
[d] 16S rRNA gene sequences from Angert et al. (1998).
[e] 16S rRNA gene sequences from Engel and Porter (unpublished data).
[f] 16S rRNA gene sequences from Engel et al. (2001) and Engel and Porter (unpublished data).
[g] 16S rRNA gene sequences from Barton and Luizer (2005), and Engel and Porter (unpublished data).

extra-cellular sulfur globules (Equation 5); these intermediates can be further oxidized to sulfate (Equations 4 and 6) (Fig. 2):

$$S_2O_3^{2-} + H_2O + 2O_2 \leftrightarrow 2SO_4^{2-} + 2H^+ \tag{4}$$

$$HS^- + \tfrac{1}{2}O_2 + H^+ \leftrightarrow S^0 + H_2O \tag{5}$$

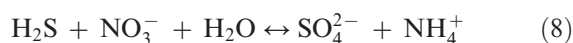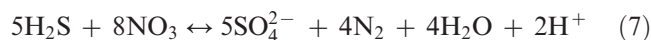$$4S^0 + 6O_2 + 4H_2O \leftrightarrow 4SO_4^{2-} + 8H^+ \tag{6}$$

Because of the large cell size and filamentous nature of some species (Fig. 3A), sulfur-oxidizing bacteria can be readily observed in conspicuous and sometimes extensive microbial mats that either attach to substrata or float in the water column in sulfidic cave streams (Fig. 3B), in karst aquifers (Fig. 3C), or in anchialine cave and stratified cenote systems (water-filled sinkholes) (Fig. 3D, in this case showing non-white mats) (e.g., Hubbard et al., 1986, 1990; Olson and Thompson, 1988; Thompson and Olson, 1988; Grubbs, 1991; Brigmon et al., 1994; Sarbu et al., 1996; Airoldi et al., 1997; Vlasceanu et al., 1997; Angert et al., 1998; Mattison et al., 1998; Humphreys, 1999; Hose et al., 2000; Sarbu et al., 2000; Gary et al., 2002; Engel et al. 2003a; Garman and Garey, 2005; Barton and Luiszer, 2005; Macalady et al., 2006; Randall, 2006). Many of the species from the alpha- ($\alpha-$), beta- ($\beta-$), gamma- ($\gamma-$), and epsilonproteobacterial ($\varepsilon-$) classes found in microbial mats from caves are associated with sulfur oxidation. Although some *Archaea* have been identified (e.g., *Thermoplasma acidophilum* from the Glenwood Hot Pool Spring, Colorado; Barton and Luiszer, 2005), *Archaea* capable of oxidizing reduced sulfur compounds (e.g., Canfield et al., 2005) have not been found from sulfidic caves to date.
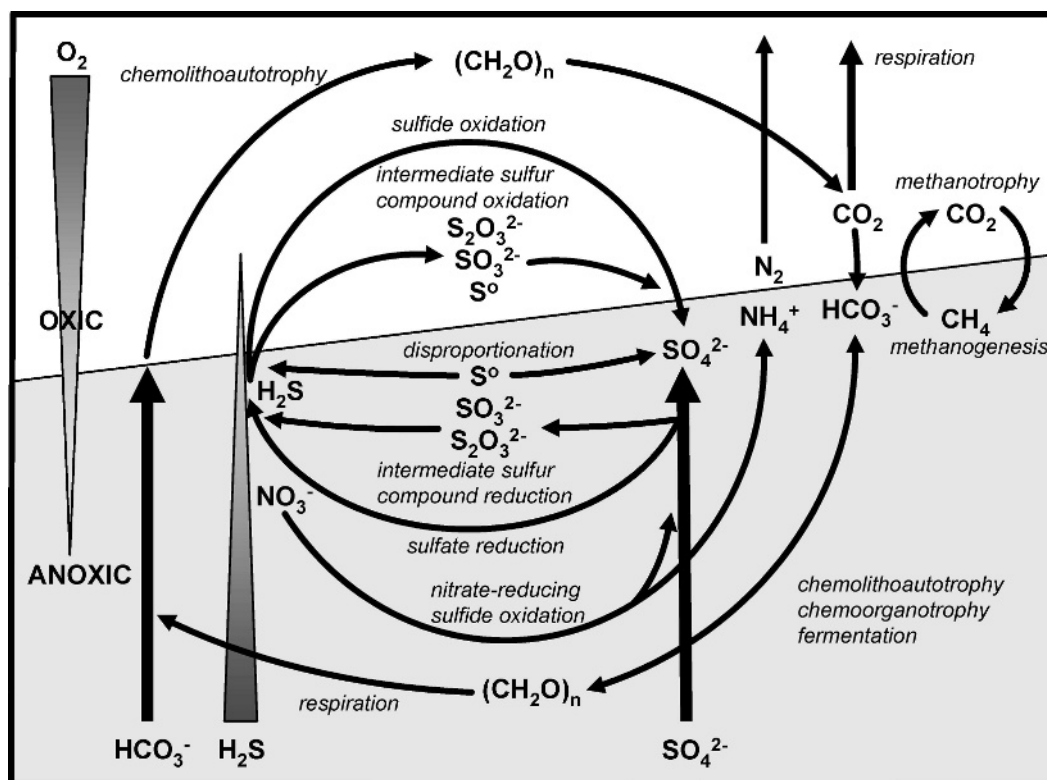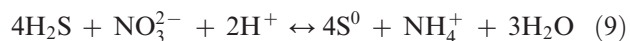
Figure 2. Schematic of integrated biogeochemical cycling in microbial ecosystems related to the sulfur, oxygen, carbon, and nitrogen elemental cycles.

Recent research demonstrates that sulfur-oxidizing bacterial communities in cave microbial mats depend on relatively stable O_2 concentrations and availability (Engel et al., 2004a), although some species can tolerate, and may even prefer, extremely low concentrations of O_2 (<1 mg L^{-1} dissolved O_2) for prolonged periods of time (e.g., Takai et al., 2003). If the concentration of O_2 is too low for growth, nitrate can be used as an electron acceptor (e.g., Sayama et al., 2005); depending on the metabolic pathway, either N_2 (Equation 7) or ammonium (NH_4^+) can form (Equation 8):

$$5H_2S + 8NO_3 \leftrightarrow 5SO_4^{2-} + 4N_2 + 4H_2O + 2H^+ \quad (7)$$

$$H_2S + NO_3^- + H_2O \leftrightarrow SO_4^{2-} + NH_4^+ \quad (8)$$

Some microbes, such as *Beggiatoa* spp., form S^0 from the oxidation of H_2S with nitrate (Equation 9), which can be further oxidized with nitrate (Equation 10) (e.g., Sayama et al., 2005):

$$4H_2S + NO_3^{2-} + 2H^+ \leftrightarrow 4S^0 + NH_4^+ + 3H_2O \quad (9)$$

$$3NO_3^- + 4S^0 + 7H_2O \leftrightarrow 3NH_4^+ + SO_4^{2-} + 2H^+ \quad (10)$$

Because many fresh water systems are nitrogen-limited, the nitrate-reducing sulfur-oxidizing bacteria (NRSOB) generate nitrogen compounds that other organisms in the ecosystem can use (e.g., NH_4^+), thereby linking the sulfur cycle to the nitrogen cycle (Fig. 2). NRSOB have been identified from several cave and karst aquifers (e.g., Lawrence and Foster, 1986; Mattison et al., 1998), and these organisms may extend the depths to which sulfur, and consequently carbon and nitrogen, are cycled in oxygen-depleted waters of sulfidic karst aquifers (Engel et al., 2004b).

