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Sulfidic spring in the gypsum karst system of Monte Conca (Italy): chemistry and microbiological evidences

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Abstract: Monte Conca Cave is a karst system placed in Messinian evaporites, consisting of an active cave and a resurgence located on the massif of Monte Conca, Campofranco within the "Riserva Naturale Integrale di Monte Conca". A sulfidic spring is located in the terminal gallery of the cave. To characterize the physical and chemical parameters of the Monte Conca Cave and of the sulfidic spring, air temperature, relative humidity, water pH, and concentrations of dissolved sulfides, nitrates and sulfates were monitored. The high sulfide consumption rate in the sulfidic spring, evaluated by a kinetic study, suggests that biotic consumption is dominant. Moreover, snottites and filamentous floating mats, rich in sulfur and nitrate suggest a microbial activity related to the sulfur cycle. Iron content was also evaluated in water and snottites, given its involvement in microbial activity. The microbial mats could be the source of an autotrophic system in close correlation with the biological cycle of many species of living organisms found near the spring. Some of them show typical troglitic characteristics, while the presence and abundance of others depends on the water amount. The greater abundance of taxa found close to the sulfidic spring suggests a complex food web associated with it. The monitoring lasted a year and half has highlighted the difference between chemical- physical parameters of the cave and the sulfidic spring, emphasizing its typical microenvironment.

Keywords: sulfidic spring; cave; snottites; microbial mats; monitoring; Monte Conca; Italy

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INTRODUCTION

The explorations and the scientific investigations of sulfide-rich caves have advanced the knowledge on the development of karst systems. Multi-disciplinary teams have recognized the importance of hydrogen-sulfide on the geochemistry and microbiology of these systems.

Principi (1931) first proposed sulfuric acid-driven speleogenesis and suggested that some caves were created by the interaction of sulfidic waters with limestone. Galdenzi & Maruoka (2003) reported that many limestone caves contain small gypsum deposits formed by evaporation of sulfate-rich water on cave fills or walls. Moreover, they described the morphologic effects of the oxidation of H₂S for the Frasassi Cave, where the limestone walls, exposed to the H₂S vapors, are highly corroded and partially or completely covered by gypsum crusts. Hose & Pisarowicz (1999) reported

Cueva de Villa Luz as a striking example of sulfur-related speleogenesis and a chemoautotrophic ecosystem. In Movile Cave, Romania (Sarbu et al., 1996), performed the first complex study of a sulfidic underground ecosystem based on chemosynthesis, and report sulfuric acid speleogenesis, including sulfur stable isotope data.

Other authors described sulfuric acid speleogenesis and presence of microbial mats also in U.S. caves (Angert et al., 1998; Engel et al., 2001; Engel et al., 2003; Engel et al., 2004). Moreover, Galdenzi et al. (2008) reported an interesting study on the influence of the cave environment and speleogenesis by composition of sulfidic groundwater. By monitoring the chemical parameters of the water, they explained how the seasonal changes in water chemistry interact with the cave speleogenesis. The authors showed that sulfidic groundwater contains both oxidized and reduced sulfur species with significant variation

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during the year. The drastic increase of the H_2S concentration in the spring water was also correlated to the activity of sulfate-reducing-bacteria, identified by Macalady et al. (2006). These bacteria live closely associated with sulfide oxidizing bacteria in the microbial films that cover the water surface.

Engel (2000) published a list of known active sulfidic caves, documented sulfur-oxidizing microorganisms, and explored the biodiversity of sulfidic caves and karst ecosystems distributed worldwide and containing sulfidic springs (Engel, 2007). The same author reported that a wide range of microorganisms live in sulfidic waters and a vast majority of them are sulfur-oxidizing bacteria inhabiting the cave spring and the streams, as well as the cave walls. Some of these bacteria are shown to be acidophilic due to the production of sulfuric acid. All sites with microbial evidences and containing sulfidic springs reported in literature are limestone cave systems, whereas very few gypsum caves are known to have microbial evidences. Recently, Cacchio et al. (2012) investigated the involvement of bacteria in the formation of a new type of speleothems from Grave Grubbo Cave (Italy). This is the first report on sulfidic spring water and its environment in a gypsum karst system: Monte Conca Cave (Italy).

The intense explorations of the Monte Conca Cave in the last two decades and the discovery of its sulfidic spring, prompted us to evaluate their chemical-physical parameters in various hydrogeological and climate conditions (wet and dry seasons). The investigation also provided some evidence of the presence of microbial communities associated with the sulfidic conditions. The presence of snottites, typical structures of the sulfidic spring environment, has been investigated and documented for the first time in the Monte Conca Cave. Furthermore, chemical analysis has shown the presence of iron and sulfur deposits that could be involved in microbiological activity, as described in literature (Hose et al., 2000; Dopson & Johnson, 2012).

The preliminary results of the faunistic monitoring have shown the presence of invertebrate species closely correlated with the microbial life of the sulfidic spring. The experimental results documented the presence of more than 50 species belonging to different phyla. Among these, some show typical troglobitic characteristics, while the presence of others depends on the cave water amount.

SITE DESCRIPTION

The Monte Conca Cave is the longest and deepest gypsum karst system in Sicily. It is located in Messinian evaporites in central-western Sicily and consist of an active sink cave (Inghiottitoio di Monte Conca),

a resurgence (Risorgenza di Monte Conca) and a relict resurgence (Paleorisorgenza di Monte Conca), as described by Madonia and Vattano (2011). The “Inghiottitoio di Monte Conca” is formed by an upper gallery, about 100 m long, developed along the N-S and NW-SE oriented fractures. Following this gallery there is a sequence of four waterfalls: 11, 12, 35, and 26 deep respectively. The lower gallery, about 450 m long, is developed from the base of the last shaft. Additional sub-horizontal galleries, about 1.5 km long, were discovered between 2003 and 2008 (Madonia & Vattano 2011). Figures 1 and 2 show the location of the sulfidic-spring on the plan and the profile views of the cave.

Inghiottitoio di Monte Conca (Si/CL 3000)
Municipality: Campofranco (CL)
Entrance coordinates: 37°29'23"N - 13°42'49"E
Total surveyed length: 2062,5m. Depth: -132m.
Entrance elevation: 275m a.s.l.
Topographic survey: V. Biancone, M. Vattano (2004/2008)
Collaboration: P. Arico, A. Barresi, G. Biondillo, C. Bitetto, M. Calò, R. Davi, C. Lo Cascio, G. Madonia, M. Nicolosi, S. Ruffino.

Fig. 1. Cave map of the Inghiottitoio di Monte Conca: plan. In red dash circle the location of the sulfide-spring (from Madonia & Vattano, 2011).

