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High Levels of Antibiotic Resistance but No Antibiotic Production Detected Along a Gypsum Gradient in Great Onyx Cave, KY, USA

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Abstract: A preliminary study of antibiotic production and antibiotic resistance was conducted in Great Onyx Cave in Mammoth Cave National Park, KY, to determine if gypsum ($CaSO_4 \cdot 2H_2O$) affects these bacterial activities. The cave crosses through the width of Flint Ridge, and passages under the sandstone caprock are dry with different amounts of gypsum. The Great Kentucky Desert hypothesis posits that gypsum limits the distribution of invertebrates in the central areas of Great Onyx Cave. Twenty-four bacterial isolates were cultivated from swabs and soils. Using three methods (soil crumb, soil crumb with indicator bacteria, and the cross-streak method using isolated bacteria) we did not detect any production of antibiotics. Antibiotic resistance was widespread, with all 24 isolates resistant to a minimum of two antibiotics of seven tested, with three isolates resistant to all. Antibiotic resistance was high and not correlated with depth into the cave or the amount of gypsum. The Great Kentucky Desert hypothesis of the negative effects of gypsum seems to have no impact on bacterial activity.

Keywords: gypsum; antibiotic production; antibiotic resistance; Great Onyx Cave; Great Kentucky Desert

1. Introduction

The discovery and manufacturing of antibiotics have been our central defense against bacterial infection since antibiotics were first discovered 80 years ago, but as canny as our medical evolution has been, bacteria evolve and gain resistance quickly [1]. Both antibiotic production and antibiotic resistance are natural processes. Antibiotic resistance has been shown to be an ancient process [2], and antibiotic production must have preceded it. Gupta [3] proposed that the evolution of Gram-negative diderm cell walls was a defense against antibiotics. The World Health Organization [4] has monitored antibiotic resistance and has declared it a global health crisis. Development of new antibiotics and new strategies to fight infections are key in reducing morbidity and mortality due to infectious disease, and to reduce health care costs.

Caves are extreme environments due to high humidity, constant low temperatures, and absence of light, which results in oligotrophic conditions. The cave microbiome has great potential as a novel resource for drug discovery [5–7] due to microbial competition for limited resources. When resources are scarce, microbial production of secondary metabolites including toxins for predation of bacteria on other bacteria [8,9], ionophores, bioregulators, and signal molecules [7] could increase to create a competitive advantage [10,11]. Martinez et al. [12] reviewed antibiotic production and resistance, taking a global view that these processes play fundamental ecological roles that shape the structure of microbial communities. Antibiotic resistance is an example of bacterial adaptation to stressful situations. Secondary metabolites have a hormetic effect, where their role is concentration dependent, for example

a beneficial response at low concentrations but toxic at higher concentrations. These agents strongly influence bacterial physiology through a broad range of ecological roles going beyond traditional "weapons and shields" [12]. Many researchers believe that natural products are the most promising source of novel antibiotics, and cave microbes have great potential for the development of new bioactive antimicrobial metabolites [5,6,13].

Gypsum ($CaSO_4 \cdot 2H_2O$) is non-hazardous, but does pose a slight inhalation risk in powdered form [14]. Gypsum has a wide range of uses [15], and is widely used as a soil additive to counter alkaline conditions. Gypsum added to soil increases water retention and maintains a workable texture. Studies going back to the early 1900s of effects of gypsum on soil microbes generally show no negative effects on microbial growth or metabolic processes, although results vary with concentration [16]. Ajjappalavara et al. [17] showed that the application of gypsum controlled bacterial wilt disease due to *Ralstonia solanacearum* in *Solanum melongena* (an eggplant), which they suggested was due to increased calcium nutrition to the plant but the high calcium suppressed the pathogen. They did not do experiments to test these hypotheses.