The presence of ε-*proteobacteria* in all of the sulfidic caves studied thus far is exciting. A recent study of ε-*proteobacteria* by Campbell et al. (2006), using a large dataset of geographic, genetic, and ecological information, reveals that members of this class are not only in sulfidic caves, but also numerous other sulfur-rich habitats, including marine waters and sediments, deep-sea hydro-thermal-vent sites and vent-associated animals, groundwater associated with oilfields, and from terrestrial and marine sulfidic springs. The best studied terrestrial system where ε-*proteobacteria* have been described is Lower Kane Cave (Campbell et al., 2006). Quantification of different microbial groups using genetic approaches reveals that up to 100% of some samples is comprised of ε-*proteobacteria*, making Lower Kane Cave the first non-marine natural system known to be driven by the activity of filamentous ε-*proteobacteria* (Engel et al., 2003a). The majority of the 16S rRNA sequences could be assigned to two lineages distinct at the genus level, LKC group I and LKC group II (Engel et al., 2003a; Engel et al., 2004a), and LKC group II was found to be predominately responsible for sulfuric acid
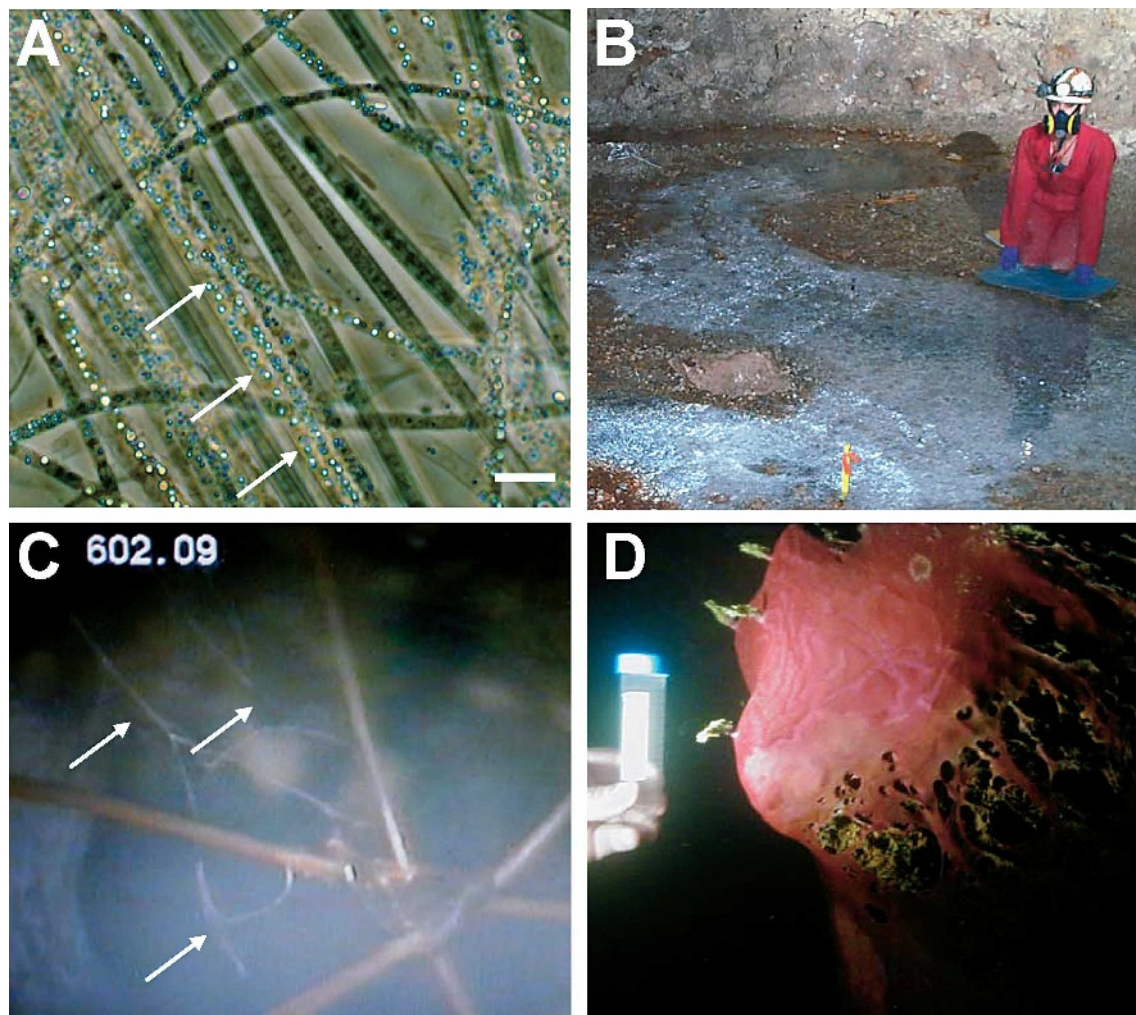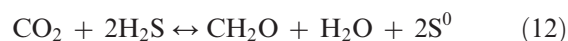
Figure 3. (A) Filamentous and rod-shaped microbial cells of sulfur-oxidizing bacteria. Arrow, sulfur globules. Scale is 10 microns. (B) White microbial mat in sulfidic stream, Lower Kane Cave, Wyoming. (C) Arrows pointing to white filaments suspended in sulfidic water of an open-hole well in the Edwards Aquifer. Field of view is ~6 inches. Number at upper left refers to well depth in feet (183.5 m) from the surface (image digitally captured from video provided by the Edwards Aquifer Authority, San Antonio, Texas). (D) Biofilm of purple sulfur bacteria covering carbonate rock in La Pilita cenote, of the Sistema Zacatón, Mexico.

dissolution of the cave host limestone (Engel et al., 2004b). Certain ε-proteobacterial groups correlated to high dissolved sulfide and low dissolved O_2 content in the cave streams, suggesting that some species prefer different geochemical conditions (Engel et al., 2004a).

Another diverse group of microbes that use H_2S (or H_2) as an electron donor during anoxygenic photosynthesis includes the purple sulfur bacteria (e.g., *Chromatium*, *Thiocapsa*, *Ectothiorhodospira*), the purple nonsulfur bacteria (e.g., *Rhodobacter*), the green sulfur bacteria (e.g., *Chlorobium*, *Pelodictyon*), the green nonsulfur bacteria (*Chloroflexus*, *Oscillochloris*), and the *Heliobacteria* (e.g., Brimblecombe, 2005; Canfield et al. 2005). Some of the species oxidize reduced sulfur completely to sulfate (Equation 11), while others form intermediate sulfur

compounds (Equation 12), where CH_2O represents organic carbon compounds made during photosynthetic CO_2 fixation:

$$3CO_2 + H_2S + 2H_2O \leftrightarrow 2CH_2O + SO_4^{2-} + 2H^+ \quad (11)$$

$$CO_2 + 2H_2S \leftrightarrow CH_2O + H_2O + 2S^0 \quad (12)$$

These organisms have been found in sulfidic springs (e.g., Elshahed et al., 2003; Barton and Luiszer, 2005) and cenotes (e.g., Stoessell et al., 1993; Humphreys, 1999; Gary et al., 2002; Herbert et al., 2005) (Fig. 3D), and are likely to be significant contributors to ecosystem sulfur and carbon cycling in those habitats. Because of the need to photosynthesize, these groups should not be found in

complete darkness; however, *Chloroflexus* spp. have been described from Lower Kane Cave (Meisinger et al., 2005) and the Frasassi Caves (Grotta Grande del Vento-Grotta de Fiume-Grotta Sulfurea), Italy (Macalady et al., 2006) (Table 1), and may be present in Cueva de Villa Luz (Cueva de las Sardinas), Mexico (Hose et al., 2000). It is suspected that these species may be able to grow in the dark using alternative pathways for energy and carbon fixation (e.g., Canfield et al., 2005).