The cave formation was explained by a morpho-evolution model proposed by Vattano and Madonia (2011). The sulfidic-spring (Fig. 3) is located in the inner part of the lower gallery and is characterized by different sediments such as clay, mud, gravel, and chemical deposits. The size of the spring varies according to the seasons. The smallest size recorded, in dry season, was about 2 m diameter and, at most, 50 cm deep. In dry season there is no flow of surface water into the cave and only small lakes are present under the shafts, therefore the sulfidic spring is not connected with these water bodies. During the wet season and after heavy rains, surface water reach the lower gallery

Fig. 2. Cave map of the Inghiottitoio di Monte Conca: extended profile. In red dash circle the location of the sulfidic-spring (from Madonia & Vattano, 2011).

and may flood the sulfidic spring. A little sulfidic stream with an estimated flow rate of about 1 l/sec is generated by the sulfidic spring and it persists during the dry season too. This stream, about 60 meters long, flows into the final sump of the lower gallery. It still maintains the main features of the sulfidic spring, such as filamentous mats, iron deposits, and suspended sulfur folia or gypsum mineralizations.

A typical smell of hydrogen sulfide is permanent in the inner part of the lower gallery.

Nearby and inside the sulfidic-spring some black and orange mineralization deposits were discovered, and their sampling and subsequent chemical analysis have confirmed the presence of iron as the main element. The genesis of the mineralization deposits is under investigation at present. Peck (1986) described the presence of microbial species in iron and manganese oxides within the caves and proposed the possibility of the chemolithotrophic primary producers in these systems. The presence of microbial species in the Monte Conca Cave was also confirmed by "organic stalactites" commonly known as Snottites, present on the walls and the ceilings, as also described by Galdenzi & Maruoka (2003) in the Frasassi Cave and by Hose and Pisarowich (1999) in the Cueva de Villa Luz. These microbial deposits, can grow and cover the walls with a layer of organic mucous matter, secreting acid drops, rich in H_2SO_4 , with pH values lower than one.

MATERIALS AND METHODS

Chemical-physical measurements

The measurements were carried out between May 2012 and November 2013, in both dry and wet seasons. Sampling and measurements were

Fig. 3. Sulfidic spring containing the characteristic white microbial mats floating on water surface, sulfur suspensions, as well as black and red deposits (photo by F. Fiorenza).

skipped during some wet periods due to the high water flow in the cave.

Air physical parameters

Cave's air temperature (T) and relative humidity (RH) measurements were performed using a HOBOWare sensor (sensitivity, s, $0.01^\circ C$). Table 1 lists average temperature values above the sulfidic spring and along the cave, excluding the entrance gallery that is affected by external temperature. Figure 4 displays an example of sensor measurement performed in July 2012 during the run of cavers.

Water chemistry and chemical-physical parameters

Water temperature was monitored by mercury thermometer (s $0.5^\circ C$) and the values are reported in Table 1. Similarly to the air monitoring, the water temperature average values were calculated excluding the water temperature values of the entrance gallery, very close to temperature of external water stream, that is channelled in the cave.

Fig. 4. Air temperature (°C) and relative humidity (RH,%) trends monitored along the entire cave in July 2012. Data points represent the principal places of the measurement.

The pH measurements *in situ* were carried out with Carlo Erba pH indicator strips ($s = 0.3$ unit of pH) and with a Mettler-Toledo FiveEasy™ pHmeter ($s = 0.01$ unit of pH) in laboratory.

The sulfate concentrations, expressed as barium sulfate (BaSO_4), were determined in laboratory by turbidimetry (Molecular Devices SpectraMax® spectrophotometer) using a linear regression method and confirmed by ionic chromatography (Metrohm). For turbidimetric analysis, amounts of barium chloride solution were added *in situ* to water samples, after acidification at $\text{pH} < 2$ to prevent BaCO_3 precipitation, in order to obtain the stable compound barium sulfate.

Table 1 describes the average values of sulfate amounts for the sulfidic-spring and the cave.

The concentrations of sulfide, expressed as hydrogen sulfide (H_2S), were measured in the laboratory using the spectrophotometric methylene blue method reviewed by Cline (1969) ($\text{LOD} = 0.0084$ ppm, $\text{LOQ} = 0.028$ ppm) with a Molecular Devices SpectraMax® spectrophotometer. The water samples were collected from June 2012 to November 2013.

Two aliquots were collected for each sampling point. The first aliquot was left as is. Zinc acetate (Sigma) was added to the other one in order to precipitate zinc sulfide (ZnS), a sulfur stable compound that can be easily transported and analysed in the laboratory for quantitative determinations. Furthermore, in order to investigate the sulfide distribution versus the spring water depth, three different sub-aliquots were sampled at three different depths (at the surface, 15 cm, and 30 cm deep). H_2S concentration for the stabilized water samples are reported in Table 1. The water samples that were not stabilized showed negligible amount of H_2S .

From May to July 2012, the sulfide sampling and measurements were performed in different points of the cave: entrance, small lakes at the base of the four shafts, in the middle of lower gallery and in the

sulfidic-spring. As no sulfide was detected for the first four sampling points, for the following months we focused our efforts to sample and measure only in the lake at the base of last shaft, in the middle of lower gallery and in the sulfidic-spring.

In July 2013, water samples were collected throughout the cave and in the sulfidic spring in order to evaluate the water hardness and conductivity as well as the concentrations of the main ions, by ionic chromatography (Table 2). The presence of nitrate and sulfur in microbial mats was also evaluated by mass spectrometry. Quadrupole mass spectrometer (WATERS) equipped with electrospray ionization (ESI) interface were used for data acquisition.

Microbiological evidences

As widely described in literature for different caves containing sulfidic-spring the presence of biofilms that hang from the walls and ceilings above spring and the filamentous white mats that float on the sulfidic water surface, are evidence of the presence of bacteria (Hose et al., 2000; Barton & Northup, 2005; Hose & Macalady, 2006; Engel, 2007; Jones et al., 2008; Jones et al., 2011; Dopson & Johnson, 2012). Iron deposits, elemental sulfur suspension or folia, gypsum deposits, peculiar microclimate and chemical parameters were also reported as combination of typical features that indicate microbiological activity. (Hose et al., 2000; Hose & Macalady, 2006; Engel, 2007; Galdenzi et al., 2008; Dopson & Johnson, 2012).

Iron deposits

Black deposits and reddish-orange percolates in the sulfidic-spring area have suggested the presence of iron compounds, respectively, as iron (II) sulfide and iron (III) oxides (Fig. 5). Ionic iron identification was carried out by specific thiocyanate spectrophotometric assay.

Table 1. Monitoring of chemical-physical parameters in the cave and spring of Monte Conca.

