Discovery, Characterization and Ecological Impact of a Predatory Bacterium of Cyanobacteria Responsible for Epidemics in Biocrusts

by

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ABSTRACT

Predatory bacteria are a guild of heterotrophs that feed directly on other living bacteria. They belong to several bacterial lineages that evolved this mode of life independently and occur in many microbiomes and environments. Current knowledge of predatory bacteria is based on culture studies and simple detection in natural systems. The ecological consequences of their activity, unlike those of other populational loss factors like viral infection or grazing by protists, are yet to be assessed. During large-scale cultivation of biological soil crusts intended for arid soil rehabilitation, episodes of catastrophic failure were observed in cyanobacterial growth that could be ascribed to the action of an unknown predatory bacterium using bioassays. This predatory bacterium was also present in natural biocrust communities, where it formed clearings (plaques) up to 9 cm in diameter that were visible to the naked eye. Enrichment cultivation and purification by cell-sorting were used to obtain co-cultures of the predator with its cyanobacterial prey, as well as to identify and characterize it genomically, physiologically and ultrastructurally. A *Bacteroidetes* bacterium, unrelated to any known isolate at the family level, it is endobiotic, non-motile, obligately predatory, displays a complex life cycle and very unusual ultrastructure. Extracellular propagules are small (0.8-1.0 μm) Gram-negative cocci with internal two-membrane-bound compartmentalization. These gain entry to the prey likely using a suite of hydrolytic enzymes, localizing to the cyanobacterial cytoplasm, where growth begins into non-compartmentalized pseudofilaments that undergo secretion of vesicles and simultaneous multiple division to yield new propagules. I formally describe it as *Candidatus* Cyanoraptor togatus, hereafter Cyanoraptor. Its prey range is restricted to

biocrust-forming, filamentous, non-heterocystous, gliding, bundle-making cyanobacteria. Molecular meta-analyses showed its worldwide distribution in biocrusts. Biogeochemical analyses of Cyanoraptor plaques revealed that it causes a complete loss of primary productivity, and significant decreases in other biocrusts properties such as water-retention and dust-trapping capacity. Extensive field surveys in the US Southwest revealed its ubiquity and its dispersal-limited, aggregated spatial distribution and incidence. Overall, its activity reduces biocrust productivity by 10% at the ecosystem scale. My research points to predatory bacteria as a significant, but overlooked, ecological force in shaping soil microbiomes.

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1 - INTRODUCTION

Following a fortuitous discovery of pathogenic agents in the large-scale production of cyanobacterial inoculum intended for restoration of arid soils, I studied a novel predatory bacterium, *Candidatus* Cyanoraptor togatus in the *Bacteroidetes* phylum that attacks soil cyanobacterial populations from biocrusts, establishing its life cycle, unusual cell morphology, genomics, basic ecophysiological traits, and providing a formal taxonomic description for it. I also conducted an in-depth study of the consequences of Cyanoraptor's predation for the ecology of biocrusts in the Sonoran and Chihuahuan deserts and detected it in a variety of biocrusts beyond these biogeographical provinces. At the cm-scale, its activity leads to devastating losses in biomass, productivity, and other biocrust properties that form the base of typical biocrust "ecosystem services". At the landscape and regional scales, it translates to a decimation of biocrust's productivity. This dissertation is divided into this introductory chapter, three additional research chapters and a conclusions chapter containing an outlook section. In chapter two, I provide suggestions for biocrust restoration inoculum production and report on the discovery of a predatory bacterium of cyanobacteria. In chapter 3, I describe the predatory bacterium's life cycle and basic interactions with its prey, as well as provide a genomic analysis and a taxonomic classification. In chapter 4, I determine the spatial distribution of this bacterium, describe its symptomology in its natural environment and quantitatively assess its impact on biocrusts' ecological contributions.

Predatory bacteria

Predatory bacteria are a functional guild of heterotrophs that utilize other living bacteria as prey[1] in order to obtain nutrients and grow[2]. While originally thought to be restricted to the *Proteobacteria*, they have since been identified in many phyla[1] including the *Firmicutes*[3,4], *Bacteriodetes*[5], *Melainibacteria*[6] and *Chloroflexi*[7]. Some are obligately predatory and others only facultatively so[2]; however, they tend to be generalist feeders that can target both Gram-positive and negative bacteria[1]. Their strategic approach to predation varies across and within taxonomic boundaries, which consequently has led to their categorization on the basis of their hunting strategy. Wolf-pack predators (Fig. 1d), such as *Myxobacteria*[8], work in concert to hunt their prey, releasing hydrolytic enzymes in close proximity to or directly into their target[9]. Epibiotic predators, such as *Vampirococcus*[10] attach but remain external to their prey, injecting hydrolytic enzymes into their quarry before assimilating the resulting hydrolysates and dividing via simple binary fission (Fig. 1c). In contrast, endobiotic predators breach the cell wall and lodge themselves most commonly in the prey's periplasm[11] (Fig. 1a), or, at least in one instance, within the, cytoplasm as has been described in *Daptobacter*[10] (Fig. 1b).
The most studied endobiont is the small, 0.3-0.5 by 0.5-1.4 μm[12], Gram-negative *Proteobacterium Bdellovibrio bacteriovorus*, which has served for many decades as the model organism for bacterial predation. *B. bacteriovorus* has a complex life cycle, with a free-living attack phase, a short transitory phase beginning with penetration of the host and an intracellular growth phase[13]. During the attack phase, the predator is exceptionally motile and hunts its prey at high speeds, up to 160 μm per second[14], ten times that of *E. coli*[15], which is necessary as it only has a few hours to locate its prey before starving[16].

During this phase, it cannot replicate its DNA or perform cell division, being entirely reliant upon the intracellular growth phase for these functions[17]. Once the predator has located potential prey via detection of amino acids[18] and high bacterial biomass[19] it reversibly attaches[20] to its host, likely assessing the prey's nutritional value, before irreversibly attaching and using hydrolytic enzymes[21,22] to form a pore in the prey envelope, entering and then shedding its flagellum[15]. The entry pore is resealed, restricting access to any further predators[23]. The predator then enters a 3–4 hour growth phase[14], using prey hydrolysate for growth, and produces a filament that septates into 3-6 progeny, depending on prey size[24], before forming pores in the prey's cell wall enabling escape[25].

In general, genomic studies of predatory bacteria demonstrate the loss of genes to synthesize some amino acids[6,26–28], vitamins and nucleotides[29], indicating reliance on prey biomass for growth. However, unlike symbionts[30,31], there is no large-scale reduction in genome size when compared to non-predators. The only difference is between epibiotic and endobiotic predators, with epibiotic predators having on average a smaller genome (2.5 Mbp versus 3.5 Mbp)[9]. The maintenance of larger genomes is likely due to increases in the number of proteases[28,29,32], necessary for accessing prey hydrolysates and for movement of both hydrolytic enzymes and nutrients. Adhesion proteins, requisite for attachment to prey, are more abundant in *B. bacterivorous*[33]. Transcriptional profiling indicates there are distinct shifts in *B. bacterivorous*'s attack and growth phases. The production of cell surface proteins and taxis proteins increase during the attack phase. The shift to the growth phase coincides with an immediate increase in

protein synthesis, specifically in transporters, hydrolytic enzymes and enzymes responsible for peptidoglycan remodeling[32,33].

Ecology of predatory bacteria

Predatory bacteria have been isolated from a multitude of environments, including marine[34] and freshwater[35] systems, sewage[36,37], soil[38–40] and host-associated microbiomes[41]. Many have yet to be rendered into pure culture because of their absolute reliance on appropriate prey organisms[42]. Cultivation in axenic form or co-culture with the prey can be hindered by a) the potentially unculturable nature of the prey themselves, or by b) the predators' slow growth, which allows other, faster growing bacteria to overtake them during isolation attempts[8]. Yet, much of what is currently known is culture based. Environmental studies have been primarily descriptive[42] and focused on marine systems rather than freshwater systems or soils[43]. Furthermore, whole community interactions and functional impacts have yet to be fully investigated, leaving gaps in knowledge in such areas as how predators differentiate between prey and non-prey, what factors influence prey preference, what characteristics distinguish distinct phylotypes under similar environmental conditions, and how predators interact with other predators (phages and protists)[42]. A single recent study describes whole community interactions and the impact of predation on nutrient cycling, wherein predators grow faster and consume more resources than non-predators in soils[44]. In stark contrast, there has been a great deal of research on the impact of other population loss factors such as phages and protists on cyanobacterial mortality; both can modify population structure, dynamics and

primary production in aquatic blooms[45–49]. However, it is unknown for any environment if the impact of bacterial predation is comparable to these other biotic loss factors[43].