Generally, abiotic conditions influence the types of organisms that a habitat can support. Most sulfur-oxidizers require neutral pH conditions to buffer metabolic acidity (Ulrich et al., 1998; Brimblecombe, 2005), and the buffering capacity of dissolving carbonates may be one reason why sulfur-oxidizers are prevalent in karst. Yet, some sulfur-oxidizers (e.g., *Acidothiobacillus*) thrive in low pH environments as acidophiles (acid-lovers). In active sulfidic caves, such as in Cueva de Villa Luz, extremely low pH habitats have been described, especially on subaerial cave-wall surfaces. Biofilms on subaerial, cave-wall surfaces (also described as snottites, mucotites, microbial draperies, or cave-wall biofilms by different investigators over the years) have been described from active sulfidic caves and mines (Johnson, 1998; Vlasceanu et al., 2000; Engel et al., 2001; Engel et al., 2003b). In Cueva de Villa Luz, for example, measured cave-wall pH associated with 'snottites' was 0 (Hose et al., 2000). Culture-dependent and culture-independent studies revealed diverse populations of *Thiobacillus, Sulfobacillus*, *Acidimicrobium*, and other groups, such as the *Firmicutes* (Hose et al., 2000; Vlasceanu et al., 2000; Engel et al., 2001; Engel et al., 2003b).

SULFATE REDUCTION AND SULFUR DISPROPORTIONATION

Reduced sulfur compounds originate from several sources, including abiotic processes (e.g., volcanism), the degradation of organics (e.g., proteins), or dissimilatory sulfate reduction whereby oxidized compounds (e.g., SO_4^{2-}) serve as electron acceptors under anaerobic conditions; elemental sulfur can also be reduced to H_2S (Fig. 2). Sulfate (or S^0) can be reduced using H_2 as the electron donor (Equation 13) or using organic compounds, such as acetate (Equation 14) or lactate (although numerous organic compounds can be used):

$$4H_2 + SO_4^{2-} + H^+ \leftrightarrow 4H_2O + HS \qquad (13)$$

$$CH_3COO + SO_4^{2-} \leftrightarrow 2HCO_3^- + H_2S \qquad (14)$$
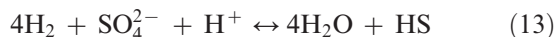
The utilization of organic compounds by sulfate-reducers, either as complete oxidation (e.g., acetate) to CO_2 or the incomplete oxidation of other compounds, again links the sulfur and carbon cycles.

Molecular investigations of some sulfidic aquifers, including those associated with oilfields, have documented sulfate-reducers (Voordouw et al., 1996; Ulrich et al.,

1998); thus far, studies identifying these organisms in active sulfidic caves have been limited to Lower Kane Cave and the Frasassi Caves (Engel et al., 2004a; Meisinger et al., 2005; Macalady et al., 2006). A genetically varied group of microbes are known to carry out dissimilatory sulfate reduction, but the sulfate-reducers that have been found in sulfidic karst systems predominately fall within the δ-*proteobacteria* class (Table 1). The other groups of sulfate-reducers grow at 70 to 105 °C (Brimblecombe, 2005; Canfield et al., 2005), well above the temperatures of currently explored, active sulfidic cave and deep aquifer systems.

Another recently recognized, environmentally significant sulfur transformation pathway is disproportionation (e.g., Brimblecombe, 2005; Canfield et al., 2005). During disproportionation, intermediate sulfur compounds that were produced during incomplete oxidation, such as S^0 or $S_2O_3^{2-}$ (Equation 15), form both reduced and oxidized forms of sulfur (Fig. 2):

$$S_2O_3^{2-} + H_2O \leftrightarrow H_2S + 3SO_4^{2-} \qquad (15)$$
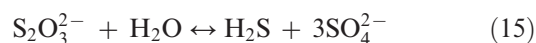
Several groups of microbes disproportionate sulfur compounds, including anoxygenic phototrophs, some sulfate-reducers (e.g., *Desulfovibrio* and *Desulfobulbus* spp.), and sulfate-reducing bacteria that perform sulfur disproportionation as their sole metabolism (e.g., *Desulfocapsa* spp.). In general, characterization of sulfate- and S^0-reducing or sulfur-disproportionating microbes from sulfidic caves and aquifers has not been thoroughly done, although *Desulfocapsa thiozymoxenes* has been found in Lower Kane Cave and the Frasassi Caves (Engel et al., 2004a; Meisinger et al., 2005; Macalady et al., 2006). Where O_2 can abiotically oxidize reduced sulfur compounds, the reductive and disproportionation pathways generate supplemental sulfide that sulfur-oxidizing bacteria within the microbial mats can use (Engel et al., 2004a).

FAUNAL INVENTORIES

The fauna of cave and karst aquifer ecosystems have not been exhaustively sampled nor characterized (i.e. large, conspicuous animals are easy to see and describe), and obligate cave fauna have been inadequately identified (e.g., Culver et al., 2004). Similarly, microscopic eukaryotes (e.g., fungi, molds, protozoa) and micro-invertebrates (e.g., copepods) are almost virtually unknown for most subterranean systems, despite the extensive work done on microbes involved in sulfur cycling and descriptions of the chemolithoautotrophic microbial communities (see previous section of text) (e.g., Angert et al., 1998; Engel et al., 2004a; Hutchens et al., 2004; Barton and Luiszer, 2005; Macalady et al., 2006) (Table 1). Nevertheless, Culver and Sket (2000) illustrate that some of the most biologically diverse karst ecosystems (based on the numbers of species, exclusively) are associated with sulfidic waters, especially

when considering systems with a high number of endemic populations. Such systems include the Movile Cave, the Edwards Aquifer in Texas, and the anchialine Washingham Caves in Bermuda. Most notable on their list is the chemolithoautotrophically-based ecosystem of the Movile Cave, with 30 terrestrial species (24 are cave-adapted and endemic) and 18 aquatic species (9 cave-adapted and endemic) (Sarbu et al., 1996; Culver and Sket, 2000). However, not all sulfidic caves or aquifers are known for high species numbers, as is the case for Lower Kane Cave with only four identified species (Porter, unpublished data) (Table 2). Part of this difference in the number of higher trophic level species in sulfidic cave and karst systems may be attributed to the invasion history of animals in the region (e.g., Christman and Culver, 2001) and the age of the system, as Lower Kane Cave is likely to be quite young geologically (e.g., Stock et al., 2006) compared to the other caves (e.g., Longley, 1986; Oetting et al., 1996; Engel, 1997; Groscehen and Buszka, 1997; Sarbu et al., 2000).