	Air T (°C)		Water T (°C)		pH		H ₂ S ppm		BaSO ₄ ppm	
	cave	spring	cave	spring	cave	spring	cave	spring	cave	spring
May 12	15.5	16.8	13.0	16.0	-	-	-	-	-	-
Jun 12	15.26	16.68	13.5	16.0	7.6	7.4	0.18	9.62	-	-
Jul 12	16.21	17.67	14.0	16.5	7.4	6.8	-	20.21	-	-
Aug 12	16.0	17.2	14.0	16.0	7.4	6.8	0.19	23.69	1,456	1,916
Sep 12	16.02	17.37	14.0	16.0	7.6	6.9	0.19	27.81	1,427	1,901
Jan 13	14.30	16.40	11.0	12.5	7.70	7.65	0.035	0.27	1,548	1,444
May 13	15.3	16.3	15.5	16.0	7.70	7.61	0.032	0.47	1,517	1,442
Jun 13	15.1	16.5	14.5	16.5	7.45	6.93	0.088	7.45	1,665	1,887
Jul 13	15.7	17.0	15.0	17.0	7.39	6.77	0.13	16.21	1,415	1,762
Sep 13	15.85	17.10	15.0	17.0	7.68	6.95	0.16	16.93	2,042	1,372
Nov 13	15.90	17.30	15.0	17.0	-	-	-	1.19	-	-

Table 2. Main chemical parameters for cave and spring water of Monte Conca Cave.

	pH (unit)	Conductivity (μS/cm)	Hardness (°F)	SO ₄ ²⁻ (ppm)	S ²⁻ (ppm)	Cl ⁻ (ppm)	NO ₃ ⁻ (ppm)	Ca ²⁺ (ppm)	Mg ²⁺ (ppm)	Na ⁺ (ppm)	K ⁺ (ppm)
cave	7.5	3,341	178.6	1,435	<0.1	162	3	601	69	139	4
spring	6.9	3,415	208.4	1,862	<0.1	149	<0.1	656	108	113	25

Fig. 5. left) Black iron deposit of FeS in sulfidic spring; right) Reddish iron deposits on walls and ceiling nearby the sulfidic spring (photo by F. Fiorenza).

Sulfur deposits

During the dry seasons, the water surface of the sulfidic spring accommodates a white-yellow deposit identified as elemental sulfur. The sulfur identification was carried out by specific test-tube with characteristic red vapor formation on the tube walls as a consequence of sulfur heating and later, formation of yellow sulfur deposits once the temperature drops. Moreover, in order to evaluate elemental sulfur in microbial mats, mass-spectrometry measurements were carried out. Elemental sulfur extraction from microbial mats was performed using the method reported by Hinch et al. (2007), Preisler et al. (2007), and properly revised. The extracted sulfur was identified as polysulfide intermediates by mass spectrometry as reported by Gun et al. (2004).

Microbial mats

White filamentous mats are floating on the surface of the sulfidic-spring (Fig. 6). These mats are floating on the surface of the sulfidic stream and they also cover the surface sediments. In some parts, of the sulfidic-spring, filamentous mats are present to a water depth of about 10 cm.

Fig. 6. Filamentous white microbial mat floating on the surface of the sulfidic-spring (photo by F. Fiorenza).

Snottites

Many limestone caves containing sulfidic water, show peculiar organic “stalactites” called snottites. They are usually few centimetres long, consisting of viscous microbial biofilms that grow and cover the walls and ceilings, secreting acidic drops, rich

in sulfuric acid (Galdenzi et al., 1999a; Galdenzi et al., 1999b; Hose et al., 2000; Galdenzi & Maruoka, 2003; Hose & Macalady, 2006; Barton & Northup, 2007; Macalady et al., 2007; Jones et al., 2011). In the Monte Conca Cave, snottites were found on the walls and ceiling around the sulfidic-spring area

and the measured pH value of their drops ranged from 0.5 to 1.0 (Fig. 7). The presence of sulfuric acid in the distillate was confirmed by its precipitation as barium sulfate and by mass spectrometry measurements as reported by Campana & Risby (1980) for sulfuric acid solutions.

Fig. 7. Snottites on Monte Conca sulfidic spring: left) Snottites hanging from the ceiling; right) Sulfuric acid (pH ~1) dripped from snottites (photo by F. Fiorenza).

Sulfide consumption: kinetic studies

The sulfide consumption in aqueous solutions can occur through two different pathways, abiotic ($\text{H}_2\text{S} + 2\text{O}_2 \rightarrow \text{H}_2\text{SO}_4$) and biotic (microbially-assisted) with different kinetics. In order to evaluate the biotic or abiotic sulfide consumption, two experiments were carried out. In the experiment, the spring water samples, and for comparison, a standard sulfide solutions were monitored at a different time. Aliquots of 40 ml of standard sodium sulfide and spring water samples (both not stabilized) were stored in falcon tubes for 30 days, nearby the sulfidic spring, at the same temperature and darkness conditions. Additional aliquots of spring water and standard sodium sulfide were stabilized with zincum acetate after the sampling, as reference at time zero. The standard sodium sulfide solution concentration was 18.5 ppm, within the sulfide concentrations range for the sulfidic-spring water.

In the second experiment (September 2013) a detailed kinetic study was performed to evaluate the sulfide consumption at different times. In particular eight spring water aliquotes were sampled and zinc acetate was added, respectively, after 2, 4, 8, 16, 24, 32, and 44 hours.

RESULTS AND DISCUSSION

Monte Conca Cave is the first reported example of karst system developed in gypsum, that contains an active sulfidic spring accompanied by a rich microbiota. The characterization of the sulfidic-spring and its peculiar morphologies, as snottites and sulfur suspensions, indicate the presence of sulfur bacteria, which might represent the food source for a chemo-autotrophically based ecosystem. The fauna monitoring in the cave has shown the presence of more than 50 invertebrate species, belonging to different phyla. The most part of them are troglaxene species, which are present in

function of the water flow in the cave. The observations seem to suggest that many troglaxene species are able to survive within the cave and in some cases to reproduce underground, due to the abundance of trophic resources. Some of the observed species show typical troglaxile or troglaxite characteristics, such as depigmentation and anophthalmia. The greatest abundance of taxa was found in the sulfidic spring area and it suggests a complex food web associated with it. Here, as stygobiont species, particularly abundant are Haplotaxida (Anellida), Hydrobiidae (Gasteropoda) and *Proasellus* sp. (Isopoda, Asellidae). These species are prey for *Nepa cinerea* (Hemiptera, Nepidae) and at least three species of *Agabus* (Coleoptera, Dytiscidae), troglaxene species that are able to survive in the sulfidic water. Among the rotting plant detritus material near the spring, we observed detritivore troglaxile or troglaxite species, such as *Mastigonodesmus destefani* (Diplopoda, Polydesmida), *Haplophthalmus danicus* (Isopoda, Trichoniscidae) and *Folsomia candida* (Collembola, Isotomidae). Currently we are working on a new paper focused on the fauna of Monte Conca Cave. Table 3 and Fig. 8 report some examples of the fauna identified in the cave.

Air and water temperature

The air temperature in the sulfidic spring area (zone is displayed by a red dash circle in Figs. 1 and 2) was at least 1°C higher than the average temperature of the cave, up to the highest value of 2.1°C recorded in January 2013 (Table 1). The mean value of air temperature in the cave was 15.6°C, with a seasonal trend in agreement to the outside air temperature measured by SIAS (Servizio Informativo Agrometeorologico Siciliano). The correlation between cave and outside temperature was also described in literature for other caves (Sarbu et al., 2000; Galdenzi et al., 2008). Indeed the mean value of air temperature measured in the sulfide spring zone was 16.9°C, with a minimum of 16.4°C in January 2013 and a maximum of 17.6°C in July 2012.