Of the environmental conditions that have been explored, temperature and salinity seem to be the main drivers of shifts in bacterial predator community composition. Culture-based studies of marine bacteria indicate that predators have a preferred temperature range dependent on their tolerance to salinity; non-halotolerant predators grow best between 30 and 37 °C whereas the halotolerant *Halobacteriovorax* grow best between 20 and 30 °C [50,51], with overall growth limit bracketed between 5 and 40 °C[52]. Field studies correlating seasonal changes with water temperature suggest that colder winter waters restrict the abundance of predatory bacteria when compared to the warmer spring months[53,54].

Salinity levels also have an impact on the presence and phylotype of predatory bacteria. A study by Marbach et. al (1976)[50] found the optimal salinity range for ten strains of marine *B. bacteriovorus* to be between 11 and 60%, while other non-marine species were unable to survive the lowest salinity tested, 11%. Studies of *Halobacteriovorax* found in the Chesapeake and Delaware Bays (United States) demonstrate that there is a segregation of particular phylotypes along a salinity gradient; however, further investigation of characteristics that may be responsible for this environmental segregation was not carried out[55,56].

As all predatory bacteria are dependent on their prey, it logically follows that the presence of preferred prey and their abundance will impact a predators' survival. Predators' partiality has been comprehensively studied using isolates, predominantly sourced from soils, and indicates that predatory bacteria tend to be generalists[57] targeting

primarily Gram-negative bacteria[42], and that in at least one genus, *Halobacteriovorax,* they preferentially prey on hosts sourced from the predator's native habitat[58]. Predatory bacteria are highly dependent on the distribution and abundance of their prey[59], which thus determine niches[60] available to predators. This is illustrated by the high numbers of predators found in biofilms with their dense food stock[61]. Prey therefore have the ability to shape not only the community composition[61–64] but also the possible environments available to predators. Prey may also be unwillingly responsible for triggering predatory attack. *Myxococcus xanthus,* a wolf pack predator, is able to detect compounds released by its prey[65,66]. *M. xanthus* constitutively releases toxins into its immediate environment damaging nearby prey which causes prey to release an unknown compound that in turn triggers predatory attack[66].

Predators can also significantly shape their prey community. Importantly, known predators only ever consume a fraction of the available prey[67], with prey populations reaching stable levels containing predation-resistant strains and slower growers[68], a plastic response that reverts after the removal of predation pressure[69]. Predation rates and success in part depend on physical barriers in the environment. Fragmented spaces, typically found in soils with their irregular distribution of soil particles and air pockets that provide refuge, result in high variation in predation rates and significant amounts of prey persisting in comparison to continuous spaces, those without internal barriers[70], such as aquatic environments.

Biological soil crusts

Biological soil crusts, biocrusts, are topsoil communities of photosynthetic microorganisms primarily composed of cyanobacteria[71], heterotrophic bacteria[72], fungi[73] and archaea[74] (Fig. 2b). In successionally mature crusts with suitable precipitation, lichens[75] and mosses[76] may also be present. Biocrusts are found in plant interspaces in arid and semiarid environments worldwide, covering an estimated 12% of land[77]. Given that drylands cover nearly 45%[78] of terrestrial environments, biocrust communities provide crucial ecosystem services globally, such as supplying fixed carbon[79,80] and nitrogen[81]. They are responsible for 15% of net primary productivity[77] and nearly 50% of terrestrial nitrogen fixation[79] worldwide. Biocrusts also regulate water runoff[82–84] and stabilize soils against erosion[85,86].

Soil stabilization is especially important as it is necessary for the formation of incipient communities. This service is provided by filamentous cyanobacteria such as *Microcoleus vaginatus* which forms supracellular ropes that weave together soil particles[86] and sheds its polysaccharide sheath[85] creating a glue that binds soil. After soils are stabilized, secondary cyanobacterial colonizers, such as those in the genera *Nostoc* and *Scytonema*[71] can establish. If temperature and water availability are favorable fungi and lichen can also colonize[76]. Increases in soil surface rugosity parallel the maturation process, starting with smooth biological soil crusts, 0-1 cm in height, that occur in hyper-arid deserts to rolling, 3-5 cm in height, that are present in cold deserts where frost heaving occurs[87]. Mature biocrusts provide additional ecosystem services with a greater capacity to fix carbon[88,89] and nitrogen,[90] the potential to increase water infiltration and retention due to an increase in rugosity[83], increase in exopolysaccharide production[91] and the production of the

sunscreen pigment scytonemin, protecting against UV damage[92]. However, in hyper-arid deserts, crusts may not progress beyond smooth biological soil crusts composed of microorganisms typical of incipient communities[93].

Microcoleus vaginatus

Microcoleus vaginatus is likely the most common terrestrial cyanobacterium, contributing crucially to early biocrust formation[85,86] (Fig. 2a). It is able to bind loose soil particles[85] by formation of optimally sized bundles, which are aggregates of trichomes ensconced in a polysaccharide sheath[85,86]. The polysaccharide sheaths that enclose these bundles are then left behind after *M. vaginatus* moves across and within the soil, effectively "gluing" soil particles together[85]. A third adaptation is crucial to colonization of nutrient poor bare soil. As *M. vaginatus* is unable to fix N_2[94] it relies on its diazotrophic symbionts within the cyanosphere[95] to provide this crucial nutrient in exchange for copious fixed carbon exudates[96,97].

M. vaginatus specializes in survival in harsh environments by seeking refuge within soils during periods of high temperature[98] and intense UV radiation[99]. It is phototactic[100] and hydrotactic[101,102], moving vertically within the soil profile to shade itself from intense sunlight and in response to the presence or absence of water. Once rehydrated it can begin photosynthesizing within minutes[101]. *M. vaginatus* also contains a suite of proteins key to mitigating damage caused by high light, oxidative stress and desiccation[94].

While well adapted to its native environment, *M. vaginatus* is facing anthropogenically induced challenges. Compressional stress from agriculture[103] and recreation[104] crush delicate filaments while climate change is causing their preferred thermal niche to be lost

as warming temperatures favor pioneer cyanobacteria in the *Coleofasciculaceae* family (formerly the *M. steenstrupii* complex) over *M. vaginatus*[105].

Heterotrophic microbial interactions in biocrusts

Logically, most of the studies on the biology and ecology of crust organisms have centered on the primary producers (largely cyanobacteria), as they are the main source for carbon input[88,89] and an important source of nitrogen[88,90]. And yet, biocrusts represent miniature ecosystems with diverse phylogenetic and functional components[106]. Beyond these important pioneer organisms are heterotrophic bacteria, many uncharacterized concerning their functional role within the crust[72]. One of the few such examples is a symbiosis that is the key to the formation of biocrusts. It has long been a question how *M. vaginatus* is able to colonize new, nitrogen poor soils while lacking the ability to fix nitrogen[107,108]. Recent research points to a community of closely associated diazotrophic heterotrophic bacteria (a "cyanosphere"), creating an environment that has a 100-fold concentration in nitrogen fixing potential[95]. This relationship is based on a carbon for nitrogen exchange, wherein heterotrophs provide fixed nitrogen in exchange for copious carbon exudates[96] produced by *M. vaginatus*[95,109]. Clearly heterotrophs play important roles in the biocrust community, and their contributions are worthy of further study.

Biotic loss factors

Microfaunal grazing pressure is a potential loss factor for biocrusts. While the presence of grazing protozoa, nematodes, and other microarthropods are documented in biocrusts[110], very little is known about their feeding preferences and impacts on biomass

or growth dynamics. Feeding preference conjecture is based on what is known in aquatic systems or organismal feeding apparatuses. But such studies estimate only what they *could* consume rather than what they *do* consume[13,111].

Alternatively, disease can be a significant loss factor in the population dynamics of phototrophs. Examples can be found in the literature on the severe impact of cyanophages on the population dynamics of planktonic blooms of cyanobacteria[45–48], with viral abundance ranging from 10^6 to 10^{11} per liter of sea water[45], enough to potentially regulate community structure and primary production[47]. Soils are also known to harbor significant populations of bacteriophages, approximately 10^8 per gram of soil[112]. However, within biocrusts there has been but a single report of bacteriophages targeting *Firmicutes*[113] and no reports of disease impacting primary producers.

Abiotic loss factors

Though biological soil crust organisms have many adaptations to protect against native environmental conditions, they are highly susceptible to compressional stress caused by anthropogenic factors such as agriculture[103] and recreational activities[104]. Even limited grazing results in less diverse communities and the reduction in functions provided by those components[114]. Land cultivation and addition of herbicides change water penetration[115,116]. Minimal foot traffic can entirely destroy the structure of the crust[104] and stresses from off-road vehicles crush and bury biocrusts[117].

Though biocrusts are adapted to native stressors, they are not fully impervious to these harsh conditions. Rainfall frequency[118,119] and timing of rain events[120] constricts growing season and its alteration can shift cyanobacterial community composition[121]. Similarly,

temperature gradients[105] and permanent N deficiency[122] in drylands restrict species distributions.