For the purpose of this review, the known faunal inventories for some sulfidic caves and karst aquifers are provided (Table 2); the compiled lists of species numbers (available at <http://geol.lsu.edu/Faculty/Engel/geomicrobiology_publications.htm>) result from combing through the literature, the World Wide Web (<http://www.karstwaters.org/kwidata.htm>), and by personally contacting individual research groups. To my knowledge, no summary like this has been previously assembled for sulfidic cave and karst aquifer systems. A note of caution: these lists are not inclusive and they likely contain errors because they were compiled from many different, including previously unpublished, sources. Although the biodiversity of some submarine caves has been studied (e.g., Grotta Azzura; Mattison et al., 1998), the focus of the next section is limited to continental systems.

In short, sampling caves is tricky work, but sampling sulfidic caves is definitely more complicated (see footnote 1). Similarly, sampling groundwater can also be difficult (e.g., Ghiorse and Wilson, 1988; Krumholz, 2000). Therefore, sampling biases may have caused the incomplete and inaccurate picture of species richness and distribution for sulfidic systems (e.g., Culver et al., 2004; Schneider and Culver, 2004; van Beynen and Townsend, 2005). Certainly, the novelty of the Movile Cave ecosystem may have prompted the years of investigations (e.g., Plesa, 1989; Sarbu, 1990; Georgescu and Sarbu, 1992; Decu and Georgescu, 1994; Decu et al., 1994; Georgescu, 1994; Poinar and Sarbu, 1994; Weiss and Sarbu, 1994; Sarbu et al., 1996; Vlasceanu et al., 1997; Manoleli et al., 1998; Porter, 1999; Vlasceanu, 1999; Hutchens et al., 2004). Moreover, in some faunal descriptions, organisms were only characterized to the family or order levels, and some genus- and species-level identifications have changed over the years due to more detailed systematics and molecular phylogenetics. Future work should concentrate on completing and verifying the list because these issues obviously

inhibit a thorough statistical comparison of sulfidic karst-system biodiversity and presently hinder any evaluation of the possible economic value of these systems (e.g., Fromm, 2000; Gibert and Deharveng, 2002; van Beynen and Townsend, 2005).

MICROSCOPIC EUKARYOTES

The diversity of the microbial eukaryotes (e.g., fungi, protists, etc.) in sulfidic cave and karst aquifers has been poorly measured, despite the importance of these organisms to ecosystem function. Several fungal groups have sulfur-based metabolism, like sulfur gases consumption and production, and fungi also play a role in concrete corrosion associated with methanethiol (CH_3SH) consumption. These studies suggest that fungi may be an overlooked part of the sulfur cycle in these systems, and may be important to limestone dissolution (e.g., Burford et al., 2003). Fungi, ciliated protozoa, and rotifers have been described from the sulfidic waters in Grotta di Fiume Coperto, Italy (Latella et al., 1999a; Maggi et al., 2002) (Tables 1 and 2). Not shown in Table 2, however, are the results from a survey from the Sulphur River passage of Parker's Cave, Kentucky, which identified 13 genera of protozoa (from eight orders), including species common to sulfidic habitats and associated with grazing (Thompson and Olson, 1988). Fungi and rotifers (also unclassified) have been reported from Movile Cave (Sarbu, 1990).

INVERTEBRATES
Phylum Platyhelminthes

Although the diversity of the flatworms is high in non-sulfidic subterranean settings, only *Dendrocoelum* sp. has been reported from Movile Cave (Sarbu, 1990). Flatworms have also been observed in Lower Kane Cave and Cueva de Villa Luz, but no identification was done.

Phylum Nematoda

Several new species of stygobitic nematodes have been described from sulfidic karst aquifers (e.g., Moravec and Huffman, 1988; Poinar and Sarbu, 1994). Although *Chronogaster troglodytes* sp. n. from Movile Cave is bacterivorous, *Rhabdochona longleyi* sp. n. from the Edwards Aquifer was found infecting the intestines of the two blind catfishes, *Trogloglanis pattersoni* Hubbs & Bailey 1947 and *Satan eurystomus* Eigenmann 1919 (Moravec and Huffman, 1988).

Phylum Annelida

This group is represented by aquatic worms and leeches, both of which have been described from just two sulfidic cave systems (Table 2). Most notable is *Haemopis caeca* Manoleli, Klemm & Sarbu 1994, the cave leech endemic to Movile Cave and the surrounding sulfidic karst aquifer (Manoleli et al., 1998). Annelids have been reported from the Sulphur River passage of Parker's Cave, but no details are given (Thompson and Olson, 1988). Tubificid worms

Table 2. Estimated number of species reported for sulfidic caves and karst aquifers, including accidentals (i.e. surface invaders); bats and salamanders are not included in the aquatic counts. * refers to a possible occurrence, but no formal description (the symbol is also used if an origin could not be verified). Note that some organisms are not limited to the sulfidic portions of the systems, but have been described from the freshwater component of a cave or aquifer (e.g., in the case of the Frasassi Caves). For the Edwards Aquifer, however, taxa recorded from only the sulfidic part of the system are enumerated. Full species lists can be found at <http://geol.lsu.edu/Faculty/Engel/geomicrobiology_publications.htm>.

	Movile Cave (Romania)		Frasassi Cave (Italy)		Grotta di Fiume Coperto (Italy)		Cueva de Villa Luz (Mexico)		Edwards Aquifer (Texas)	Lower Kane Cave (Wyoming)	
	terrestrial	aquatic	terrestrial	aquatic	terrestrial	aquatic	terrestrial	aquatic	aquatic	terrestrial	aquatic
Oligohymenophorea (ciliate protozoa)						1					
Rotifera		2				1					
Platyhelminthes (flat worms)		1						*			*
Nematoda (round worms)		3							1		
Annelida											
Oligochaeta (aquatic worms)		3		1							
Hirudinea (leeches)		1				1					
Mollusca											
Gastropoda (snails)		1		1	2	1	*	1			1
Archnida											
Acari (mites, ticks)	1		1				~80				
Araneae (spiders)	5		5		3		13			1	
Pseudoscorpiones (false scorpions)	3		1		2		6				
Schizomida (whip scorpions)							1				
Scorpiones (scorpions)					1		*				
Crustacea											
Copepoda (copepods)		3				3		*			
Ostracoda (ostracods)		1									
Amphipoda (amphipods)		2		1		1					
Isopoda (isopods)	4	1	1		3	1		*	*		
Decapoda (shrimp, crayfish, crabs)						1		1			
Myriapoda											
Chilopoda (centipedes)	3				3						
Diplopoda (millipedes)	1				1						
Symphyla (garden centipedes)	1						*				
Hexapoda											
Ellipura (collembola)	3		2				23				
Insecta											
Coleoptera (beetles)	4		2		3		6			1	
Diptera (flies)	2				2		*	1			
Hymenoptera (wasps, ants, bees)							10				
Orthoptera (crickets, cockroaches)					2		*				
Lepidoptera (moths, butterflies)					1		*				
Hemiptera (bugs, aphids)							1				
Heteroptera (true bugs)	1					1		2			
Psocoptera (barklice, booklice)							1				
Thysanura (silverfish)							2				
Vertebrata											
Osteichthyes (fish)								1	2		
Anguilliformes (eel)				*				*			
Totals	27	19	12	3	23	10	~143	~6	3	2	2

have also been described from sediments in sulfidic cave streams where surface water can back-flood into the cave passages (e.g., Lower Kane Cave), although no formal descriptions have been made.