Table 3. Preliminary list of taxa observed nearby the sulfidic spring.

Phylum	Class	Order	Family	Species
Anellida	Oligochaeta	Haplotaxida	Tubificidae	nd
Artropoda	Entognatha	Collembola	Isotomidae	<i>Folsomia candida</i> Willem 1902
Artropoda	Insecta	Coleoptera	Dytiscidae	<i>Agabus biguttatus</i> (Olivier, 1795)
Artropoda	Insecta	Coleoptera	Dytiscidae	<i>Agabus bipustulatus</i> (Linnaeus, 1767)
Artropoda	Insecta	Coleoptera	Dytiscidae	<i>Agabus brunneus</i> Fabricius, 1768.
Artropoda	Insecta	Hemiptera	Nepidae	<i>Nepa cinerea</i> Linnaeus, 1758
Artropoda	Diplopoda	Polydesmida	Polydesmidae	<i>Mastigonodesmus destefani</i> Silvestri, 1898
Artropoda	Aracnida	Araneae	Nesticidae	<i>Nesticus eremita</i> Simon 1879
Artropoda	Malacostraca	Isopoda	Asellidae	<i>Proasellus</i> sp.
Artropoda	Malacostraca	Isopoda	Trichoniscidae	<i>Haplophthalmus danicus</i> (Budde-Lund, 1880)

Fig. 8. left) *Nepa cinerea* (photo by P. Valenti); right) Oligochaeta Haplotaxida and *Proasellus* sp. in sulfidic water (photo by F. Fiorenza).

The sulfidic spring with an air temperature variation of just 0.5°C could be considered as an ecosystem able to counteract the external air temperature variation, more efficiently than the entire cave system which has shown a temperature variation of 1.3°C. The relative humidity was about 90% throughout the cave.

Water temperature measurements showed that the spring water was always warmer than the cave water by about 2°C. It is in agreement with the temperature data reported for the main sulfidic caves, except for the Acquasanta Terme Cave (Jones et al., 2010).

The cave water temperature decreased significantly in January 2013 as consequence of a large volume of cold water (outside air temperature 7.7°C) that entered from the surface stream, while the spring water maintained a higher temperature. In May 2013, probably due to the very large volume of warm water that entered the cave (425 mm of rain at 19°C), the smallest gap between the temperatures of the spring water and the water cave was observed. Cave water temperature increased up to the highest value of 15.3°C while the spring water still maintained its typical temperature of 16°C. Figure 9 describes the temperatures trend for cave and spring water and for outside air temperature as a function of the millimeters of rain during the monitoring period.

The spring area has always shown air and water temperature higher than the air and water cave temperature. Herbet et al. (2005) describe a water temperature increase coincident with the microbial layer in the South Andrea Black Hole and Galdenzi et al. (2008) describe sulfidic ground water in the

Frasassi Cave slightly warmer than the seepage water. These features may be related to the bacterial life cycle. Indeed, according to Lehninger et al. (1993), living organisms can be considered as open thermodynamic systems that preserve their internal order by taking from their surroundings free energy as nutrients or sunlight and returning to the same surroundings an equal amount of energy as heat, compensating the order created within cells ($\Delta S_{\text{system}} < 0$) with the disorder created in their surrounds ($\Delta S_{\text{surroundings}} > 0$).

In this way the total entropy still remain positive: $\Delta S_{\text{total}} = \Delta S_{\text{system}} + \Delta S_{\text{surroundings}} > 0$.

Meantime, many chemical reactions at the basis of biological processes occur with negative enthalpy that means releasing of heat in the surround and, consequentially, surrounding temperature increase. Negative value of enthalpy and positive value of entropy contribute to the decrease of the Gibbs free energy of the system, as described by the Gibbs free energy equation: $\Delta G = \Delta H - T\Delta S < 0$.

pH measurements

During the wet season (January-May 2013), the pH values for cave and spring water were quite similar with an average value of about 7.5, probably due to the large volume of stream water that flowed into the cave, diluting the spring water (Table 1).

The pH recorded in the dry season (June-September 2012 and June-September 2013) for the cave water was about 7.5, while for spring water was about 6.8 (Table 1). As well as due to the H₂S content, the pH

Fig. 9. The plot of water spring, cave, and surface temperatures versus the amount of rain (mm), during the monitored period.

decrease found for spring water might be related to microbial activity. It is well known that different *Beggiatoa* species are predominantly distributed in the suboxic and oxic zone, in which O_2 is used as the electron acceptor for the oxidation of H_2S to S^0 in a first step and to SO_4^{2-} in a second one (Sayama et al., 2005; Kamp et al., 2006; Preisler et al., 2007). One of the hydrolytic species at the pH value measured is HS^- , so the biological aerobic oxidation could be summarized by the following reactions:

The second oxidation step is an acidogenic process that produces sulfuric acid, which contributes to the pH decrease and the increase of sulfate amount in spring water, according to our results.

H₂S measurements

The peculiar feature to demonstrate the sulfidic nature of a spring water is the presence of H_2S amount at concentration higher than 1 ppm. The spring water of Monte Conca is well known to the cavers for the bad smell of rotten eggs that suggests the presence of sulfidric acid. Our monitoring, for the first time, highlighted the high sulfide content in the spring water of the lower gallery. The data reported in Table 1 indicates, a negligible H_2S amount, ranging from 0.03 ppm to 0.18 ppm in the cave water; while for the spring water a H_2S amount one and two order of magnitude higher was found. Although the H_2S level in spring water was always higher than cave water, its values were strictly dependent on the seasons. Changes in sulfide concentration, correlated with the relative dilution of groundwater and caused by the amount of seepage water during the wet seasons, was also described by Galdenzi et al. (2008) for Frasassi Cave.

Noteworthy was the gradient of sulfide concentrations monitored in the spring water at three different

depth levels, water surface, depths of 15 and 30 cm. The results are shown in Fig. 10. The data indicate a lower sulfide concentration for the first level and similar values for the other two levels, always higher than the first one. This difference is in agreement to the presence of clearly visible microbial mats at the surface and in the shallow water, that consume the sulfide for their metabolism.

Table 1 reports only the mean values of H_2S content for the three depth levels. The H_2S concentration values were obtained by water samples stabilized with zinc acetate because the same samples unstabilized have shown a negligible H_2S amount. All sulfide quantitative measurements were carried out in laboratory, 20 hours after sampling. The evident presence of filamentous microbial mats in sulfide spring water and the fast sulfide consumption suggests that the microbial mats could be composed of sulfur oxidant bacteria, such as *Beggiatoa* and *Acidithiobacillus*.