The combination of natural limitations and increasing anthropogenic pressures has resulted in the demise of biocrust cover in a large portion of arid lands[117]. Damaged mature biocrusts revert to the simpler assemblages characteristic of early successional stages with phototroph niche replacement by cyanobacteria[123] or become sources for aerialized dust particles, which in turn may bury adjacent crusts, preventing primary producers from photosynthesizing[124,125], and causing further losses of crusts along with the ecosystem services they provide.

Restoration

Intensifying effects of abiotic loss factors have spearheaded a surge in attempts to actively restore biocrusts. Generally unassisted recovery is slow, with the most arid locations presenting the slowest recovery rates, taking anywhere from decades to centuries[126]. In order to speed recovery, early restoration approaches transplanted intact biocrusts to damaged locations, leaving behind areas devoid of biocrusts equal to that of restored patches[127–129]. While this did speed recovery, the amount of crust needed for harvesting was unsustainable. Later attempts involved inoculation with isolated and mass-cultured components, both cyanobacteria[130–137] and mosses[138]. More recent attempts include inoculation using the "mixed-community" approach, where a small amount of remnant biocrust from a disturbed site is used to grow large amounts of compositionally similar inoculum in greenhouses or "microbial nurseries"[139,140].

The mixed-community approach results in community composition that mirrors that of the source material, inoculum that is conditioned to native conditions and quality controlled[139]. Current restoration techniques utilize heterotrophic symbionts of *M. vaginatus,* inoculating both with and without cyanobacterial isolates. Co-inoculation of heterotrophs and isolates on sterilized soil double cyanobacterial biomass and soils with incipient to moderate levels of phototrophic biomass benefit from the addition of cyanosphere heterotrophs alone[109]. Restoration has proven to shorten recovery time[132,141,142] and increase biomass and biocrust coverage[141]. Inoculation with cyanobacteria increases the diversity of the community overall, heterotrophs included[143], and restores essential ecosystem functions. Soils are stabilized[142] and sediment loss is decreased[144]. Fertility is also increased, with available P doubling and available N quadrupling in just five years in one study[135].

Dissertation Objective

The discovery of a predatory bacterium on cyanobacteria while optimizing biocrust restoration practices prompted my investigation of this disease agent. My overarching objective was to identify, characterize and determine the ecological impact of this predatory bacterium. Recent studies indicate that heterotrophic microbes can play a fundamental role in the establishment of biocrusts; however, deleterious interactions have yet to be explored. Investigation of this organism provides a more complete picture of biocrust function and serves as a warning when using mixed-community approaches for restoration. Given biocrusts' extensive coverage in drylands and their sizeable contributions to ecosystem function, discovery of a predator of cyanobacteria has

potential worldwide implications for the overall health and ecosystem contributions of biocrusts. Additionally, identifying the existence of a predatorial bacterium in biocrusts and understanding how it interacts with other community members provides a more holistic view of biocrust microbiomes.

Approach

I first set out to determine if a disease agent was responsible for severe losses in cyanobacterial growth in restoration inoculum. Once the presence of a predatory bacterium was confirmed, I enriched for, identified and characterized this organism, elucidating its life cycle and phylogenetic position. Identification allowed me to determine its worldwide presence. I then investigated whether potential signs of infection in natural settings were in fact symptoms of disease caused by predatory bacteria. Verification of this allowed me to evaluate Cyanoraptor's impact on ecological contributions of cyanobacteria within biocrusts.

Figures

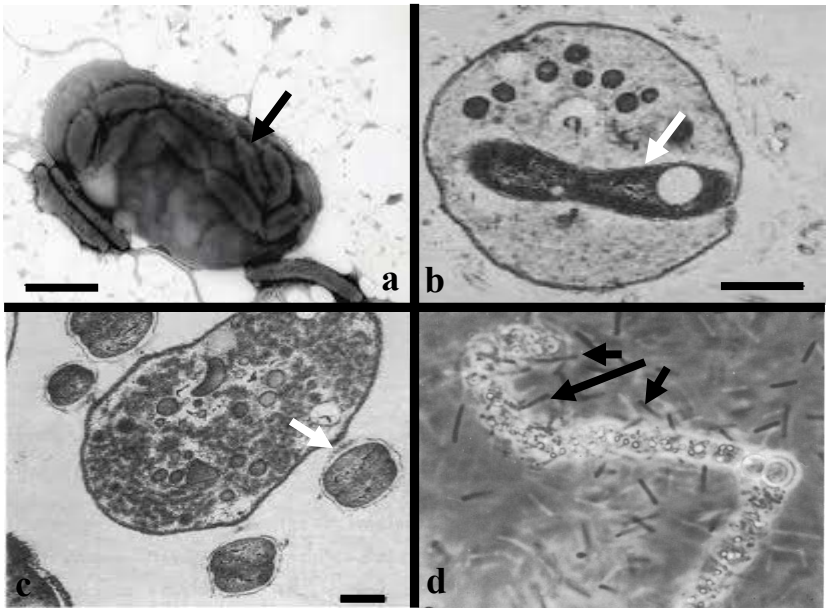

Figure 1. Predatory bacteria attack strategies. a) Filamentous replication of *Bdellovibrio bacteriovorus* within a prey's periplasm, demonstrating endobiotic attack. Arrows indicate newly replicated predator cells. Scale bar 1 µm. Modified from Sockett 2009[145] b) The endobiotic predator *Daptobacter* localizes within the prey's cytoplasm. Arrows indicate *Daptobacter*. Scale bar 1 µm. Modified from Guerrero et. al 1986[10]. c) Exhibiting epibiotic predation, *Vampirococcus* docks at prey's outer membrane. Arrow indicates docking and release of hydrolytic enzymes. Scale bar 0.5 µm. Modified from Guerrero et. al 1986[10]. d) *M. xanthus* exhibiting swarming behavior or wolf pack predation. Arrows indicate predatory cells. Modified from Shilo 1970[146].

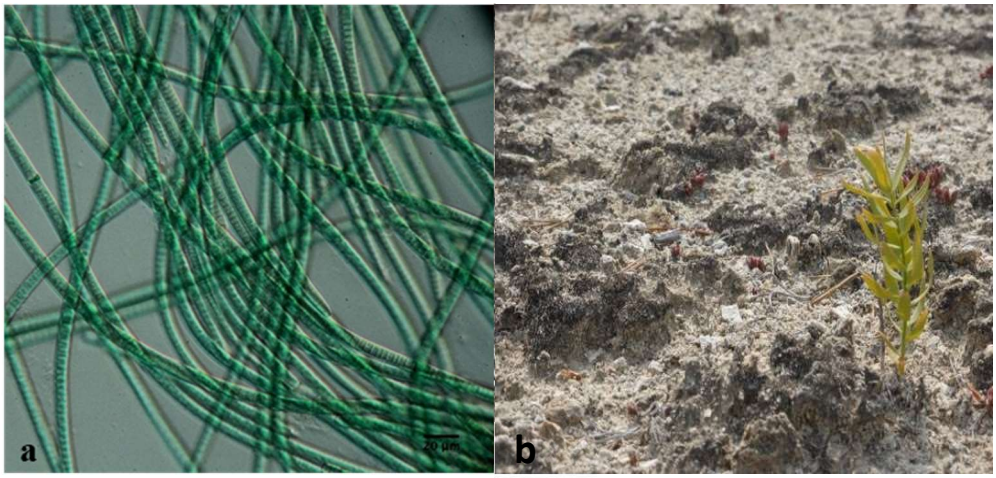

Figure 2. a) Filaments of *Microcoleus vaginatus*. Scale bar 20 µm. Photo credit: Ana Giraldo-Silva. b) "Dark" biological soil crust. Photo credit: Sergio Velasco Ayuso.

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2 – OPTIMIZING THE PRODUCTION OF NURSERY-BASED BIOLOGICAL SOIL CRUSTS FOR RESTORATION OF ARID LAND SOILS

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Abstract

Biological soil crusts (biocrusts) are topsoil communities formed by cyanobacteria or other microbial primary producers and are typical of arid and semi-arid environments. Biocrusts promote a range of ecosystem services, such as erosion resistance and soil fertility, but their degradation by often anthropogenic disturbance brings about the loss of these services. This has prompted interest in developing restoration techniques. One approach is to source biocrust remnants from the area of interest for scale-up cultivation in a microbial "nursery" that produces large quantities of high-quality inoculum for field deployment. However, growth dynamics and the ability to reuse the produced inoculum for continued production have not been assessed. To optimize production, I followed nursery growth dynamics of biocrusts from cold (Great Basin) and hot (Chihuahuan) deserts. Peak phototrophic biomass was attained between 3 and 7 weeks in cold desert biocrusts, and at 12 weeks in those from hot deserts. I also re-used the resultant biocrust inoculum to seed successive incubations, tracking both phototroph biomass and cyanobacterial community structure using 16S rRNA gene amplicon sequencing. Hot desert biocrusts showed little to no viability upon re-inoculation, while cold desert biocrusts continued to grow, but at the expense of progressive shifts in species composition. This leads me to discourage the reuse of nursery-grown inoculum. Surprisingly, growth was highly variable among replicates, and overall yields were low, a fact that I attribute to the demonstrable presence of virulent and stochastically distributed but hitherto unknown cyanobacterial pathogens. I provide recommendations to avoid pathogen incidence in the process.