Mammoth Cave National Park (MCNP) in Kentucky is part of the Interior Low Plateau region that is characterized as an area of low relief karst that covers an area of about 800 km^2 [18]. The caves form by dissolution in limestone protected by the overlying Big Clifty caprock, made up of sandstones and shales [18]. There are cracks and breaks in the caprock that allow for infiltration of water. Despite its large size, complexity, and high biodiversity, there are very few microbiology studies done in the region [19]. This preliminary study was done in Great Onyx Cave (Figure 1) in MCNP. Great Onyx Cave is the only major cave in the Park that is not part of the more than 644 km (400+ mile) long Flint Ridge-Mammoth Cave system [20]. Great Onyx Cave crosses from one side of Flint Ridge to the other, and the center portion of the cave is under the sandstone caprock with limited water inputs, allowing for the development of gypsum (Table 1). It has a main passage, Edwards Avenue, and one major branch, Cox Avenue. Gypsum deposits in protected passages from evaporation of moisture from calcium sulfate-rich water, leaving behind a crust and gypsum flowers. Gypsum is not found at the ends of passages within ~300–1000 m of cave entrances. Poulson noted the relative absence of invertebrates in the gypsum areas, especially the lack of *Hadenoecus subterraneus* cave crickets, describing it as "The Great Kentucky Desert" [21]. He attributed the absence of crickets to the hygroscopic effect of gypsum. We therefore decided to sample bacteria along the length of the cave and the gypsum gradient to see if the Great Kentucky Desert designation applies to microbes and their production of antibiotics and levels of resistance.

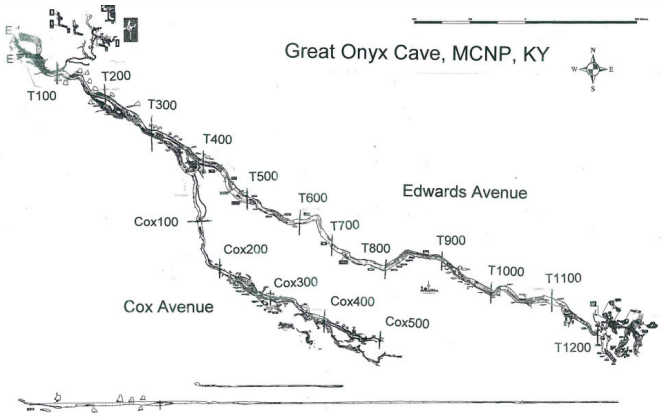

Figure 1. Map of Great Onyx Cave showing sampling sites by 100 m intervals. Gypsum first appears in Edwards Avenue at 310 m, and continues through 1000 m. Locally thick crusts and gypsum flowers occur from approximately 400–900 m. Cox Avenue has much less gypsum throughout the length of the passage, with thinner crusts and no flowers. (Map modified from Cave Research Foundation, NPS).

Table 1. Location of samples and extent of gypsum from the man-made entrance (E in Figure 1) along Edwards Avenue main cave passage and the Cox Avenue side passage. Location includes the number of meters from the entrance for Edwards Avenue and from the branch point from Edwards for Cox Avenue.

Location	Gypsum Content
Entrance, 10 m	None
30 m	None
Natural Entrance, 20 m	None
Natural Entrance, 40 m	None
50 m	None
70 m	None
90 m	None
Edwards, 110 m	None
Left Hand Tunnel	None
Edwards, 200 m	Start gypsum
Edwards, 300 m	Gypsum crust
Edwards, 400 m	Gypsum flowers
Edwards, 500 m	Gypsum crust
Edwards, 600 m	Gypsum crust
Edwards, 700 m	Gypsum crust
Edwards, 800 m	Gypsum crust
Edwards, 900 m	Limited gypsum
Edwards, 1000 m	Limited gypsum
Edwards, 1100 m	Limited gypsum
Cox Avenue, 100 m	Limited gypsum
Cox Avenue, 200 m	Gypsum crust
Cox Avenue, 300 m	None
Cox Avenue, 400 m	None
Cox Avenue, 500 m	None

Understanding how bacteria are able to survive in harsh conditions like gypsum-crusted caves could provide insight into their physiological tolerances. We hypothesize that gypsum makes the already extreme cave conditions even harsher for microorganisms, and, under these stressful conditions, bacteria may respond with increased production of secondary metabolites [10], including antibiotics. If the bacteria are producing more antibiotics, then we also expect to see high levels of antibiotic resistance to counter the possible advantage of the antibiotic producers. We also expected greater resistance to naturally occurring antibiotics than modified or synthetic antimicrobial agents.