H₂S consumption rate

In order to evaluate the biotic H_2S consumption in spring water samples a kinetic comparison with a pure abiotic consumption in aqueous sodium sulfide solution was performed. The experimental results for not stabilized aliquots of spring water samples (stored for 30 days) have reported the complete disappearance of sulfide, whereas no significant change occurred for the sodium sulfide samples (Table 4). These experiments were replicated at two different times. Our results are in agreement with the literature data which reports that abiotic oxidation rates are generally very slow (Millero et al., 1987). Recently, Luther et al. (2013) report an abiotic sulfide consumption rate of about 1 μM per day despite a biotic rate of about one thousand time higher was recently reported.

Based on our experimental evidences and literature data we have performed detailed kinetic studies to evaluate the sulfide consumption at different time periods. In particular, in September 2013 have been sampled 8 spring water aliquotes. In the first one

zinc acetate was added immediately, in the seven remains aliquots zincum acetate was added after 2, 4, 8, 16, 24, 32, and 44 hours respectively. The results

indicate a drastic decrease of sulfide concentration after just two hours (Fig. 11) according to the high sulfide consumption rate typically for biotic oxidation.

Fig. 10. Sulfide concentrations (ppm) for the three different levels monitored in sulfidic spring. In inset, the data for January and May 2013.

Table 4. Sulfur amount for stabilized and unstabilized samples at different storage times.

		Spring water		Aqueous sulfide solution (Na ₂ S)	
		Stabilized sample (with Zn-acetate)	Not stabilized sample	Stabilized sample (with Zn-acetate)	Not stabilized sample
July 2012	Time zero *	27.8	0.20	18.5	18.5
	30 days	27.6	0.16	18.4	18.3
July 2013	Time zero *	16.2	0.15	18.5	18.5
	30 days	16.1	0.14	18.5	18.1

* samples analyzed in laboratory after 20 hrs

Fig. 11. Sulfur consumption for unstabilized spring water sample at different storage times. Inset the detailed kinetic after 8 hrs storage time.

Water chemistry: nitrate measurements by MS

As reported in Table 2 the cave and spring water showed different ion composition and chemical-physical parameters. The data have confirmed the abundance of sulfate in the spring water and the total sulfide consumption (for not stabilized samples). The different nitrate amount between the two waters, respectively 3 and <0.1 μM , might be a further evidence of thiobacterial activity. Low nitrate concentrations were found in sulfidic spring of Acquasanta Terme Cave and in Frasassi Cave, respectively <1 μM and <0.7 μM , suggesting that nitrate is scavenged by anaerobic microorganisms (Macalady et al., 2006; Jones et al., 2010). Indeed, as widely reported in literature, different *Beggiatoa* species employ nitrate in their metabolism,

especially, in the anoxic water zones (Mußmann et al., 2003; Kamp et al., 2006; Engel, 2007; Hinck et al., 2007; Preisler et al., 2007; Yekta, 2011). These *Beggiatoa* species contain nitrate reductase which enables them to use nitrate as electron acceptor to sulfide oxidation (MacHatton et al., 1996). They are able to internally accumulate nitrate in levels up to some order of magnitude higher than the nitrate concentration of their surrounding water (Kamp et al., 2006) using this intracellular stored nitrate for sulfide oxidation with spatially separated pathways. As proposed by Sayama et al. (2005) the *Beggiatoa* filaments stored nitrate in vacuoles and transport it downwards to anoxic zone, with their gliding mechanism. This stored nitrate is used for anaerobic sulfide oxidation to elemental sulfur, which will be stored in vacuoles for aerobic oxidation to sulfate in oxic zone. In *Beggiatoa* cultures the nitrate amount decrease from the initial amount, with a consequently nitrate enrichment on bacteria vacuoles (Kamp et al., 2006).

Considering the different nitrate amounts found into the two water samples (Table 2) and supported by literature data, additional experiments were performed to evaluate the presence of nitrate in filamentous mats of Monte Conca Cave. For this purpose triplicate samples of the microbial mats were analyzed using the method described by Hinck et al. (2007) and Preisler et al. (2007) revised by us. The samples were transferred into 250 µl of deionized water and frozen at -20°C for 2 days, causing cell rupture. After thawing, the samples were centrifuged (5 minutes at 4000 x g) and the supernatants were analyzed in electrospray mass spectrometry (ESI-MS) in negative ion mode to search nitrate. The obtained results (Fig. 12) show the characteristic mass peaks for nitrate (62.01 m/z, base peak) and for nitrite (46.02 m/z). The found nitrate amounts were determined within the mats of the Monte Conca Cave and include the porewater nitrate as well as intracellular nitrate, released from the bacterial filaments (as described for *Beggiatoa*) during freezing and thawing. Therefore, in order to evaluate nitrate presence in spring water using mass spectrometry, an ESI spectrum of spring water sample, after filtering, was recorded at the same instrumental conditions. In this case the most abundant peaks refer to sulfate anion (Campana & Risby, 1980) and nitrate peak is present under 10% of base peak.

Fig. 12. Mass spectra analysis of filamentous mats with the characteristic peaks for nitrate (62.01 m/z) and nitrite (46.02 m/z).

Water chemistry: sulfur measurements by MS

Elemental sulfur content is closely related to bacterial activity because it is widely described that metabolism of various sulfur bacteria produce elemental sulfur as intermediate of the sulfide oxidation (Hose et al.,

2000; Barton & Northup, 2005; Kamp et al., 2006; Engel, 2007; Hinck et al., 2007; Schwedt et al., 2012). In order to evaluate the elemental sulfur in microbial mats (reaction 4), the remaining pellet (after centrifugation) was dried in air and dissolved in 500 µl

of pure methanol for 3 days, therefore few μl of 0.1M NaOH solution were added up to pH 10. After 4 hours, the sample was centrifuged (5 minutes at 4000 x) and the supernatant was analyzed by ESI-MS in negative ion mode in order to evaluate polysulfide signals. At basic pH, the disproportionation of polysulfide is induced (equation 6), thus analytical species can be analyzed in ESI-MS (Gun et al., 2004).

Figure 13 reports the mass spectra that show the characteristic peaks for thiosulfate and polysulfides chains (inserted spectrum from 160 to 275 m/z). Significant changes in signal enhancement were recorded by varying the cone voltage from 30 to 80 V and the spray voltage from 2.5 to 4.5 kV, so, in order to enhance the linear polysulfide peaks respect to the polysulfide adducts, compromise values of 35 V and 3.5 kV were respectively chosen. Table 5 reports the main assigned peaks.

Fig. 13. Microbial mat mass spectra for sulfur, which show peaks for thiosulfate poly-sulfides chains peaks (see table 5 for the assignment of peaks).

Table 5. Characteristic mass peaks for sulfidic spring water, snottites, and filamentous microbial mats treated for nitrate or sulfur.