Introduction

Biological soil crusts ('biocrusts') are topsoil microbial communities that include populations of cyanobacteria or microalgae as primary producers[1], as well as bacteria[2], archaea[3] and fungi[4] as heterotrophs. In some well-developed biocrusts, mosses[5] or lichens[6] are also primary producers. Biocrusts generally occur in the interspaces between plants throughout arid and semi-arid environments and provide a variety of ecosystem services. Among them is the mitigation of erosion due to the action of pioneer filamentous non-heterocystous cyanobacteria, such as those in the genus *Microcoleus*, that adhere to and stabilize soil particles due to their large size[7] and polysaccharide sheath[8]. Biological soil crusts fix and release key nutrients, such as carbon[9,10] and nitrogen[11], and a variety of other micronutrients[12] that increase soil fertility.

In spite of their remarkable resilience to climatic extremes, biocrusts are subject to damage and even destruction by compressional stress associated with anthropogenic activities such as agriculture, especially livestock grazing[13], and recreational activities[14]. Large portions of arid lands are impacted by these pressures and have become devoid of their once natural biocrust cover. Damaged lichen and moss biocrusts revert to the simpler assemblages characteristic of early successional stages with phototroph niche replacement by cyanobacteria[15]. Natural recovery rates vary widely for biocrust communities, although generally unassisted recovery is slow, with the most arid locations presenting the slowest rates[16]. Unassisted full recovery can take from multiple decades to centuries[17,18]. And yet, compositionally simple cyanobacterial crusts can recover relatively quickly, in periods of months to several years[14,19] if conditions are conducive to

growth and propagules are present. This scenario has spearheaded a recent surge in attempts to actively restore biocrusts.

Early biocrust restoration attempts relied on transplanting intact biocrusts to crustless locations. While this proved that restoration was possible[20–22], it represents an unsustainable, net-zero approach. Current alternative foci include inoculation with mass-cultured biocrust organisms, typically cyanobacteria[23–30] or mosses[31], and the so-called biocrust "mixed-community" approach[32], where a small amount of remnant biocrust from a disturbed site is used as a seed to grow large amounts of compositionally-mixed inoculum in greenhouses or "microbial nurseries". The advantages and shortcomings of each have been recently discussed[33]. The mixed community approach results in an inoculum that is, i) location-specific, ii) pre-conditioned to native edaphic factors and iii) amenable to quality control of microbial community composition. Optimally, conditions are set so that while overall growth is promoted, species composition is kept as close as possible to that of the field sites of origin. However, the effort associated with the mixed-community nursery approach is still subject to optimization. For example, aspects of temporal growth dynamics or the feasibility of utilizing the nursery-reared product as a sustainable seed for recurrent continuous production were not addressed in the original work[32]. With this in mind, and following protocol established in the original work[32], I set out to first evaluate in detail nursery biocrust growth dynamics to minimize incubation time needed to attain the biomass carrying capacity of particular soils, as well as its variability among different soils. In a second objective, I wanted to test the possibility of re-using nursery-grown biocrusts for several growth rounds while maintaining high

growth potential and a stable community composition, so as to further reduce the need for often meager field biocrust remnants.

Materials and Methods

Field locations and sampling

Four types of remnant biocrust that differed in substrate soil type and climate/region of origin, as well as bulk soil from each of the locations were collected. Hot desert, coarse soils (loamy sand) were from the Chihuahuan Desert (S Texas, Fort Bliss military base; lat 32.431069°, long -105.984151°) and hot desert, fine soils (clay loam) from S New Mexico (Jornada Basin Long Term Ecological Research Site; lat 32.545580°, long -106.723240°). Cold desert coarse soil (sandy clay loam, lat 41.104198°, long -113.008204°) and fine soil (clay loam, lat 41.104211°, -113.008204°) were collected from the Great Basin Desert, at Hill Air Force Base-Utah Test and Training Range. Hot desert crusts were at a LOD (level of development)[34] of 1, a light cyanobacterial crust and cold desert crusts at a LOD of 6, with pedicels ranging from 0.5 mm to 7.5 mm. Sampling and storage protocols followed Velasco Ayuso et al.[32].

Greenhouse incubations

Greenhouse incubations were carried out in discrete, 12 x 12 x 5 cm Tupperware-type plastic containers containing bulk native soil (250ml) that was used as the filler in the containers upon which the biocrust was inoculated. Temperature and watering regimes roughly mimicked those of the sites of origin: hot desert conditions were simulated at an

Arizona State University greenhouse in Tempe, Arizona from October 2016 through April 2017 with an average outdoor temperature of 23.1°C (ranging from 3.9°C to 37.2°C)[35] and cold desert conditions were simulated at a Northern Arizona University greenhouse in Flagstaff, Arizona, from November 2016 through May 2017 with an average indoor temperature of 16.6°C (ranging from 12.5°C to 30.2°C)[35]. Each container then served as a sacrificial, independent sample for my time-course experiments. A wicking watering system[36], was used to avoid flooding while wetting the biocrusts. Deionized water was delivered to 80% soil holding capacity every two or three days, for hot and cold desert locations, respectively, allowing the soil to dry naturally. To optimize growth, test containers received initial nutrient supplements, delivered as either 4.7 ml each of a N+P solution (357 mM NH_4NO_3, 80.5 mM K_2HPO_4, 80.6 mM KH_2PO_4 in double deionized water), or a P-only solution (80.6 mM KH_2PO_4 in double deionized water) (see Fig. S1 for details). Whether to supplement a soil for both N and P or just for P was according to prior determinations[32]. For inoculation, natural biocrusts from the corresponding location were gently crushed, homogenized and spread over the surface of the bulk native soil, so as to inoculate at roughly 5% of the population density (based on areal chlorophyll *a*, see below) in the original field biocrusts. Cloth that reduced incoming solar radiation by 60% was placed on top each of the containers and positioned approximately 2 cm above the soil surface[32].

Experimental Design

I set up 4 treatments, one for each of my four field biocrusts using its bulk native soil, each having two phases. First, I followed biomass development weekly (Phase A), to

later probe the potential to use the biomass obtained in phase A to serve as inoculum for successive rounds of growth (Phase B). Each container was used as an independent harvested sample, and three containers were randomly sampled per time point. Additional containers containing bulk native soil were incubated, watered, and supplemented with nutrients, but left un-inoculated, as controls for growth based on airborne cyanobacterial propagules rather than from the inoculum[37].

Biocrusts obtained during phase A were pooled and used as inoculum for phase B, in which the biomass resulting from a round of growth (4 or 8 weeks of growth in the nursery) was used to seed the next round. To accelerate phase B, inoculation levels for cold desert sites (rounds of growth 3 and 4) and hot desert sites (all rounds of growth) were increased to the equivalent areal chlorophyll *a* cover of 15% of that existing in the biocrust used as inoculum. A flow chart is provided (Fig. S1) for tracking treatment details including inoculation levels, growth rounds, sampling and time points.

Biomass Determination

Chlorophyll *a* areal concentration was used as a proxy for photosynthetic biomass. Biocrust cores (0.5 cm deep, 0.5 cm diameter) were collected and kept at 4°C in dark, dry conditions until analysis. Two cores were collected per container, yielding a total of 6 replicates per time point. Chlorophyll *a* was extracted in the dark at 4°C for 24 hours following the Giraldo-Silva method described in Sorochkina 2018[37], after sample grinding by mortar and pestle in 90% acetone. The centrifuge-clarified samples (15° C, 10 minutes at 8437 *g*) were analyzed spectrophotometrically in a Shimadzu UV1601

spectrophotometer, following the protocol of Garcia-Pichel and Castenholz[38], which corrects for scytonemin and carotenoid interference.

Bioassay for presence of cyanobacterial pathogens

Axenic, liquid cultures of *Microcoleus vaginatus* (PCC9802)[1], grown in Jaworski's medium (JM)[39], were inoculated with 0.2 g of biocrust from 10 randomly selected Phase A containers for each crust type. Two sets of liquid *M. vaginatus* (PCC9802) cultures served as controls: one was inoculated with 0.2 g of autoclaved soil from each location, and a second set was left uninoculated. Health was visually monitored for 2 weeks, with healthy filaments appearing green and diseased or dead cells appearing brown. Biocrust inoculated cultures that resulted in the death of *M. vaginatus* were syringe-filtered (0.2 µm, 0.45 µm, 0.8 µm, 1 µm, 3 µm) and inoculated in healthy *M. vaginatus* cultures. *M. vaginatus* health was tracked to approximate the pathogen's body size.