2. Methods

2.1. Study Site

Samples were collected for microbiological studies in May 2015 in Great Onyx Cave (Figure 1) located in Mammoth Cave National Park, KY, under permit #: MACA-00143 to K. Lavoie. Great Onyx Cave was discovered in 1915, and commercialized until it was sold to Mammoth Cave National Park in 1961 [22]. Access to the cave is through the blasted entrance installed to commercialize the cave. Adjacent to the blockhouse that controls access to the blasted entrance is a very small natural entrance. The presence of cave crickets beyond 1200 m in Edwards Avenue supports that at least a small natural entrance or entrances still exist at the far end of the main passage, but any entrances are undetectable from the surface (D. Griffith, personal communication).

2.2. Isolation of Pure Cultures

Cave walls were swabbed every 20 m for the first 100 m, then every 100 m to 1100 m along Edwards Avenue, and from the branch point of Cox Avenue off Edwards Avenue from 0 to 500 m

(Figure 1). Sterile saline-moistened cotton swabs were used to swab an area of 10 cm × 10 cm, and stored in a sterile culturette (BD BBL, Franklin Lakes, NJ, USA) tube until plated within 1 h.

A total of ten soil samples were collected from Edwards Avenue (at E400, E500, E650, E800, E900, E1000 and E1100) and from Cox Avenue (at COX200A, COX200B and COX400) by aseptically scooping 50–100 g of soil into sterile culture tubes. A series of 1:10 dilutions were made in sterile dH_2O and spread-plated in triplicate. Swabs and soil dilutions of the samples were spread-plated on R2A (Criterion Media, Hardy Diagnostics, Santa Maria, CA, USA), a medium for the isolation of bacteria from oligotrophic potable water samples [23] and half-strength Nutrient Agar (Difco, Becton Dickson, Detroit, MI, USA) incubated for 1 week at room temperature, and selected for distinct colonial morphology, yielding a total of 24 pure cultures. Isolates were Gram stained using traditional methods with ethanol as a decolorizing agent, but further identification was not done. One isolate (Cox200B-a) was presumptively actinobacterial, based on cellular and colonial morphology, and production of the distinctive smelling chemical geosmin.

2.3. Antibiotic Production

Three methods were used to detect antibiotic producing bacteria. These tests were done sequentially as the previous test detected no antibiotic producers over the course of a month.

2.4. Soil Crumb Plate

A simple method to detect antibiotic production, the bacteria present in a soil sample grow out from the soil crumbs. Any that produce an antibiotic will be identified by a zone of inhibition around the colonies. Immediately after collection, about 0.1 g of cave sediment/soil was sprinkled aseptically over the surface of both R2A and 0.5 Nutrient Agar plates and incubated at room temperature for 48 h, then examined for colonies showing zones of inhibition. The soil crumb assay was repeated using heavier inoculums of soil.

2.5. Soil Crumb with Indicator Bacteria

Adding indicator bacteria to the soil crumb plate allows for greater detection of antibiotic producing bacteria that grow out from soil crumbs. One-half strength trypticase soy agar (TSA; Hardy Diagnostics) plates were swabbed with indicator bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Bacillus mycoides* from in-house stock cultures). Soil was sprinkled aseptically over the surface and incubated at room temperature for 48 and 96 h, then examined for zones of inhibition in the lawn of the indicator bacteria.