Class Mollusca

Even though non-sulfidic caves can be colonized by both terrestrial and aquatic snails, few descriptions of gastropods from sulfidic caves and aquifers are known (Table 2). In the case of land snails, this is most likely due to the lack of communication with the surface whereby snails can be washed into a cave. Described aquatic snails include the endemic prosobranch snail, *Heleobia dobrogica* Bernasconi 1991, from Movile Cave (Bernasconi, 1997), populations of *Islamia* spp. in the sulfidic stream portions of the Grotta di Fiume Coperto and the Frasassi Caves (Latella et al., 1999a; Sarbu et al., 2000; Maggi et al., 2002), and the endemic snail *Physella* (formerly *Physa*) *spelunca* Turner & Clench 1974 from the sulfidic streams in Lower Kane Cave (Porter et al., 2002; Wethington and Guralnick, 2004). A sister species, *Physella johnsoni* Clench 1926, has also been reported from sulfidic springs (one in a cave) on Sulphur Mountain in Banff National Park, Canada (Lepitzki, 2002; Wethington and Guralnick, 2004). The *P. spelunca* population in Lower Kane Cave is tremendous, with an estimated 6,800 individuals per square meter (Porter et al., 2002). *P. spelunca* was originally described as being troglomorphic (i.e. pigmentless, no eyes), but observations indicated there were at least two other color morphs (red and black) although genetic variation from the cave populations has not been identified to date (Porter et al., 2002). Two species of snails, with high population densities, have been observed, but not yet described, from Cueva de Villa Luz (K. Lavoie, personal communication).

Class Arachnida

Because many of the sulfidic caves are in poor communication with the surface, the colonization of these caves by arachnids (e.g., mites, spiders, scorpions) has been limited, except in the case of systems with many entrances or with large bat populations. These have high arachnid diversity (Table 2). Most notable are the numbers of different arachnid species reported from Cueva de Villa Luz, and the ~80 species of acarians, representing five orders. The microarthropods have been the subject of extensive research by one group and of several Masters theses (Palacios-Vargas et al., 1998; Palacios-Vargas et al., 2001;Estrada, 2005; Pastrana, 2006); the account of the arachnids in this one cave is likely due to that concentrated effort. Moreover, seven species of bats representing three different families have been described from Cueva de Villa Luz, and most of the microarthropods were found associated with bat guano or surface-derived material proximal to cave entrances (Palacios-Vargas and Estrada, personal communication). Several acarians (*Sejus* sp., *Gamasellodes* sp., *Protolaelaps* sp.) are found near the

sulfidic cave stream and the microbial mats (Palacios-Vargas and Estrada, personal communication). Two possibly new species of mites, *Dactyloscirus* sp. and *Neoscirula* sp. (Cunaxidae family), have been found near the microbial mats in the sulfidic water of Cueva de Villa Luz (Estrada and Mejía-Recamier, 2005). Undescribed acarians have also been reported from the Sulphur River passage of Parker's Cave (Thompson and Olson, 1988). Troglobitic spiders have been described from both Movile Cave (five species, each representing their own order) and the Frasassi Caves (two species from one order) (e.g., Georgescu and Sarbu, 1992; Georgescu, 1994; Weiss and Sarbu, 1994; Sarbu et al., 2000). *Nesticus* spp. have been reported from acidic cave walls in both of these caves, and spider webs commonly have low pH droplets hanging from them. Drops also form on webs from the linyphiid spider, *Phanetta subterranea* Emerton 1875, in the Sulphur River passage in Parker's Cave (Thompson and Olson, 1988).

Subphylum Crustacea

Much like the microbial eukaryotes, micro-invertebrates have been poorly studied from sulfidic caves and karst aquifers. Several species of copepods and ostracods have been described from only two caves (Table 2). Movile Cave hosts an endemic copepod and ostracod (Plesa, 1989). Additionally, within the Order Amphipoda there are few described species from sulfidic caves or aquifers (Table 2); however, given the prevalence and diversity of amphipods in non-sulfidic caves globally (e.g., Culver and Sket, 2000; Gibert and Deharveng, 2002), and their metabolic flexibility and high tolerance of hypoxia (e.g., Macneil et al., 1997; Hervant et al., 1999a; Hervant et al., 1999b; Kelly et al., 2002; Lefébure et al., 2006), it is surprising that more amphipods have not been identified. A few stygobitic isopods have been characterized from sulfidic systems, although comparatively more troglobitic isopods have been described (Table 2).

One habitat that has high potential for crustaceans is sulfidic groundwater (despite the fact that even fresh groundwater has not been adequately sampled). Longley (1981) asserted that the Edwards Aquifer in Central Texas had the potential to be the most diverse subterranean biological community on earth, although little work has been done to verify the proclamation. The sulfidic (badwater) portion (Rye et al., 1981; Oetting et al. 1996; Ewing, 2000) of the aquifer has been virtually unexplored biologically and has the potential to host a unique fauna (see description below of the Osteichthyes), including microbes (e.g., Grubbs, 1991). The non-fungal microbiology has recently been described for a portion of the sulfidic aquifer in the San Antonio area (Randall, 2006; Engel, unpublished data). Overall, ~91 species or subspecies of animals have been described from the entire Edwards Aquifer, including 44 endemic stygobites (Ourso and Horning, 2000). One sampled artesian well in San Marcos, Texas, reportedly has ~10 species of amphipods, from

numerous families (Holsinger, 1980). Several descriptions of stygobitic amphipods indicate that some species, such as *Artesia subterranea* Holsinger 1980, were retrieved from warm mineral water from artesian wells (Holsinger, 1966, 1980), which may be taken to mean that the species was retrieved from a sulfidic well. This phenomenal crustacean diversity deserves attention, and verification is needed if any of these species are living in the sulfidic portion of the aquifer.

Superclass Hexapoda

The types of hexapods described from sulfidic cave and karst systems include collembolans and insects, and the group is dominated by terrestrial species (Table 2). Among the species described, endemic troglobites have been reported from Movile Cave (e.g., Decu and Georgescu, 1994) and the Frasassi Caves (Sarbu et al., 2000). Numerous hexapods, particularly among collembolans and hymenopterans, have been inventoried from Cueva de Villa Luz as part of thesis research (Estrada, 2005; Pastrana, 2006). Although considered a terrestrial taxon, the larva stage of chironomid midge is found in high abundance in the sulfidic waters in Cueva de Villa Luz (Lavoie and Evans, 2002). Many hexapods are considered to be grazers in the cave food webs, predominantly consuming microbial biofilms; some may also be omnivorous. One notable heteropteran is the endemic, stygobitic water scorpion, *Nepa anophthalma* Decu et al. 1994, from Movile Cave (Decu et al., 1994); *Nepa cinerea* Linnaeus 1758 has been identified from Grotta di Fiume Coperto (Latella et al., 1999a).

VERTEBRATES

Among the organisms found in caves, perhaps the vertebrates have elicited the most attention, even though many are accidental in caves (from birds to skunks). Bats are frequent visitors to sulfidic caves with entrances to the surface, such as Cueva de Villa Luz and the Frasassi Caves (Hose et al., 2000; Sarbu et al., 2000) (a species list is provided in the supplement at <http://geol.lsu.edu/Faculty/Engel/geomicrobiology_publications.htm>, but not in Table 2). For this review, only aquatic vertebrates are described in detail.