Cyanobacteria Microbial Community Structure

A first screening for key community members was performed from additional discrete samples by bright field microscopy using a compound microscope (Nikon Labphot-2). Additionally, biocrust cores (0.5 cm deep, 0.3 cm diameter) were taken for assessment via 16S rRNA gene amplicon. Field biocrusts used to seed Phase A growth were sampled in triplicate and nursery grown biocrusts were sampled in replicates of 9 at the end of each growth round for each of the 4 biocrust types. All cores were stored at -80° C until the bacterial community composition was determined via high throughput Illumina sequencing of PCR amplified 16S rRNA gene amplicons. The cores for each growth

round were randomly pooled and homogenized into three composite samples, so that three independent sequencing reactions per growth round and biocrust type were ran. DNA was extracted from the three composite samples via the Qiagen DNeasy PowerSoil Kit, reference number 12888-100, following manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified using the barcoded primer set 515F and 806R[40]. Triplicate PCR reactions included the following: denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 64°C for 45 seconds, annealing at 50°C for 50 seconds, extension at 72°C for 90 seconds and a final extension at 72°C for 10 minutes. PCR amplifications for each composite sample were pooled and DNA yield quantified with Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, New York, USA). 240 ng of DNA per sample were used for library preparation after purification via QIA Quick PCR Purification kit (QIAGEN, Valencia, CA, USA). The DNA concentration of the PCR pooled library was quantified by the Illumina library quantification kit ABI Prism (Kapa Biosystems, Wilmington, MA, USA). The PCR pooled library was diluted to a final concentration of 4nM and denatured before being mixed with 30% (vol/vol) of 4 pM denatured Phi X viral DNA. Finally, the PCR pooled library and Phi X mixture was loaded in the MiSeq Illumina sequencer cartridge, and the run was performed using chemistry version 2 (2 x 150 paired-end) following the recommendations of the manufacturer (Illumina, San Diego, CA, USA). Sequencing was performed in the Microbiome Analysis Laboratory at Arizona State University (Tempe, AZ, USA), yielding raw FASTQ sequence files.

Data Availability

Raw sequence data have been submitted to NCBI and are publicly available under the BioProject number PRJNA515304.

Bioinformatic Analysis

The raw FASTQ file was de-multiplexed within the MiSeq Illumina workflow under default parameters. Paired sequences were de-multiplexed and analyzed via Qiime2.10[41], using the DADA2 plugin[42] to create a feature table with representative sequences (features) and their frequency of occurrence. To remove highly variable positions, sequences were aligned with the MAFFT program[43]. FastTree[44] was used to generate a tree. Taxonomy was assigned with the Naive Bayes classifier trained on the Greengenes 13.8 release, where sequences were trimmed to include 250 bases from the V4 region, bound by 515F/806R primers[40]. I chose to focus on cyanobacteria because they are the pioneers of biocrust formation[7,8] and thus an optimal target for restoration. As such, I selected them from the master feature table using the filter_taxa_from_otu_table.py function in Qiime1[41]. Given the poor taxonomic resolution obtained with Greengenes[45], cyanobacteria sequences that attained at least 0.005% of the total number of cyanobacterial features were then phylogenetically assigned using a curated cyanobacteria database/tree version-0.22 (https://github.com/FGPLab/cydrasil/tree/0.22a) via RAxML[46] and displayed using ITOL[47].

Statistical Analyses

In phase A, I define peak biomass as the largest average chlorophyll *a* concentration obtained at any one point. Statistically significant growth is defined as a significant difference between chlorophyll *a* concentrations at peak biomass and those at inoculation. Welch's t-tests were used for all controls and biocrust types except for the cold desert, fine soil treatment, where the non-parametric Kolmogorov-Smirnov test was used because of unequal variances. For microbial community analyses, significance in composition shifts was tested with mains PERMANOVA calculated on Bray Curtis similarity matrices of relative abundances derived from sequencing with 9999 permutations and visualized using 2-D MDS plots. Ellipses indicate 90% confidence intervals. All calculations were performed using R[48] except for shifts in specific cyanobacterial community members. Shifts in specific cyanobacterial community members (as taxa, not OTUs) were analyzed using one-way ANOSIM with 9999 permutations combined with SIMPER analyses using relative abundance of taxa (not OTUs). Taxa identified, via SIMPER, as drivers of dissimilarity were assessed for significant shifts using ANOVAs, within the PRIMER software, v6[49].

Results

Population dynamics

Each of my four treatments consisted of two phases. Phase A was a time-course designed to assess biocrust growth dynamics to minimize incubation times. I aimed to establish when nursery-grown biocrusts first reached biomass levels similar to those found in the field. Phase B was designed to determine if greenhouse grown biocrusts could be re-used

to seed recurrent inoculum production. In both phases, nursery incubation conditions roughly mimicked the natural environment of origin in terms of temperature, but were run with a watering regime and nutrient additions designed to maximize growth but minimize shifts in community structure, as determined previously[32].

In Phase A, I expected that biomass would steadily increase with time, eventually reaching a carrying capacity typical for the particular soil and setting, with biomass remaining invariant thereafter. I also expected that the carrying capacity would be roughly similar to the biomass of established biocrusts in the field. Under this scenario, establishing the minimal time required to reach this carrying capacity would be the main contribution of these experiments to process optimization. However, these expectations found little support in my experiments (Fig. 3). Overall, biocrust growth in phase A was quite variable among independent replicates, chlorophyll *a* in individual containers varying widely at any time point. While the general trends of increasing chlorophyll *a* with time held for all incubations, variability made it difficult to establish clear linear (or exponential) dynamics. In all treatments the time course of chlorophyll *a* seemed to denote complex dynamics instead.

Peak average chlorophyll *a* was reached between weeks 3 and 7 for cold desert biocrusts, but only at week 12 for hot desert biocrusts, after some events of significant chlorophyll *a* loss (Fig. 3). And yet, single independent containers could show very fast growth, attaining 10-fold increases in as little as a week, as can be seen in the single outlier point at week one in the fine, cold desert crust incubation.

In the cold desert coarse soils, chlorophyll *a* peaked at week 3 with an average level of 44 mg m^{-2} (n=6) (Fig. 3), which was still below the initial value of the remnant field biocrust

from the site. Otherwise, the dynamics of growth in this biocrust, with an initial steady increase, followed by statistical stasis, was the only case that followed expected patterns. In the cold desert fine soils, chlorophyll *a* peaked at week 7, with an average of 130 mg chlorophyll *a* m^{-2} (n=6, also significantly below its potential in the field), but the dynamics could not be clearly differentiated from those of a slow, steady increase. An apparent net loss of chlorophyll *a* occurred at week 2, but this was not significant and probably the result of high inter-container variability. In both cold desert biocrusts, chlorophyll *a* differences from initial to peak were statistically significant (either Welch's t-tests, t=-5.08, p=.0005 for coarse soil, or Kolmogorov-Smirnov tests, D=1, p=.005 for fine soil). Although chlorophyll *a* peaks could be identified in both coarse and fine soils, these were statistically lower than their respective field levels (Welch's t-tests, t=-2.77, p=.04 and Kolmogorov-Smirnov D=1, p=.005, respectively), indicating that yield was below what was observed in remnant biocrusts.

In the hot desert soils, average chlorophyll *a* took a full 12 weeks to reach levels nearing those of the field. But again here, some individual containers were still not near chlorophyll *a* inoculum levels at this time. In the coarse soil, for example, chlorophyll *a* levels consistent with field levels were reached in only four of the six replicate containers, and in fine soils only two biocrust containers reached that level. In spite of this divergence among replicates, differences in average biomass from time of inoculation to peak were statistically significant by week 12, according to Welch's t-test (t=-3.87, p=.01; t=-2.88, p=.03; for fine and coarse soil, respectively). In fine soils, biocrust chlorophyll *a* levels were indistinguishable statistically from those of the field biocrusts (t=1.66, p=.128), but in coarse soil chlorophyll *a* was significantly lower than in

the original biocrusts (t=2.54, p=.04). The population dynamics in these hot desert soils was quite complex, with frequent outlier chlorophyll *a* values, and with several cases of significant declines in chlorophyll *a* well below the level of inoculation (weeks 1 and 8 in the coarse hot biocrusts, for example).

Tracking of phototrophic growth from air-borne cyanobacteria in control containers allowed me to exclude the possibility of contamination since no controls resulted in any significant phototrophic biomass during Phase A.