2.6. Cross-Streak Assay

The failure of the soil crumb assays led us to test our isolated pure cultures of cave bacteria using the cross-streak assay [12]. Each isolate was streaked in one line across the surface of an R2A plate and incubated at room temperature for 48 h. After incubation a thin overlay of 1/2-strength TSA was poured over the surface of the pure culture plate. Any secondary metabolite produced by the cave isolate would diffuse up through the agar overlay. After the overlay solidified, the same four indicator bacteria were streaked at right angles to cross over the underlying cave isolate. The plate was re-incubated at room temperature for 48 h, and then examined for inhibition of the indicator species where they crossed over the cave isolate streak.

2.7. Antibiotic Resistance

All 24 pure cultures of cave microbes were grown to turbidity in 1/2-strength nutrient broth at room temperature for 48 h (turbidity was not standardized), and spread onto half-strength Nutrient Agar plates. Discs impregnated with antibiotics (Hardy Diagnostics) were then placed on the surface following the procedure for Kirby-Bauer. All antibiotics used (Table 2) are broad-spectrum, except for

Clindamycin which is most effective against *Staphylococcus*, *Streptococcus*, and anaerobes [24]. Three of the antibiotics used are natural, three are semi-synthetic, and one is synthetic (Table 2). Plates were incubated at room temperature for 48 h, and the diameter of the zones of inhibition measured to determine if the pure cultures were sensitive (S), intermediate (I), or resistant (R) to each antibiotic according to the manufacturer's zone interpretive standards table [25]. Our four indicator bacteria were also tested for their antibiotic resistance as described, except we used Mueller–Hinton Agar (Criterion Media, Hardy Diagnostics) and incubation for 24 h at 35 °C.

Table 2. Antibiotics used to test for antibiotic resistance, along with code, supplier, mode of action, concentration, and origin.

Code	Name	Supplier	Mode of Action	Concentration	Origin
CTX-30	Cefotaxime	Hardy	Inhibits cell wall synthesis	30 mcg	Semi-synthetic
C-30	Chloramphenicol	BD BBL™	Inhibits protein synthesis	30 mcg	Natural
CC-2	Clindamycin	BBL	Inhibits protein synthesis	2 mcg	Semi-synthetic
GM-10	Gentamicin	BBL	Inhibits protein synthesis	10 mcg	Natural
N-30	Neomycin	BD BBL™	Inhibits protein synthesis	30 mcg	Natural
PIP-100	Piperacillin	BBL	Inhibits cell wall synthesis	100 mcg	Semi-synthetic
TMP-5	Trimethoprim	BBL	Inhibits DNA synthesis	5 mcg	Synthetic

3. Results

3.1. Antibiotic Production

Antibiotic production was not detected from soil or by our pure cultures. Plating of soil crumbs yielded sparse growth per plate on initial inoculation and more growth when repeated with a larger amount of soil inoculums, but no zones of inhibition indicative of antibiotic production were evident in microbes that grew out from the soil crumbs. Likewise, no zones of inhibition were observed from soil crumbs on lawns of indicator bacteria. None of our 24 pure cultures of cave microbes evidenced any antibiotic production by inhibition of the four indicator bacteria using the cross-streak method. Tests of antibiotic resistance of our indicator bacteria showed low resistance, with only *P. aeruginosa* showing moderate levels of resistance (four of seven antibiotics tested) (Table 3).

Table 3. Sensitivity of indicator bacteria and cave isolates to seven antibiotics. Isolates were designated according to sample location (E = Edwards, C = Cox; and distance in m from the entranced or the branch point of Cox from Edwards) and Gram stain (G + is Gram Positive, − is Gram negative), as S = sensitive, I = intermediate, or R = resistant. N/A, not available. Total R is the number that were resistant out of seven antibiotics tested unless otherwise noted in parentheses.