Samples	Species (m/z)									
Sulfidic spring water	Cl ⁻ (34.58)	NO ₂ ⁻ (45.67)	SO ₃ ⁻ (79.79)	HSO ₄ ⁻ (96.70)						
Microbial mat (Nitrate/Nitrite species)	NO ₂ ⁻ (46.02)	NO ₃ ⁻ (62.01)								
Microbial mat (Sulfur species)	HS ₂ O ₃ ⁻ (112.79)	Na S ₂ O ₃ ⁻ (134.80)	Na S ₅ ⁻ (182.92)	HS ⁻ 2Na ₂ S (188.93)	HS ₆ ⁻ (192.75)	NaS ₆ ⁻ (214.98)	NaS ₄ ⁻ Na ₂ S (228.98)	NaS ₇ ⁻ (246.98)	NaS ₅ ⁻ Na ₂ S (260.88)	NaS ₂ O ₃ ⁻ Na ₂ S ₂ O ₃ (292.79)
Snottites	SO ₃ ⁻ (79.81)	HSO ₄ ⁻ (96.99)								

All these data have demonstrated that the microbial mats contain species that are able to perform anaerobic sulfide oxidation (reaction 4). The metabolism of these microbial species could use the sulfide as main electron donor to produce intermediate species of sulfur (Engel, 2007; Hinck et al., 2007; Berg et al., 2013) and, as Engel (2007) describes, the elemental sulfur would be produced intra or extracellular. Therefore, elemental sulfur could be found as solid suspensions giving a milky color to the water surface

(Nelson et al., 1986). Mass spectra of solid suspension treated as before described, have confirmed the presence of sulphur species in the microbial mat by polysulfide adducts.

Iron measurements by UV-Vis

The presence of iron ions in the sulfidic-spring area might be associated with the microbial activity. Indeed, it is well known that some bacteria species such as *Thiobacillus ferrooxidans* or *Acidimicrobium ferrooxidans*

(Hose et al., 2000; He et al., 2005; Hose & Macalady, 2006; Baskar et al., 2008; Jones et al., 2011; Dopson & Johnson, 2012), *Beggiatoa* (Nelson et al., 1986; Hose & Macalady, 2006; Preisler et al., 2007; Druschel et al., 2008) or other sulfur bacteria, can oxidize hydrogen sulfide to sulfuric acid, using Fe(III) as electron acceptor for the sulfide anaerobic oxidation. Oxidized iron is reduced by HS⁻ as described in reaction 7.

Meantime, the soluble reduced iron reacts with HS⁻ to form insoluble iron sulfide.

Black deposits of iron sulfide were largely found in water spring of Monte Conca. The thiocyanate assay, performed on these deposits, has disclosed the presence of iron. A portion of black deposit was dissolved in chloridic acid and oxidized to Fe³⁺ as reported in reactions 9-10. In presence of NaSCN, the Fe³⁺ forms a characteristic bloody red compound of FeSCN²⁺ (reaction 11) with the typical absorbance at 460 nm (Fig. 14). In Fig. 14 for comparison is reported the absorption UV-Vis spectrum of a FeSCN²⁺ reference solution (2 x 10⁻⁵ M). The same thiocyanate assay performed into a spring water sample has given a negative result according to the complete disappearance of Fe²⁺ from solution, due to its complete precipitation as FeS.

Snottites

The presence of abundant snottites (Fig. 6), described for the first time in a gypsum cave, indicates the existence of a large aerial microbial life related to the

sulfide-rich water microbial life (Fig. 7). As described in literature (Hose & Macalady, 2006; Jones et al., 2011; Dopson & Johnson, 2012) snottites have very low biodiversity and are dominated by *Acidithiobacillus thiooxidans*. Actinobacteria related to *Acidimicrobium ferrooxidans* have also been detected in Frasassi snottites. This suggests the importance of the iron ions in the bacterial activity development.

In order to investigate the presence of iron in the snottites of Monte Conca Cave, a sample was analyzed with thiocyanate assay, giving a positive result (Fig. 14). Furthermore sulfate and pH measurements on secreting acid drop were performed. The pH measured *in situ* by pH indicator strips (Fig. 7 right) and in laboratory by pH-meter, has shown a pH value ranging from 0.5 to 1.0 unit. The sulfuric acid dropped by snottites was analysed by mass spectrometry (Fig. 15) and the results reported on Table 5 indicate the presence of pure sulfuric acid.

As noted by Hose et al. (2000), *Acidithiobacillus thiooxidans* in the most abundantly species in snottites. Under low oxygen content is capable of producing a local pH of about 2, whereas in the presence of air, the pH may drop below 1. Therefore, the pH values measured for the snottites sampled in Monte Conca, suggests totally aerobic condition for the sulfide oxidation. In addition, the same authors report a strongly exothermic process for the complete oxidation of hydrogen sulfide to sulfuric acid, mediated by sulphur-oxidizing bacteria. A negative enthalpy means heat release and simultaneous temperature increase of the surroundings. In our case, the strongly exothermic process involved in snottites genesis could contribute to the increase of the air temperature in the spring area. Moreover, the presence of sulfate in snottites was further confirmed by its precipitation as barium sulfate after addition of a barium chloride solution.

CONCLUSIONS

The aims of this work have been the monitoring of the main chemical-physical parameters of the Monte Conca Cave and the identification and the characterization of its sulfidic-spring, located in the terminal gallery. The evaluation of the main chemical features that are peculiar of sulfidic-spring cave, indicates a clear evidence of bacterial activity.

The monitoring activity, conducted over 18 months, has shown the difference of chemical-physical parameters between the sulfidic spring and the rest of the cave, highlighting a microenvironment that is less influenced by external conditions.

The main features of a sulfidic spring, widely described in literature, were discovered in Monte Conca Cave. The evaluation of these features and the considering of additional evidences

Fig. 14. UV-Vis spectra of Fe(SCN)²⁺ obtained from black deposit, snottites samples and Fe³⁺ aqueous solution as reference.

Fig. 15. Mass spectrum of sulfuric acid dripping from snottites (SO_3^- 79.81 m/z and HSO_4^- 96.99 m/z).

such as biotic sulfide consumption, presence of nitrate on filamentous mats, and the different pH values between cave and spring water allowed to characterize, for the first time, a complex sulfidic system in a gypsum karst system.

Thus, Monte Conca Cave represents the only gypsum karst system described so far that includes an active sulfidic spring with an undoubtedly microbial activity. The presence of sulfur bacteria may be the source of an autotrophic system in close correlation with the biological cycle of many species of living organisms. Noteworthy is that in this study no biological measurements were carried out to ascertain the nature of the microbiota clearly present in the sulfidic spring, but only chemical and chemical-physical measurements were carried out. The greater abundance of taxa found close to the sulfidic spring seems to suggest a complex food web associated with it and biological studies are needed for a complete site descriptions. At present, a detailed investigation of the fauna living in sulfidic-spring area is in progress and in addition, future investigation will focus on molecular biological methods to identify the microbial species.