Re-inoculation potential

Biocrusts obtained during phase A were pooled and used as inoculum for phase B, in which the biomass resulting from a round of growth was used to seed the next round. Biocrust in hot desert soils did poorly when using recycled inoculum. After 4 weeks of incubation, median coarse and fine soil biocrust chlorophyll *a* levels were in fact *below* inoculum levels. For this reason, trials were ended early (Fig. 3). By contrast, phototrophic biomass in cold desert containers recurrently attained significant levels within 4 weeks of incubation, although not always to target levels. Cold desert biocrusts from coarse soils in the 2nd and 3rd growth rounds produced chlorophyll *a* (median) levels that met or exceeded that of the inoculum (Fig. 3). Fine soil biocrusts did not reach inoculum levels in the 2nd growth round; however, with increased inoculum density, median chlorophyll *a* levels were within one standard deviation of inoculum levels after 4 weeks in the 3rd growth round (Fig. 3).

Again, tracking of phototrophic growth in control containers allowed me to exclude the possibility of contamination in all but the final round of cold coarse incubations (Welch's t-test T=4.30, p=.49).

Community composition

An analysis of the phototrophic community structure of nursery produced biocrusts, run on the basis of taxa, revealed minimal shifts in the dominant species for the first growth round (Bray Curtis, pairwise PERMANOVA, p=.1) in at least three of the soils (cold coarse, hot fine, and cold fine). This is consistent with the results obtained in similar experiments by Velasco Ayuso et al.[32]. In the case of the hot coarse crust with complex growth dynamics, there were significant relative decreases (from 45% to 9%) already at the end of the first growth period for *M. vaginatus* (ANOVA, p<.001), while *M. steenstrupii* increased in relative abundance from 51% to 75% (ANOVA, p=.004). Given that the average chlorophyll *a* level decreased from 46.1 mg m^{-2} to 3.0 mg m^{-2}, this implies absolute losses for both populations. New taxa, including diatoms, undetectable in the original samples, also became important components (Fig. 5). In the cold desert incubations, where recurrent incubations yielded good growth, there were progressively more marked shifts in species composition at each round (Fig. 4, 6). The community composition in the final round of growth of all biocrust types differed from their respective field community composition based on Bray Curtis similarity indices of relative abundance (Bray Curtis, mains PERMANOVA, p=.003 cold fine, p=.004 cold coarse, p=.02 hot coarse, p=.03 hot fine) (Fig. 6). For example, *Lyngbya* and *Nostoc commune* became more prevalent in cold desert coarse soils in the final growth round

(ANOVA, p<.001, p=.005, respectively), contributing 18.2% and 14.4% of the dissimilarity, respectively, whereas *Leptolyngbya* spp. became more prevalent in the fine soil of cold desert sites (ANOVA, p=.04), contributing 39.3% of the dissimilarity as assessed by SIMPER analysis. Diatoms increased overall in both cold desert soils and hot desert fine soil (ANOVA, p=.03 coarse soil, p=.04 fine soil, p<.001 hot desert fine soil, respectively). These changes were also observed by microscopic inspection. For coarse, hot desert biocrust, the interpretation was more difficult, in that, in the absence of net phototroph population growth, shifts must have accrued through differential survival rather than by differential growth (Fig. 5). A detailed assessment of shifts based on phylotype (OTU) for organisms identified at the genus level, such as *Leptolyngbya* spp., *Lyngbya* spp., *Scytonema* spp. *Chroococcidiopsis* spp., and *Nostoc* spp. confirmed patterns observed at the taxon level, with significant shifts in community composition (mains PERMANOVA p<.001 cold fine and coarse, p=.003 hot fine, p=.004 hot coarse) (Fig. S2, S3, S4).

Two key species, *Microcoleus vaginatus* and *Microcoleus steenstrupii* are of special interest as they are the pioneer[7] and dominant biocrust organisms[50]. Within hot desert biocrusts, *M. steenstrupii* was typically a dominant community member and continued to dominate in both soil types throughout all growth rounds. Community shifts primarily introduced increased diversity in secondary community members but also losses of *Scytonema* and *Microcoleus sociatus* (Fig. 5). In cold deserts, where *M. vaginatus* is typically dominant, it remained dominant in nursery-produced biocrusts for at least 3 rounds of growth in coarse soil, and for the whole treatment in fine soil (Fig. 4).

Pathogenic agents

Given the unexpected growth dynamics, I tested for the presence of pathogenic agents of cyanobacteria in my biocrusts. Bioassays of soil pathogenicity to *Microcoleus vaginatus* PCC9802 (Fig. 7) in phase A containers resulted in mortality of 70-80% of assays from hot soils, 60% of cold coarse soil samples, but only 10% in those from cold fine soil (Table 1). No virulence was recorded in any of the assays inoculated with autoclaved soils, and uninoculated controls remained healthy in all cases. Additionally, size filtration indicated the pathogen ranged in body size from 0.8 μm to 1 μm, excluding most viral and eukaryotic predator or pathogens[51]. It is likely bacterial in nature.

Discussion

With respect to my first goal, a strict reading of my results obtained here would be that growth dynamics are heterogenous but appear to be dependent on biocrust inoculum origin, including climatic aspects, whereby biocrusts from cold deserts took 3-7 weeks to reach maximum levels whereas biocrusts from hot desert took roughly twice as long (12 weeks). My data indicate that cold biocrusts will likely develop photosynthetic biomass at a faster rate than warm biocrusts, possibly due to longer active periods enabled by more frequent wetting: warm biocrusts were only watered every three days whereas cold desert biocrusts were watered every two days. Also, significantly warmer conditions for hot desert biocrust generally result in faster soil water evaporation. Since soil biocrusts are only active when wet[52,53] these two factors may have caused a much longer (approaching double) cumulative growth period for the hot desert biocrusts. This could explain the differences in biocrust growth in the two desert soil types. Of course,

simulating the temperature and wetting frequency of the local climatic regime is considered crucial in maintaining the original community composition, and maintaining a stable community composition should be a priority as there is evidence of biogeographic patterns in the distribution of biocrust microbes[50,54,55] and inoculation with foreign microbes may introduce invasive species or low quality inoculum. I did not observe a strong effect of soil texture on growth dynamics, although previous field studies have shown a positive correlation between finer textured soil and an increase in biocrust cover[56]. Based on these results, shortening the times for the original protocols, duplicated here, (which were around 8 weeks in duration[32]) to 3-4 weeks seems like a feasible optimization for production of biocrusts from cold deserts, particularly since the community structure remained rather stable, conserving significant populations of the major biocrust components. In the case of biocrusts from warm deserts, the original 8 week growth duration[32] seems already to have been close to optimal.

However, the previous data interpretation would obviate the unexpectedly high level of variability in growth among replicates. It far exceeded that of analytical error and suggests that the variability was in the organisms themselves. It is clear that for at least some replicates (usually seen as outliers in Fig. 3), very fast biocrust growth is possible, but these growth rates were not realized in all replicates. Apparently stochastically distributed and important loss factors to the phototrophic populations were at play, and hence the dynamics failed to conform to simple models of a linear (or exponential) growth dynamics followed by stasis at carrying capacity. Previous nursery production of restoration inoculum had similar results, with high levels of growth in some containers and little to none in others; however, containers with high levels of growth were used for

restoration inoculum and containers with poor growth were excluded (Corey Nelson, personal communications). This approach was likely an unintentional selection against any native loss factors. This level of complexity, and the nature of those loss factors must be studied and understood, not only to better understand biocrust ecology in its basic sense, but also for an effective application in the production of inoculum for restoration: being able to suppress those loss factors would lead to maximal growth rates in *every* growth container. The nature of those loss factors is as yet unknown, and the literature does not offer much solace. With respect to grazing by microinvertebrates, several publications have documented their presence in soil biocrusts[57–62] even though grazing pressure has not been measured as a significant ecological factor, and there is no evidence that those microinvertebrates may be stochastically distributed. Alternatively, disease can be a significant loss factor in the population dynamics of phototrophs. Examples can be found in the literature on the severe impact of cyanophages on the population dynamics of planktonic blooms of cyanobacteria[63–66], and soils are known to harbor significant populations of bacteriophages[67,68]. However, also in the case of infectious agents, I would need to find one that is rare enough as to appear stochastically at the cm scale. The unidentified plaque-forming agent described by Sorochkina et al.[37] in similar biocrusts might be a potential contender. A retrospective examination of this possibility via bioassays with relevant cyanobacterial cultures clearly demonstrated the potential for virulence of the nursery biocrust soils. Hot desert biocrusts, which were particularly virulent, presented the most variable dynamics and the weakest yield. The presence of this biological agent(s) likely had a profound impact on biocrust growth, and the homogenization of inoculum that was carried out in Phase A and again in Phase B,

probably contributed to its spread. An effort to establish the identity via 16S rRNA gene amplicon sequencing was not successful due to increasing numbers of soil heterotrophic bacteria following cyanobacterial death; isolation, etiology and relevance in natural communities of this cyanobacterial pathogen are currently underway. Until that research is completed, it is my recommendation that a process of bioassay testing of inoculation be carried out prior to nursery incubations, selecting at each step only pathogen-free containers, and avoiding cross-container inoculum homogenization.