Indicator or Isolate	Gr	Amount Gypsum	CTX30 Cefotaxime	C30 Chloramphenicol	CC2 Clindamycin	GM10 Gentamicin	N30 Neomycin	PIP100 Piperacillin	TMP5 Trimethoprim	Total R
E. coli	−		N/A	S	R	S	S	S	S	1 (6)
B. mycoides	+		R	S	S	S	S	I	R	2
P. aeruginosa	−		R	I	R	S	R	S	R	4
S. aureus	+		N/A	S	S	S	S	S	S	0 (6)
E0110	−	None	R	R	R	R	R	I	R	6
E500	−	Crust	R	S	N/A	S	R	S	R	3 (6)
E500-1	−	Crust	I	I	R	R	R	N/A	R	4 (6)
E500-2	−	Crust	R	R	R	R	R	S	R	6
E500-3	−	Crust	R	R	R	R	I	S	R	5
E500-3	−	Crust	S	S	R	R	R	S	S	3
E500-4	−	Crust	R	R	R	S	R	S	R	5
E500-6	−	Crust	R	R	R	R	R	S	R	6
E500-7	+	Crust	S	R	R	R	R	R	R	6
E500-B	−	Crust	R	R	R	N/A	R	R	R	6 (6)
E650-A	−	Crust	R	R	R	R	R	R	R	7
E650-C	+	Crust	I	R	R	S	S	S	R	3
E800-1	−	Crust	R	R	R	R	R	S	R	6
E800-A	−	Crust	I	N/A	R	N/A	I	S	R	2 (5)
E800-2	+	Crust	I	S	S	S	I	R	R	2
E1000	−	Limited	R	R	R	R	R	R	R	7
E1000A	−	Limited	S	R	R	R	R	R	R	6
E1000-B	−	Limited	R	R	R	S	S	R	R	5
E1000C	+	Limited	R	R	R	S	R	R	R	6
E1100C	−	Limited	S	R	R	R	R	S	S	4
Cox200B-1	+	Crust	R	R	R	R	R	R	R	7
Cox200B-2	−	Crust	R	R	R	S	I	N/A	R	4 (6)
Cox 400	+	None	I	S	R	I	I	S	R	2
Cox400-1	−	None	R	N/A	R	S	R	S	R	4 (6)

3.2. Antibiotic Resistance

Antibiotic resistance, however, was common (Table 3). (Turdibities were not standardized. All liquid cultures had low turbidity, but it may have affected some of the interpretations, although few zones were intermediate.) Every pure culture isolate was resistant to a minimum of two of seven antibiotics tested and three were resistant to all seven. There was the least resistance to 100 µg Gentamicin (12 of 24 strains) and the most resistance (22 of 24 strains) to both 5 µg Trimethoprim and 2 µg Clindamycin. There is no pattern of resistance by mode of action of the antibiotics tested. A regression analysis of number of antibiotic-resistant strains with location in the cave shows no correlation between gypsum level and level of antibiotic resistance in cave isolates. The total number of isolates resistant were identical for the natural and semi-synthetic antibiotics, and the number resistant to the one synthetic antibiotic fell within the range of the others.

4. Discussion

Great Onyx Cave has been commercialized since the 1920s, although tours did not usually go beyond 450 m into Edwards Avenue, and seldom into Cox Avenue. There is widespread human impact evident in the development of a trail along both passages extended by the Civilian Conservation Corps in the 1930s after the depression [22]. Since becoming part of Mammoth Cave National Park in 1961, lantern tours were restricted to the Onyx Colonnade formation area in the first 70 m of the main cave, and even those rare tours have now ceased. The cave is currently visited occasionally by NPS personnel, explorers, and researchers.

Montano and Henderson [13] tested two hypotheses on factors that may influence antibiotic production in four carbonate caves in Carlsbad Caverns National Park, NM, USA. They found that frequency of human visitation had no effect on production of antibiotics by cave bacteria, but increasing depth into the cave and increasingly oligotrophic conditions resulted in higher levels of antibiotic production. The percent of cultures that showed antibiosis to indicator bacteria using the cross-streak method ranged from 8.62% to 21.1%. A study from a cave in the Western Caucasus [26] showed 14% of 87 isolates produced antimicrobials. The proportion increased with sampling depth into the cave, and was highest in the most remote lower levels of the cave.