Class Osteichthyes

Two different families of fishes have been described from sulfidic karst settings. *Poecilia mexicana* Steindachner 1863 (the cave molly, family Poeciliidae) is prevalent in the sulfidic waters of Cuvea de Villa Luz and nearby sulfidic springs (Langecker et al., 1996; Hose et al., 2000; Tobler et al., 2006). This small fish, having reduced eye size and pale coloration compared to surface-dwelling populations, is the center of attention for the ritual celebration of native villagers (Langecker et al., 1996; Hose et al., 2000). For probably a thousand years, kilograms of fish are sacrificed annually during the ceremony, but the population appears to be robust (Tobler et al., 2006). The sources of food for the fish are considered to be microbial mats and chironomid larvae (Langecker et al., 1996; Lavoie and Evans, 2002).

The deep sulfidic waters of the Edwards Aquifer host the two endemic blind catfishes, *T. pattersoni* and *S. eurystomus* (both from family Ictaluridae), whose origin has been traced back to the Pliocene or Miocene (Langecker and Longley, 1993). Both fish show remarkable adaptations to the deep aquifer, having been retrieved from over 400 m water depth, including the lack of pigment, loss of eyes and pineal organs, and the lack of the swim-bladder (which is typical for deep-sea fishes). Each of the aquifer species also has unique morphological features that are attributed to their respective ecological niches. *T. pattersoni* has a sucker-like mouth distinct from any other species in the family that is suggestive of grazing (Langecker and Longley, 1993), and Longley and Karnei (1978) report partially degraded fungus in the gut. The catfish was probably full of sulfur-oxidizing bacteria instead of fungus, as the bacteria form extensive biofilms on the aquifer walls (Grubbs, 1991; Randall, 2006) (Fig. 3C). In contrast, *S. eurystomus* had gut contents resembling stygobites (e.g., amphipods), suggesting that it was probably a predator (Langecker and Longley, 1993).

Class Anguilliformes

Hundreds of well-preserved, 30–70 cm long, adult eel fossils (*Anguilla anguilla*) have been found in the Frasassi caves, ~5 m above the present day water table (Mariani et al., 2004). Isotopic comparisons between the eels and river and cave animals indicated that the eels were not endemic to the sulfidic cave waters, but instead to the surface river. Reconstructed ^{14}C ages were consistent to the cave paleolevels, dating back as far as 9,000 years ago. An eel has been reported from Cueva de Villa Luz (Hose et al., 2000), although it is unclear whether it is endemic or accidental.

THE ROLE OF CHEMOLITHOAUTOTROPHY IN SHAPING THE BIODIVERSITY OF SULFIDIC KARST ECOSYSTEMS

As previously discussed, the major energy and food sources in most cave and karst aquifers are from photosynthetically-produced organic matter that is brought into the system from the surface by air, water, or animals. Prolonged periods of limited to no food can cause widespread starvation (e.g., Hüppop, 2005), which undoubtedly results in stress (see discussion below) (Howarth, 1993). Accordingly, individuals who are stressed may expend greater energy for survival and would require more food in order to cope with habitat-induced pressures (e.g., Howarth, 1993; Hüppop, 2005; Parsons, 2005). For sulfidic systems, one of the consequences of chemolithoautotrophic primary productivity is an increase in the quality and quantity of organic carbon (Poulson and Lavoie, 2000;

Engel et al., 2004a). This rich and abundant food source may have a significant impact on biodiversity and an organism's ability to endure habitat stresses.

The carbon to nitrogen ratio (C:N) of microbial biomass can be related to food quality. The lower the ratio (~3–5), the better the quality because of limited influx and processing of surface-derived material that would increase the nitrogen content of the organic matter (Engel et al., 2004a). Microbial biomass from Movile Cave and Lower Kane Cave have C:N values of ~5 and are comparable to periphyton in surface streams and bacteria from deep-sea vents (Kinkle and Kane, 2000; Engel et al., 2004a). In contrast, high C:N ratios indicate that there is an abundant carbon supply, likely due to storage of biomass, but a reduction in nitrogen availability.

Stable isotope ratio analyses (SIRA) and radiolabeled-carbon assimilation studies confirmed that chemolithoautotrophic primary productivity was prevalent in the microbial mats from various caves (Sarbu et al., 1996; Airoldi et al., 1997; Pohlman et al., 1997; Mattison et al., 1998; Humphreys, 1999; Porter, 1999; Kinkle and Kane, 2000; Sarbu et al., 2000; Vlasceanu et al., 2000; Engel et al., 2004a; Hutchens et al., 2004). For carbon isotope systematics, the two carbon isotopes of importance are ^{12}C and ^{13}C, whereby the incorporation of carbon into living tissues invokes significant kinetic isotope fractionation. Specifically, biological (e.g., enzymatic) processes discriminate for the lighter isotope (^{12}C), leaving the heavier isotope (^{13}C) behind. Differences in the isotopic composition are expressed in terms of the delta (δ)-notation of a ratio of the heavy versus the light isotopic values for a sample relative to a standard, measured in per mil (‰). In general, biogenic carbon is isotopically lighter (more negative) than the inorganic reservoir (e.g., CO_2 or dissolved HCO_3^-); chemolithoautotrophic carbon fixation pathways have some of the largest fractionation effects, with resulting $\delta^{13}C$ values of chemolithoautotroph-dominated microbial biomass ranging between −30 and −45‰ compared to surface organic matter at ~−20‰ (Fig. 4). Variations in the $\delta^{13}C$ composition of microbial biomass are due to the taxonomic groups present and different compositions of dissolved inorganic carbon. Excretion, respiration, and heterotrophic carbon cycling are (for the most part) considered negligible carbon isotope fractionation processes, and the isotopic composition of heterotrophic organic matter will be the same as, or slightly higher than, the source organic carbon (essentially, in SIRA, the you-are-what-you-eat motto prevails).

The literature describing elaborate food webs is extensive for surface ecosystems (e.g., forests, soils, lakes), but studies of chemolithoautotrophically-based ecosystems and the structure and dynamics of their food webs are fairly limited (Sarbu et al., 1996; Pohlman et al., 1997; Vlasceanu et al., 2000; Sarbu et al., 2000). Trophic structure of most cave ecosystems is characterized by a lack of predators and extensive omnivory (Gibert and Dehar-
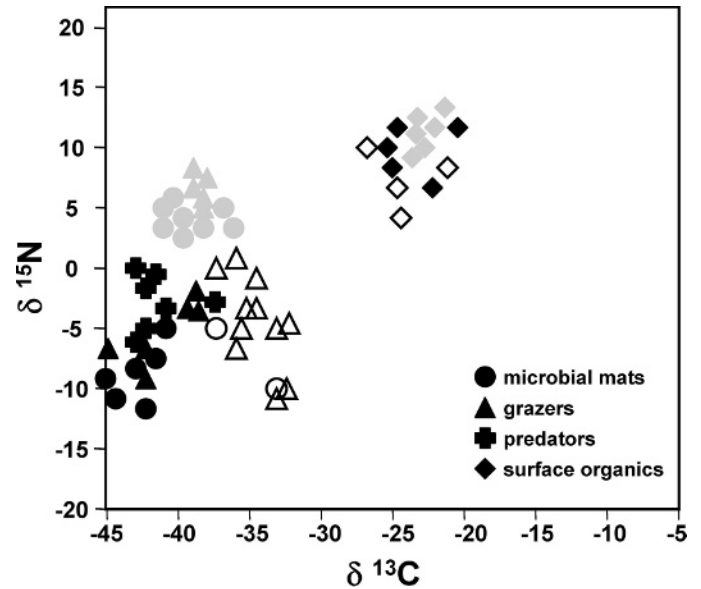
Figure 4. Stable carbon and nitrogen isotope ratio analyses for Movile Cave (solid symbols) the Frasassi Caves (open symbols), and Lower Kane Cave (shaded symbols) from Sarbu et al. (1996), Vlasceanu et al. (2000), and Engel et al. (2004a), respectively. Circles are microbial mat samples from each cave; triangles are for grazers; crosses are for predators (only Movile Cave has predators); diamonds are surface organics. Variations in the $\delta^{13}C$ composition of microbial biomass for the caves are due to the taxonomic groups present, which may have distinct carbon fixation pathways, and different starting compositions of dissolved inorganic carbon.