With respect to my second goal to establish if the recurrent use of inoculum was feasible in nursery biocrust production, I can safely say that in my treatments this was not an advisable strategy for cold desert biocrusts. While it was possible in some cases to re-use inoculum in a second, or even third incubation without major community shifts, there was evidence for a clear cumulative divergence (Fig. 4, 6). Based on my treatments, if recycled inoculum is used, I would suggest it be closely monitored. Beyond the issue with community structure, the cumulative lack of fitness for growth of the recycled inoculum in warm deserts was rather unexpected. If my ideas with respect to the importance of randomly distributed deleterious agents as the cause of sluggish and inconsistent growth hold, then the homogenization of the inoculum from prior recurrent inoculation may have in fact ensured the presence of those agents in all Phase B containers and the eventual cessation of growth in all replicates. This risk was clearly unanticipated and provides a warning for mixed-community approaches, adding to the advantages of biocrust inoculum production through mass cultivation of isolated biocrust organisms[33]. Caution must also be exercised when choosing the source inoculum from the field. Minimally, inoculum should appear healthy. At the very least, careful

monitoring of the growth trends of nursery biocrusts will allow any “diseased” biocrusts to be removed from production.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the *Applied and Environmental Microbiology* journal's website.

Tables

Location	Soil Type	Treatment	Replication	Strong	Weak
Hot (NM)	Coarse	Autoclaved	3	0	0
		Live	10	8	0
	Fine	Autoclaved	3	0	0
		Live	10	7	0
Cold (UT)	Coarse	Autoclaved	3	0	0
		Live	10	4	2
	Fine	Autoclaved	3	0	0
		Live	10	1	0

Table 1. Virulence of biocrust according to *M. vaginatus* killing assay. Strong virulence denoted by death at 5 days of incubation, weak virulence by death at 12 days.

Figures

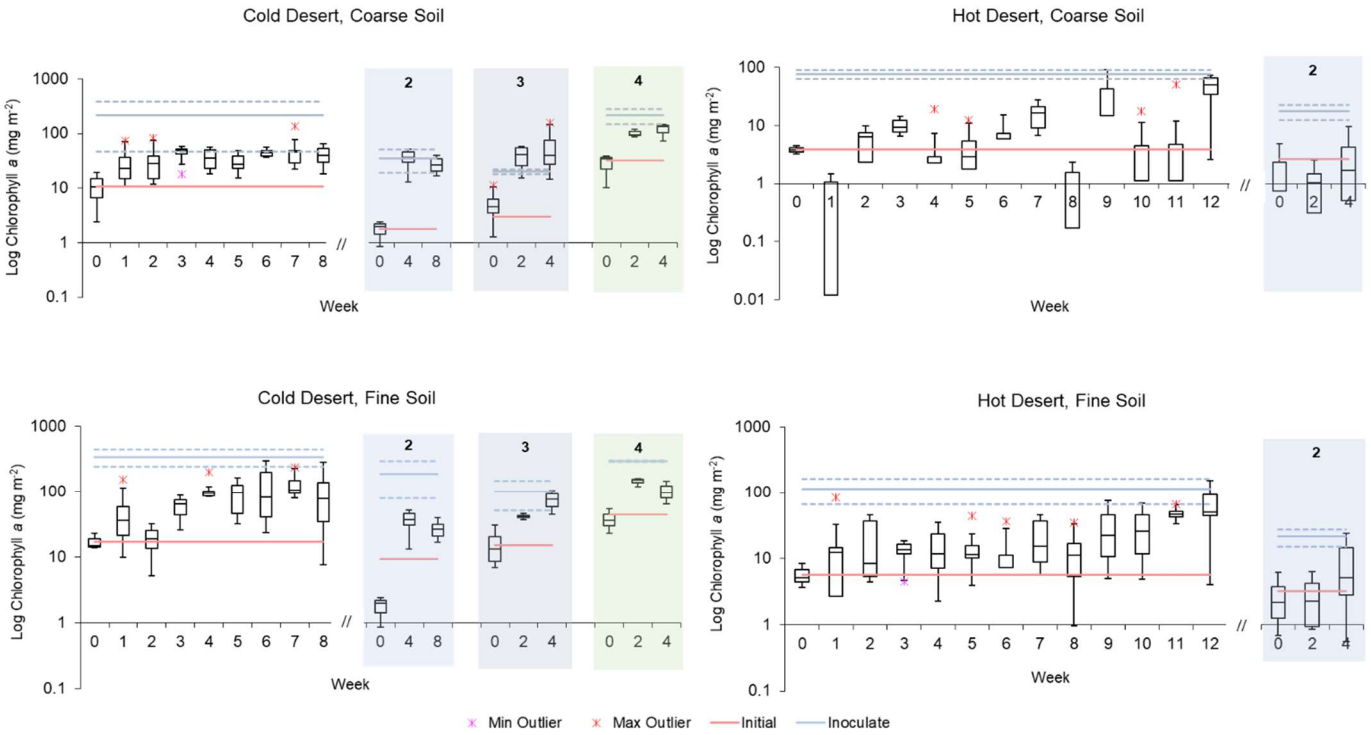


Figure 3. Growth dynamics of phototrophic biomass (as chlorophyll *a* area concentration) during biocrust incubations. Phase A is shown on the far left of each panel. Successive Phase B re-inoculations are shown under shading and numbered. For each time point, box-plots indicate upper and lower quartiles and median values are shown as solid lines within the boxes. Whiskers denote upper and lower range, and asterisks denote outliers. For Phase A, n=6, except at t=0, where n=3. For re-inoculation # 2, n=6, except at t=0, where n=3. For re-inoculation #3 n=4 and for #4, n=3. Blue lines denote chlorophyll *a* content of field biocrusts used as original inoculum (n=3) and red lines, chlorophyll *a* content at t=0 (n=3). Solid colored lines indicate mean values and dashed lines indicate one upper and one lower standard deviation of field biocrusts (n=3).