Moisture parameters and the effects on fungal growth on gypsum drywall has received a lot of study due to the impacts of uncontrolled moisture on structural damage, material degradation, health concerns from mold, and changes in the microbial communities [27]. Van Laarhoven et al. [28] showed that moisture content and water activity have separate effects on hyphal extension of *Penicillium* mold on gypsum drywall. RH is a measure of water vapor, and controls available water, which is a property of liquid water. The growth rate of hypha on gypsum wallboard decreased with both available water and moisture content. In cave environments, RH is typically close to saturation, but available water could be influenced by the hygroscopic properties of gypsum, which was the basis of our hypothesis that gypsum might influence bacterial activities.

We hypothesized that the presence of gypsum puts additional stress on bacterial cells living in the highly oligotrophic cave environment, resulting in increased production of antibiotics to reduce competition or enhance predation. Although low overall, Poulson et al. [21] reported one colony producing antibiotic using the soil crumb plate from one area with gypsum compared to a clay-silt area with no gypsum in Great Onyx Cave. However, we were unable to detect antibiotic production from our soil samples using soil crumb, even with repeated attempts using higher inoculums. Colonies grew from our soil crumbs, but no inhibition was observed. This lack of inhibition may be due to the necessity of both antibiotic producer and sensitive competing strain to be in close proximity, or due to our high level of observed resistance among cave isolates. We attempted to account for this eventuality by also exposing bacterial lawns of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. mycoides* to soil crumbs, but despite outgrowth of colonies from soil crumbs, the lack of zones of inhibition suggests little antibiotic production from soil/sediment sample microbes.

Another reason for the lack of detection of antibiotic production may be the typical low density of bacteria from our swabs and soil samples. Soil dilutions averaged in the 10^2–10^5 per gram of soil which is typical of cave soil bacterial density. Population densities of cave microbes may not reach high enough levels to trigger quorum sensing-mediated production of metabolites such as antibiotics [29]. In addition, the pure cultures isolated from the cave may not be representative of the natural cave microbiota, whose slow growth rate, temperature tolerance, and nutrient profile likely was not met in our laboratory as is common with environmental isolates.

Although our pure cultures were isolated to study the proportion showing antibiotic resistance, and not part of our original experimental design for antibiotic production, we tested our culturable bacteria from Great Onyx Cave using the cross-streak method with the same indicator bacteria. Our cave isolates were not good candidates for antibiotic production. Only one (Cox200B-1) was presumptive actinobacterial, and the majority of the rest were Gram-negative. We did not detect any antibiosis.

Our culturable cave isolates did, however, show high levels of antibiotic resistance to up to seven antibiotics, including natural, semi-synthetic, and synthetic. Antibiotic resistant bacteria are widespread in many environments. D'Costa et al. [2] used targeted metagenomics of ancient DNA from permafrost sediment cores from the Yukon. Samples showed DNA sequences from Late Pleistocene megafauna and plants with no Halocene vertebrates or plants, confirming the age of the core samples. They used assays to amplify, detect, and sequence diverse genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics, and for vancomycin resistance. Genes were similar to modern genetic variants, confirming that antibiotic resistance predates modern clinical use of antibiotics and is a natural phenomenon. Antibiotic resistance was widespread in Lechuguilla Cave in Carlsbad Caverns National Park, NM. Bhullar et al. [30] sampled culturable bacteria from a region of the caves isolated from surface influences for 4 million years. Some strains were resistant to 14 of 26 different commercially available antibiotics. Enzymes were detected for both natural and semi-synthetic antibiotics. They conclude that antibiotic resistance is natural, widespread, and "hard-wired" into the bacterial pangenome.