veng, 2002). There has been relatively little done with regard to evaluating the biogeochemical and ecological roles of the dominant groups in the sulfidic ecosystems. The limited SIRA studies do support that predators are lacking in sulfidic systems, although it is possible that the predators are not known or that the identified species have not previously been considered predators (e.g., amphipods; Kelly et al., 2002). True predators (e.g., spiders) are present in the Movile Cave ecosystem and the $\delta^{13}C$ values for those organisms (−37 to −44‰) demonstrate that they eat grazers, who in turn have $\delta^{13}C$ values consistent with consumption of the microbial mats (Fig. 4). The $\delta^{13}C$ compositions of the microbial mats suggest chemolithoautotrophic productivity, and are distinct from surface organic matter (Fig. 4). Langecker et al. (1996) explored the food web of Cueva de Villa Luz using sulfur SIRA and found that the analyses could only partially define energy flow within the food web.

Assimilation studies have been done with microbial biomass from several sulfidic cave systems using ^{14}C-bicarbonate to estimate chemolithoautotrophic productivity and ^{14}C-leucine to estimate heterotrophic productivity (Porter, 1999; Engel et al., 2001) (Fig. 5). Autotrophic rates
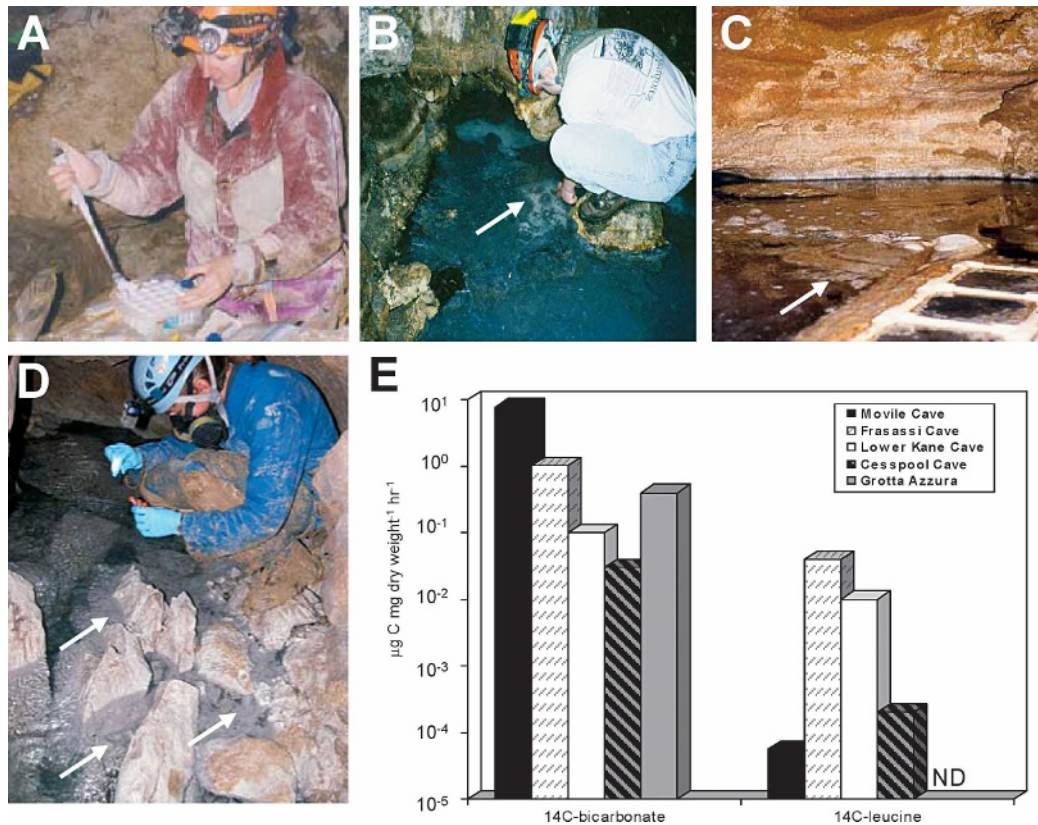
Figure 5. (A) Set-up for radiolabelled-isotope experiment in the Frasassi Caves, Italy. (B) Sampling in Cesspool Cave, Virginia; arrow pointing to mats. (C) Floating microbial mats (arrow) in Movile Cave, Romania. Grid is ~10 cm on a side. (D) Sampling stream mats in the Frasassi Caves, Italy; arrows pointing to mats. (E) Comparison between cave microbial mats for ^{14}C-bicarbonate and ^{14}C-leucine incubations to estimate chemolithoautotrophic primary productivity and heterotrophic productivity, respectively (Porter, 1999). Autotrophic productivity only was estimated for Grotta Azzura, Italy (ND = no data for leucine test) (Mattison et al., 1998).

were highest for the Movile Cave and lowest for Cesspool Cave, but in all of the caves examined, autotrophic productivity was significantly greater (from one to five times) than heterotrophic activities (Porter, 1999; Engel et al., 2001). Similar rates of autotrophic productivity were estimated for microbial mats from the submarine cave, Grotta Azzura, at Cape Palinuro, Italy (Mattison et al., 1998). Microbial heterotrophic processing of autotrophic biomass was low, with heterotrophs processing a minor fraction of the available autotrophic productivity (Porter, 1999; Engel et al., 2001). For Lower Kane Cave, the estimate is ~30% of the autotrophic productivity is processed through heterotrophy in Lower Kane Cave, which compares well with estimates of ~20–40% of autotrophic productivity processing by heterotrophy for the open oceans (Porter, 1999).

The consequences of a rich and abundant food source relate to biodiversity (as the number of species), ecosystem function, and food web dynamics. First, nutritional stress may simply be negligible because members of the ecosystem do not need to rely on outside food or energy (e.g., Howarth, 1993). Organisms consuming the chemolithoautotrophically-produced food may also have a greater ability to endure habitat stresses, such as low O_2 and high H_2S (see discussion below). Moreover, the low C:N ratios and low heterotrophic productivity indicate that there is a limited microbial detrital loop and that nutritional quality of the biomass is high. These factors should correlate to a high number of grazers and other trophic levels that could be supported by microbial mat consumption (Engel et al., 2001). However, one argument asserts that a rich and plentiful food source may increase functional redundancy at various trophic levels (thereby increasing the total number of species in an ecosystem; e.g., Wohl et al., 2004; Hooper et al., 2005), if the food can not be accessed by high trophic levels. Another argument suggests that the stability of the overall habitat and the rich food source may support lower diversity (Gibert and Deharveng, 2002; Wohl et al., 2004), especially if there is a limited influx of surface organisms to replenish the gene pool or to increase competition (e.g., Barr and Holsinger, 1985; Hooper et al., 2005). As is apparent in the preceding

faunal inventories, more thorough descriptions of the functional roles of the organisms in sulfidic karst systems are needed to address these arguments fully.