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REFERENCES

- Angert E.R., Northup D.E., Reysenbach A., Peek A.S., Goebel B.M. & Pace N.R., 1998 - *Molecular phylogenetic analysis of a bacterial community in Sulphur River, Parker Cave, Kentucky*. *American Mineralogist*, **83**: 1583–1592.
- Barton H.A. & Northup D.E., 2005 - *Geomicrobiology in a cave environments: Past, current and future perspectives*. *Journal of Cave and Karst Studies*, **69** (1): 163–178.
- Baskar S., Baskar R., Lee N., Kaushik A. & Theophilus P.K., 2008 - *Precipitation of iron in microbial mats of the spring waters of Borra Caves, Vishakapatnam, India: some geomicrobiological aspects*. *Environmental Geology*, **56**: 237–243.
<http://dx.doi.org/10.1007/s00254-007-1159-y>
- Berg S.J., Schwedt A., Kreutzmann A.C., Kuypers M.M.M. & Milucka J., 2013 - *Polysulfides as intermediates in the oxidation of sulfide to sulfate by Beggiatoa*. *Applied and Environmental Microbiology*, **80** (2): 629–636.
<http://dx.doi.org/10.1128/AEM.02852-13>
- Cacchio P., Ercole C., Contento R., Cappuccio M., Preite Martinez M., Del Gallo M. & Lepidi A., 2012 - *Involvement of bacteria in the origin of a newly described speleothem in the gypsum cave of Grave Grubbo (Crotone, Italy)*. *Journal of Cave and Karst Studies*, **74** (1): 7–18.
<http://dx.doi.org/10.4311/2010MB0136R>
- Campana J.E. & Risby T.H., 1980 - *Analysis of sulfuric acid aerosol by negative ion chemical ionization mass spectrometry*. *Analytical Chemistry*, **52**: 398–400.
<http://dx.doi.org/10.1021/ac50053a007>
- Cline J.D., 1969 - *Spectrophotometric determination of hydrogen sulfide in natural waters*. *Limnology and Oceanography*, **14** (3): 454–458.
<http://dx.doi.org/10.4319/lo.1969.14.3.0454>
- Dopson M. & Johnson D.B., 2012 - *Biodiversity, metabolism and applications of acidophilic sulfur-metabolizing microorganisms*. *Environmental Microbiology*, **14** (10): 2620–2631.
<http://dx.doi.org/10.1111/j.1462-2920.2012.02749.x>

- Druschel G.K., Emerson D., Sutka R., Suchecki P. & Luther III G.W., 2008 - *Low-oxygen and chemical kinetic constraints on the geochemical niche of neutrophilic iron(II) oxidizing microorganisms*. *Geochimica et Cosmochimica Acta*, **72**: 3358–3370.
<http://dx.doi.org/10.1016/j.gca.2008.04.035>
- Dunker R., Røy H., Kamp A. & Jørgensen B.B., 2011 - *Motility patterns of filamentous sulfur bacteria, Beggiatoa spp.* *Federation of European Microbiological Societies - Microbiology Ecology*, **77**: 176–185.
- Engel A.S., 2000 - *Microbially-enhanced weathering in subsurface habitats: sulfur-oxidizing bacteria and the cave environment*. *Proceedings of the Friends of Karst Meeting, Cluj, Romania - B. Onac and T. Tamas (eds.) Karst Studies and Problems, 2000 and Beyond*, 130–134.
- Engel A.S., Porter L.M., Kinkle B.K. & Kane T.C., 2001 - *Ecological assessment and geological significance of microbial communities from Cesspool Cave, Virginia*. *Geomicrobiology Journal*, **18**: 259–274.
<http://dx.doi.org/10.1080/01490450152467787>
- Engel A.S., Lee N., Porter L.M., Stern A.L., Bennett C.P. & Wagner M., 2003 - *Filamentous "Epsilonproteobacteria" dominate microbial mats from sulfidic cave springs*. *Applied and Environmental Microbiology*, **69** (9): 5503–5511.
<http://dx.doi.org/10.1128/AEM.69.9.5503-5511.2003>
- Engel A.S., Stern L.A. & Bennett P.C., 2004 - *Microbial contributions to cave formation: new insights into sulfuric acid speleogenesis*. *Geology*, **32** (5): 369–372.
<http://dx.doi.org/10.1130/G20288.1>
- Engel A.S., 2007 - *Observation on the Biodiversity of sulfidic karst habitats*. *Journal of Cave and Karst Studies*, **69** (1): 187–206.
- Galdenzi S., Forti P. & Menichetti M., 1999 - *L'acquifero sulfureo di Frasassi: aspetti idrogeologici e naturalistici*. *Atti "Conv. Naz. sull'inquinamento delle grotte e degli acquiferi carsici e possibili ricadute sulla collettività"* Ponte di Brenta, PD 1998, Ed. Imprimatur: 181–193.
- Galdenzi S., Menichetti M., Sarbu S. & Rossi A., 1999 - *Frasassi caves: a biogenic hypogean karst system?* *Proceedings European Conference Karst 99, Etudes de Géographie physique*. CAGEP, Université de Provence, Suppl. XXVIII: 101–106.
- Galdenzi S. & Maruoka T., 2003 - *Gypsum deposits in the Frasassi caves, central Italy*. *Journal of Cave and Karst Studies*, **65** (2): 111–125.
- Galdenzi S., Cocchioni M., Morichetti L., Amici V. & Scuri S., 2008 - *Sulfidic ground-water chemistry in the Frasassi Caves, Italy*. *Journal of Cave and Karst Studies*, **70** (2): 94–107.
- Gun J., Modestov A.D., Kamyshny Jr.A., Ryzkov D., Gitis V., Golfman A., Lev O., Hultsch V., Grishchek T. & Worch E., 2004 - *Electrospray ionization mass spectrometric analysis of aqueous polysulfide solutions*. *Microchimica Acta*, **146**: 229–237.
<http://dx.doi.org/10.1007/s00604-004-0179-5>
- He Z., Hu Y., Zhong H., Hu W. & Xu J., 2005 - *Preliminary proteomic analysis of Thiobacillus ferrooxidans growing on elemental sulphur and Fe²⁺ separately*. *Journal of Biochemistry and Molecular Biology*, **38** (3): 307–313.
<http://dx.doi.org/10.5483/BMBRep.2005.38.3.307>
- Herbert R.A., Ranchou-Peyruse A., Duran R., Guyoneaud R. & Schwabe S., 2005 - *Characterization of purple sulfur bacteria from South Andros Black Hole cave system: highlights taxonomic problems for ecological studies among the genera Allochromatium and Thiocapsa*. *Environmental Microbiology*, **7** (8): 1260–1268.
<http://dx.doi.org/10.1111/j.1462-2920.2005.00815.x>
- Hinck S., Neu T.R., Lavik G., Musmann M., de Beer D. & Jonkers H.M., 2007 - *Physiological adaptation of nitrate-storing Beggiatoa sp. to diel cycling in a phototrophic hypersaline mat*. *Applied and Environmental Microbiology*, **73** (21): 7013–7022.
<http://dx.doi.org/10.1128/AEM.00548-07>
- Hose L.D. & Pissarowicz J.A., 1999 - *Cueva de Villa Luz, Tabasco, Mexico: reconnaissance studies of an active sulfur spring cave*. *Journal of Cave and Karst Studies*, **61** (1): 13–21.
- Hose L.D., Palmer A.N., Palmer M.V., Northup D.E., Boston P.J. & DuChene, H.R., 2000 - *Microbiology and geochemistry in a hydrogen-sulfide-rich karst environment*. *Chemical Geology*, **169**: 399–423.
[http://dx.doi.org/10.1016/S0009-2541\(00\)00217-5](http://dx.doi.org/10.1016/S0009-2541(00)00217-5)
- Hose L.D. & Macalady J.L., 2006 - *Observations from active sulfidic karst systems: Is the present the key to understanding Guadalupe mountain speleogenesis?* *New Mexico Geological Society Guidebook, 57th Field Conference, Caves and Karst of Southeastern New Mexico*: 185–194.
- Jones D.S., Lyon E.H. & Macalady J.L., 2008 - *Geomicrobiology of biovermiculations from the Frasassi Cave System, Italy*. *Journal of Cave and Karst Studies*, **70** (2): 78–93.
- Jones D.S., Tobler D.J., Schaperdorth I., Mainiero M. & Macalady J.L., 2010 - *Community structure of subsurface biofilm in the sulfidic caves of Acquasanta Terme, Italy*. *Applied and Environmental Microbiology*, **76** (17): 5902–5910.
<http://dx.doi.org/10.1128/AEM.00647-10>
- Jones D.S., Albrecht H.L., Dawson K.S., Schaperdorth I., Freeman K.H., Pi, Y., Pearson A. & Macalady J.L., 2012 - *Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm*. *ISME Journal*, **6** (1): 158–170.
- Kamp A., Stief P. & Schulz-Vogt H.N., 2006 - *Anaerobic sulfide oxidation with nitrate by a freshwater Beggiatoa enrichment culture*. *Applied Environmental Microbiology*, **72** (7): 4755–4760.
<http://dx.doi.org/10.1128/AEM.00163-06>
- Lehninger A., Nelson D.L. & Cox M.L., 1993 - *Principles of Biochemistry*, Worth Publishers Inc., U.S.
- Luther III G.W., Findlay A.J., MacDonald D.J., Owings S.M., Hanson T.E., Beinart R.A. & Girguis P.R., 2013 - *Thermodynamics and kinetics of sulfide oxidation by oxygen: a look at inorganically controlled reactions and biologically mediated processes in the environment*. *Frontiers in Microbiology*, **2**: 1–9.
- Macalady J.L., Lyon E.H., Koffmann B., Alberton L.K., Meyer K., Galdenzi S. & Mariani S., 2006 - *Dominant microbial populations in limestone-corroding stream biofilm, Frasassi cave system, Italy*. *Applied Environmental Microbiology*, **72** (8): 5596–5609.
<http://dx.doi.org/10.1128/AEM.00715-06>
- Macalady J.L., Jones D.S. & Lyon E.H., 2007 - *Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy*. *Environmental Microbiology*, **9** (6): 1402–1414.
<http://dx.doi.org/10.1111/j.1462-2920.2007.01256.x>
- MacHatton S.C., Barry J.P., Jannasch H.W. & Nelson D.C., 1996 - *High nitrate concentrations in vacuolate, autotrophic marine Beggiatoa spp.* *Applied and Environmental Microbiology*, **62**: 954–958.
- Madonia G. & Vattano M., 2011 - *New Knowledge on the Monte Conca gypsum karst system (central-western Sicily, Italy)*. *Acta Carsologica*, **40** (1): 53–64.
- Millero F.J., Hubinger S., Fernandez M. & Garnett S., 1987 - *Oxidation of H₂S in seawater as a function of temperature, pH and ionic strength*. *Environmental Science & Technology*, **21**: 439–443.
<http://dx.doi.org/10.1021/es00159a003>