In the present preliminary study, we examined antibiotics with generally broad effectiveness against a range of common human pathogens, and overlap with those used by Bhullar [30]. Few of our cave isolates were sensitive to these antibiotics, showing a high level of resistance to antimicrobials in these bacteria. At least for culturable microbes from Great Onyx Cave, antibiotic resistance does not correlate with gypsum level or depth into the cave

5. Conclusions

We hypothesized that gypsum may act to suppress microbial growth and stress microbial cells, resulting in high levels of production of antimicrobials and corresponding high resistance. We were unable to detect antibiotic production by bacteria along a gypsum gradient from areas with no gypsum to areas with extensive crusts and gypsum flowers. Antibiotic resistance was widespread among our culturable bacteria, and did not show any correlations with depth into the cave, the amount of gypsum, or with antibiotic origin as natural, semi-synthetic, or synthetic. We conclude that the Great Kentucky Desert hypothesis of Poulson does not apply to bacterial antibiotic resistance. We plan on extending our studies to detect antibiotic production.

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References

1. Karam, G.; Chastre, J.; Wilcox, M.H.; Vincent, J.L. Antibiotic strategies in the era of multidrug resistance. *Crit. Care* **2016**, *20*, 136. [CrossRef] [PubMed]
2. D'Costa, V.; King, C.; Kalan, L.; Morar, M.; Sung, W.; Schwartz, C.; Froese, D.; Zazula, G.; Calmels., F.; Debruyne, R.; et al. Antibiotic resistance is ancient. *Nature* **2011**, *477*, 457–461. [CrossRef]
3. Gupta, R.S. Origin of diderm (Gram-negative) bacteria: Antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes. *Antonie Van Leeuwenhoek* **2011**, *100*, 171–182. [CrossRef] [PubMed]
4. WHO. Antimicrobial Resistance: Global Report on Surveillance 2014. Available online: http://www.who.int/drugresistance/documents/surveillancereport/en/ (accessed on 13 September 2016).
5. Pelaez, F. The historical delivery of antibiotics from microbial natural products—Can history repeat? *Biochem. Pharm.* **2006**, *71*, 981–990. [CrossRef] [PubMed]
6. Gabriel, C.R.; Northup, D.E. Microbial Ecology: Caves as an extreme habitat. In *Cave Microbiomes: A Novel Resource for Drug Discovery*; Cheeptham, N., Ed.; Springer: New York, NY, USA, 2013; pp. 85–108. ISBN 978-1-4614-5206-5.
7. Zhang, L. Integrated approach for discovery of novel drugs from microbial natural products. In *Natural Products. Drug Discovery and Therapeutic Medicine*; Zhang, L., Demain, A.L., Eds.; Humana Press: Totowa, NJ, USA, 2005; pp. 30–55. ISBN 1-58829-383-1.
8. Leisner, J.J.; Haaber, J. Intraguild predation provides a selection mechanism for bacterial antagonistic compounds. *Proc. R. Soc. Lond. B. Biol. Sci.* **2012**, *279*, 4513–4521. [CrossRef] [PubMed]
9. Leisner, J.J.; Jorgensen, N.O.G.; Middleboe, M. Predation and selection for antibiotic resistance in natural environments. *Evol. Appl.* **2016**, *9*, 427–434. [CrossRef] [PubMed]
10. Dapkevicius, M.L.E. Cave biofilms and their potential for novel antibiotic discovery. In *Cave Microbiomes: A Novel Resource for Drug Discovery*; Springer: New York, NY, USA, 2013; pp. 35–45. ISBN 978-1-4614-5206-5.
11. Khumbar, C.; Watwe, M. Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. *Nat. Sci.* **2013**, *5*, 26–40. [CrossRef]
12. Martinez, J.L.; Fajardo, A.; Garmendia, L.; Hernandez, A.; Linares, J.F.; Martinez-Solano, L.; Sánchez, M.B. A global view of antibiotic resistance. *FEMS Microbiol. Rev.* **2009**, *33*, 44–65. [CrossRef] [PubMed]
13. OSHA. Safety Data Sheet, OSHA Occupational Chemical Database. Available online: www.osha.gov/chemicaldata/ (accessed on 21 September 2017).
14. Montano, E.T.; Henderson, L.O. Studies of antibiotic production by cave bacteria. In *Cave Microbiomes: A Novel Resource for Drug Discovery*; Cheeptham, N., Ed.; Springer: New York, NY, USA, 2012; pp. 109–130. ISBN 978-1-4614-5206-5.
15. Gypsum. Available online: https://www.gypsum.org/about/gypsum-101/uses-gypsum/ (accessed on 12 September 2016).
16. Singh, T.M. The Effect of Gypsum on Bacterial Activities in Soils. Ph.D. Thesis, Iowa State University, Ames, IA, USA, 1920. Retrospective Theses and Dissertations 14298. Available online: http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=15297&context=rtd (accessed on 21 September 2016).
17. Ajjappalavara, P.S.; Dharmatti, P.R.; Salimath, P.M.; Patil, R.V.; Patil, M.S.; Krishnaraj, P.U. Effect of gypsum application on the control of bacterial wilt in *Brinjal* (*Solanum. melongena* L.). *Karnataka J. Agric. Sci.* **2008**, *21*, 600–601.
18. Palmer, A.N. The Mammoth Cave Region, Kentucky. In *Caves and Karst of the USA*; Palmer, A.N., Palmer, M.V., Eds.; National Speleological Society: Huntsville, AL, USA, 2009; pp. 108–113.
19. Lavoie, K.H. A grand, gloomy, and peculiar place: Microbiology in the Mammoth Cave Region. In *Life in Extreme Environments: Microbial Life of Cave Systems*; Engel, A.S., Ed.; De Gruyter: Berlin, Germany, 2015; pp. 47–78. ISBN 978-3-1103-3499-9.
20. Carson, V. Mammoth Cave Hits 400 Miles. Available online: https://www.nps.gov/maca/learn/news/mammoth-cave-400-miles.htm (accessed on 5 July 2017).