THE ROLE OF HABITAT STRESS IN SULFIDIC KARST ECOSYSTEMS

Subsurface environments can be highly stressful habitats for life, with stress defined as a potentially damaging condition in the biological system (e.g., Howarth, 1993). The ability of subterranean organisms to tolerate, adapt, and evolve under stressful habitat conditions has been the subject of recent research (e.g., Howarth, 1993; Hüppop, 2005). For most organisms, stress avoidance is probably the first line of defense (e.g., Badyaev, 2005; Parsons, 2005). However, obligate troglobites and stygobites have conspicuous adaptations to subsurface conditions (i.e. darkness, limited food, etc.), including the reduction in and loss of structures (eyes, pigments, wings, etc.), loss of time-keeping abilities (and circadian rhythm), slower metabolic rates, and reduced fecundity, but also the elongation of appendages, enhanced sensory structures, etc. Organisms living in the sulfidic conditions not only manifest similar morphological, behavioral, and physiological adaptations compared to non-sulfidic subsurface animals, but they also must deal with different environmental stresses, such as toxic levels of gases like H_2S, CO_2, and CH_4, and variable pH.

Excluding nutritional stress, one of the most significant stresses for organisms living in sulfidic habitats is hypoxia (dissolved O_2 concentrations <2.0 mg L^{-1}) (Hervant et al., 1997; Malard and Hervant, 1999; Hervant and Malard, 2005). Note: the solubility of oxygen is complicated by temperature, pressure, elevation, and salinity, but in general the solubility decreases with increasing temperature and salinity; so in mesothermal (>10 °C) waters that are common for continental sulfidic systems, dissolved O_2 levels can be <0.01 mg L^{-1}, or considered anoxic. Because darkness precludes photosynthesis, O_2 is not produced *in situ*, and abiotic and biotic consumption, particularly if organic carbon is plentiful, can rapidly diminish the concentration of O_2. Moreover, slow to negligible air exchange with groundwater, or limited air circulation in cave passages, not only results in atmospheric stagnation, but also causes the accumulation of noxious gases, such as CO_2, CH_4, and H_2S. Utilization of O_2 as an electron acceptor for metabolic processes (e.g., through sulfur oxidation or heterotrophy) would also keep the concentration of dissolved O_2 exceedingly low. Therefore, microbial communities play a fundamental role in maintaining habitat physicochemistry, such as possibly causing and maintaining hypoxia in sulfidic aquifers.

Although it seems that microbes can easily and readily adapt to extreme habitat conditions, and that chemolithoautotrophy provides a rich and plentiful energy source for animals, one question remains: how do higher organisms live in such a harsh habitat? Much like the dogma that all life on earth is dependent on sunlight, there has been an ecological tenet that all life on earth requires O_2, and a lot of it, to live. Clearly, the biological diversity of groundwater systems in general, and sulfidic cave and karst habitats specifically (Table 2), points toward the fact that life certainly has adaptive strategies to living in these extreme environments (e.g., Howarth, 1993; Badyaev, 2005; Parsons, 2005).

Numerous studies have shown that groundwater crustaceans can live and grow under hypoxic conditions for several months and can survive anoxia for >48 hr. This is in stark comparison with surface-dwelling crustaceans who could survive for only a few hours to one day (Malard and Hervant, 1999; Hervant and Malard, 2005). Moreover, Bishop et al. (2004) found that the respiration rates of seven orders of stygobitic crustaceans living at dissolved O_2 levels of <0.6 mg L^{-1} in anchialine caves were lower than surface-dwelling organisms or similar to organisms living at slightly higher O_2 levels. Metabolic strategies and adaptations have been examined for stygobites and troglobites (e.g., Hervant and Malard, 2005), whereby the activity of various enzymes, and specifically high levels of malate dehydrogenase, indicate that some stygobites are poised for anaerobic metabolism (Bishop et al., 2004). The research also demonstrates that organisms rapidly recover from prolonged hypoxia by efficient removal of lactate and other anaerobic waste products (Hervant et al., 1999a; Hervant and Malard, 2005). Similar results have been reported for deep-sea vent organisms, in that those animals use anaerobic metabolism to support activity at low O_2 levels, while regulating O_2 consumption, and maintaining efficient circulatory systems and high-affinity hemoglobin.

Despite these adaptations, however, living at hypoxia still brings noxious gases, such as H_2S, into an organism's body. Tolerance of, and survival in, high H_2S concentrations for cave animals in sulfidic settings (such as anchialine caves) have not been studied in detail. For some organisms, like those at the deep-sea vents, symbiosis with microbes may be an evolutionary mechanism to deal with high H_2S levels (e.g., Somero et al., 1989). However, some studies of polychaete tube worms demonstrate the animals can survive up to four days when they switch to anaerobic metabolism under anoxic conditions with high sulfide (up to millimolar levels), which may be aided by special epidermal tissue structures independent of bacterial symbiosis (Hourdez et al., 2002; Menon et al., 2003).

CLOSING REMARKS

Cave-adapted organisms have the potential to be some of the rarest and most threatened species on Earth (e.g., van Beynen and Townsend, 2005). Subterranean biodiversity is quite high globally (Gibert and Deharveng, 2002), and is considered to be strongly linked to the (hydro)geologic age and permanence of the karst setting

(e.g., Culver, 1976; Barr and Holsinger, 1985; Jones et al., 1992). With continual isolation from the surface, organisms disperse and migrate, and populations can become separated from each other and speciation can occur. For non-sulfidic karst systems, it has been estimated that >50% of obligate cave-adapted species can be found in <1% of the land, at least for the United States (Culver et al., 2000). For sulfidic habitats, local geological and hydrostraigraphic controls (e.g., Christman and Culver, 2001) will impact the distribution of organisms endemic to sulfidic systems, as conditions that lead to sulfide production are needed. Consequently, the distribution of species in sulfidic karst aquifers may be even more restricted. How does one actually measure the spatial distribution of an animal whose potential habitat is a 100 km^2 aquifer? Is this a small distribution, or a large distribution?

Although it is evident that the intimate dependence of subsurface ecosystems on surface-derived nutrients and energy has catalyzed the mandatory protection of many karst systems from above-ground, usually anthropogenic, disturbances (van Beynen and Townsend, 2005), sulfidic ecosystems may not rely on surface-derived organics and may be potentially buffered from such disturbances. To attempt to understand more fully the vulnerability, management, and sustainability challenges facing these systems, as well as the potential that these systems may have a monetary value, the amount of future work is considerable. I suggest that exciting avenues for future research will not only be in the exploration of new systems, but in the rediscovery of old systems. We have known about some of the sulfidic caves and karst aquifers for nearly 100 years, but we still must shed light on many outstanding questions, including: what is the true nature of species diversity and distribution, how are the ecosystems structured, what are the ecological functions of organisms within the system, how do species adapt to habitat stresses, how does habitat stress affect ecosystem diversity and structure, and what are the roles of geochemistry and geology on habitat development and modification of these subterranean sulfidic ecosystems? Uncovering the answers to these questions will certainly provide years of fruitful study.

ACKNOWLEDGEMENTS

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