- Mußmann M., Schulz H.N., Strotmann B., Kjar T., Nielsen L.P., Rossellò-Mora R.A., Amann R.I. & Jorgensen B.B., 2003 - *Phylogeny and distribution of nitrate-storing Beggiatoa spp. in coastal marine sediments*. Environmental Microbiology, **5**: 523-533.
- Nelson D.C., Jorgensen B.B. & Revsbech N.P., 1986 - *Growth pattern and yield of chemoautotrophic Beggiatoa sp. in oxygen-sulfide microgradients*. Applied and Environmental Microbiology, **52**: 225-233.
- Peck S.B., 1986 - *Bacterial deposition of iron and manganese oxides in north American caves*. Speleological Society Bulletin, **48**: 26-30.
- Preisler A., de Beer D., Lichtschlag A., Lavik G., Boetius A. & Barker Jorgensen B., 2007 - *Biological and chemical sulfide oxidation in a Beggiatoa inhabited marine sediment*. The ISME Journal, **1**: 341-353.
- Principi P., 1931 - *Fenomeni di idrologia sotterranea nei dintorni di Triponzo (Umbria)*. Grotte d'Italia, **5**: 1-4.
- Sarbu S.M., Kane T.C. & Kinkle B.K., 1996 - *A chemoautotrophically based cave ecosystem*. Science, **28**: 1953-1955.
<http://dx.doi.org/10.1126/science.272.5270.1953>
- Sarbu S.M., Galdenzi S., Menichetti M. & Gentile G., 2000 - *Geology and biology of the Frasassi Caves in Central Italy, an ecological multidisciplinary study of a hypogenic underground ecosystem*. In: Wilkens D., Culver D. & Humphreys W.F. (Eds), *Ecosystem of the World, Subterranean Ecosystem*. New York, Elsevier: 359-378.
- Sayama M., Risgaard-Petersen N., Nielsen L.P., Fossing H. & Christensen P.B., 2005 - *Impact of bacterial NO₃⁻ transport on sediment biogeochemistry*. Applied and Environmental Microbiology, **71**: 7575-7577.
<http://dx.doi.org/10.1128/AEM.71.11.7575-7577.2005>
- Schwedt A., Kreutzmann A.C., Polerecky L. & Schulz-Vogt H.N., 2012 - *Sulfur respiration in a marine chemolithoautotrophic Beggiatoa strain*. Frontiers in Microbiology, **2**: article 276.
- Temara A., De Ridder C., Kuenen J.G. & Robertson L.A., 1993 - *Sulfide-oxidizing bacteria in the burrowing echinoid, Echinocardium cordatum (Echinodermata)*. Marine Biology, **115**: 179-185.
<http://dx.doi.org/10.1007/BF00346333>
- Yekta S.S., 2011- *A model study of the effects of sulfide-oxidizing bacteria (Beggiatoa spp.) on phosphorus retention processes in hypoxic sediments: Implications for phosphorus management in the Baltic Sea*. Boreal Environment Research, **16**: 167-184.