21. Poulson, T.L.; Lavoie, K.H.; Keith, J.H. Biological desert under the cap rock. In *Proceedings of Annual Report for Cave Research Foundation*; Cave Books: Dayton, OH, USA, 1986; pp. 24–25.
22. Brucker, R.W.; Watson, R.A. *The Longest Cave*, 1st ed.; Southern Illinois University Press: Carbondale, IL, USA, 1976; ISBN 0-394-48793-1.
23. Reasoner, D.J.; Geldreich, E.E. A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* **1985**, *49*, 1–7.
24. Antibiotic Guides. Johns Hopkins ABX Guides. Available online: www.hopkinsguides.com/hopkins/view/Johns_Hopkins_ABX (accessed on 21 September 2017).
25. Interpretaive Standards. Available online: https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf (accessed on 21 September 2017).
26. Klusaite, A.; Vickackaite, V.; Vaitkeviciene, B.; Karnickaite, R.; Bukelskis, D.; Kieraite-Alkesandrova, I.; Kuisiene, N. Characterization of antimicrobial activity of culturable bacteria isolated from Krubera-Voronja Cave. *Int. J. Speleol.* **2016**, *45*, 275–287. [CrossRef]
27. Dedesko, S.; Siegel, J.A. Moisture parameters and fungal communities associated with gypsum drywall in buildings. *Microbiome* **2015**, *3*, 71. [CrossRef] [PubMed]
28. Van Laarhoven, K.A.; Huinink, H.P.; Segers, F.J.J.; Dijksterhuis, J.; Adan, O.C.G. Separate effects of moisture content and water activity on the hyphal extension of *Penicillium rubens* on porous media. *Environ. Microbiol.* **2015**, *17*, 5089–5099. [CrossRef] [PubMed]
29. Rutherford, S.T.; Bassler, B.L. Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspect. Med.* **2012**, *2*, a012427. [CrossRef] [PubMed]
30. Bhullar, K.; Waglechner, N.; Pawlowski, A.; Koteva, K.; Banks, E.D.; Johnston, M.D.; Barton, H.A.; Wright, G.D. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE* **2012**, *7*, e34953. [CrossRef] [PubMed]