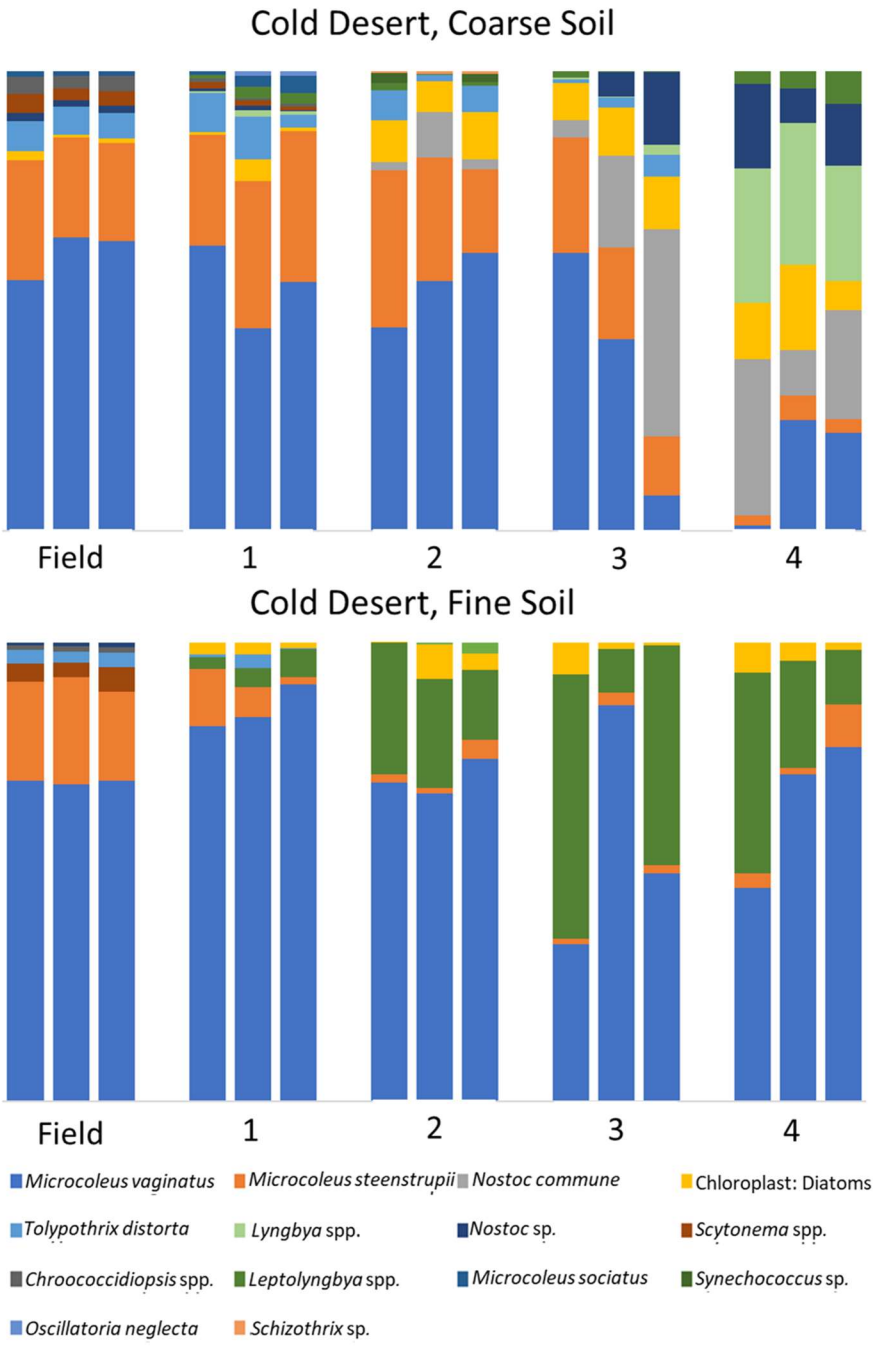


Figure 4. Cyanobacterial community composition in cold desert biocrusts, based on 16S rRNA amplicon sequencing, in biocrusts collected from the field (field), from the Phase A incubation (1) and those resulting from recurrent production according to round (2, 3 and 4).

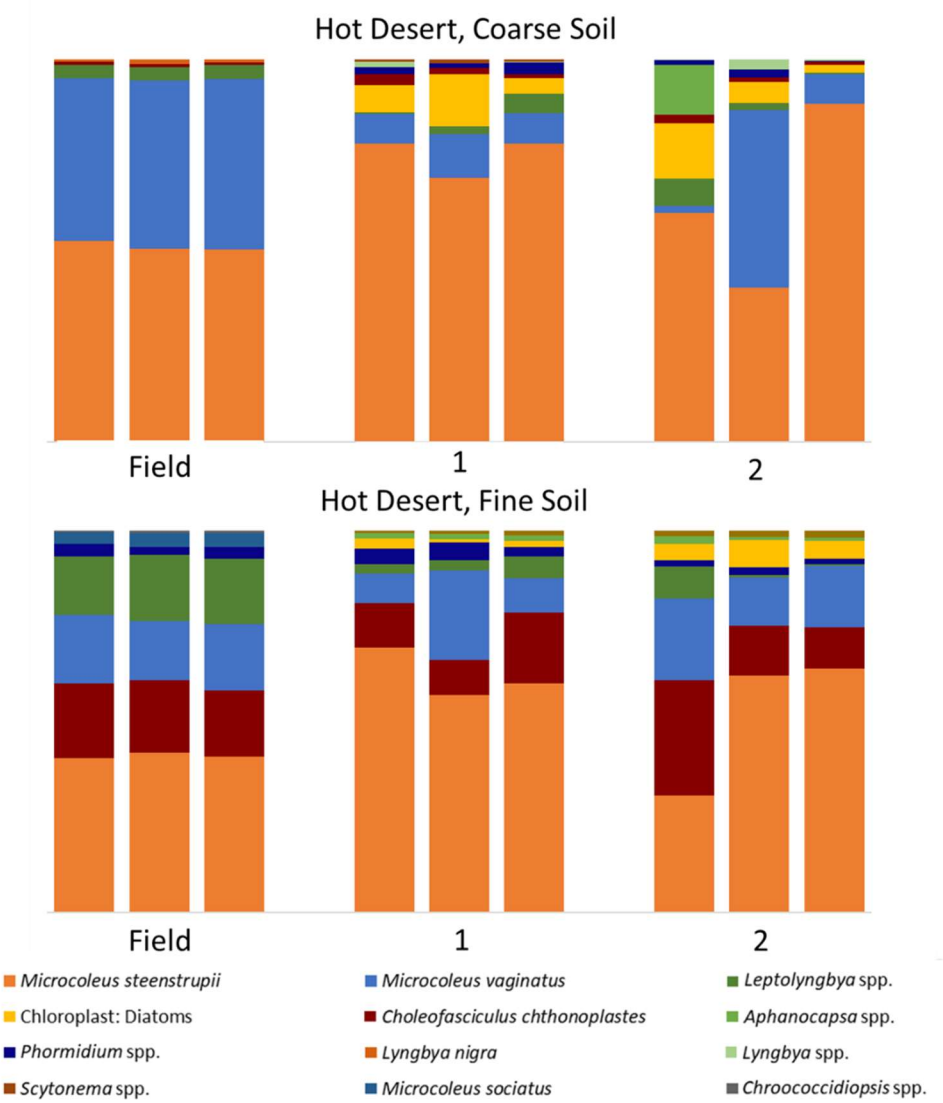


Figure 5. Cyanobacterial community composition in hot desert biocrusts, based on 16S rRNA amplicon sequencing, in biocrusts collected from the field (field), from the Phase A incubation (1) and those resulting from a second recurrent production (2).

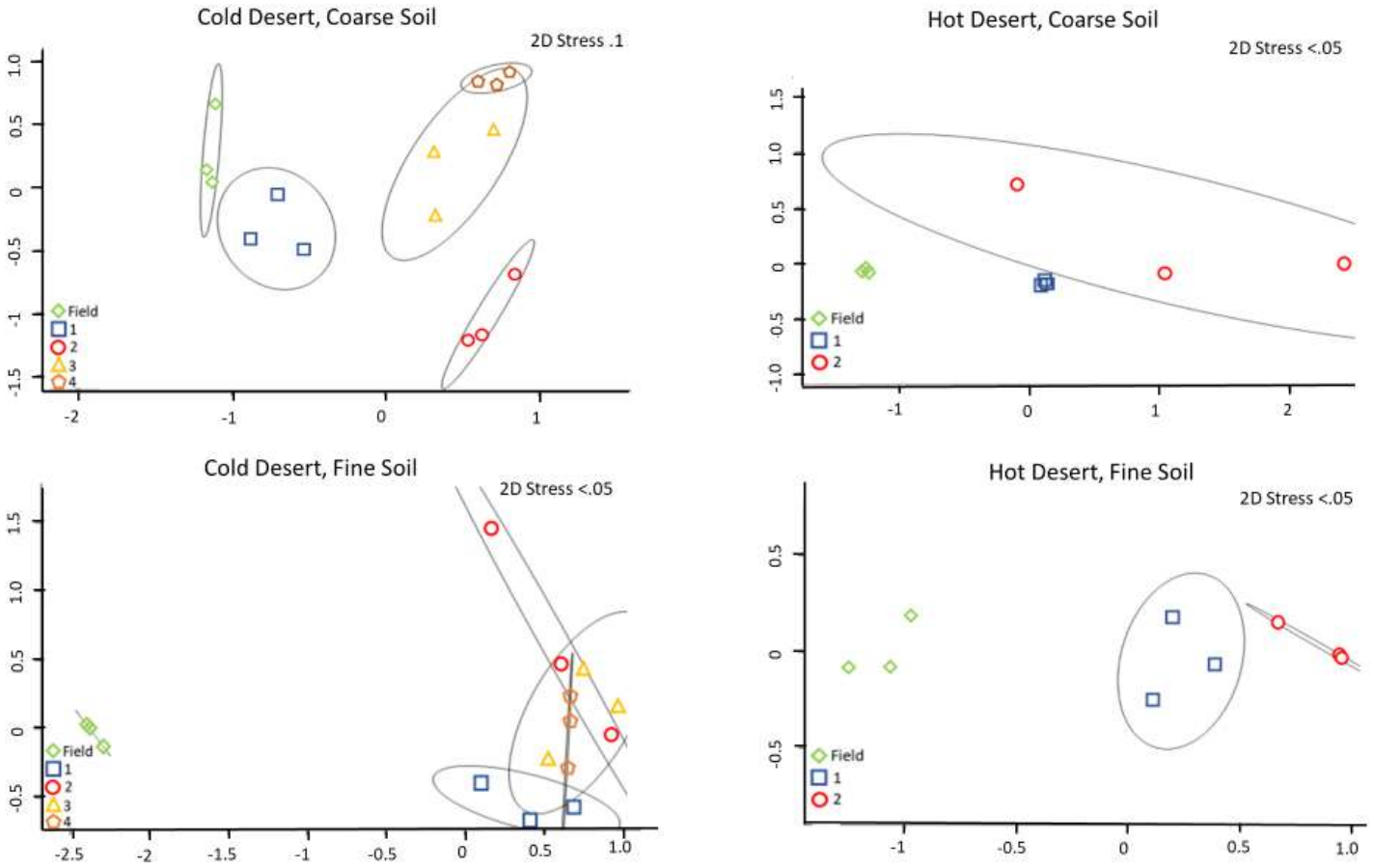
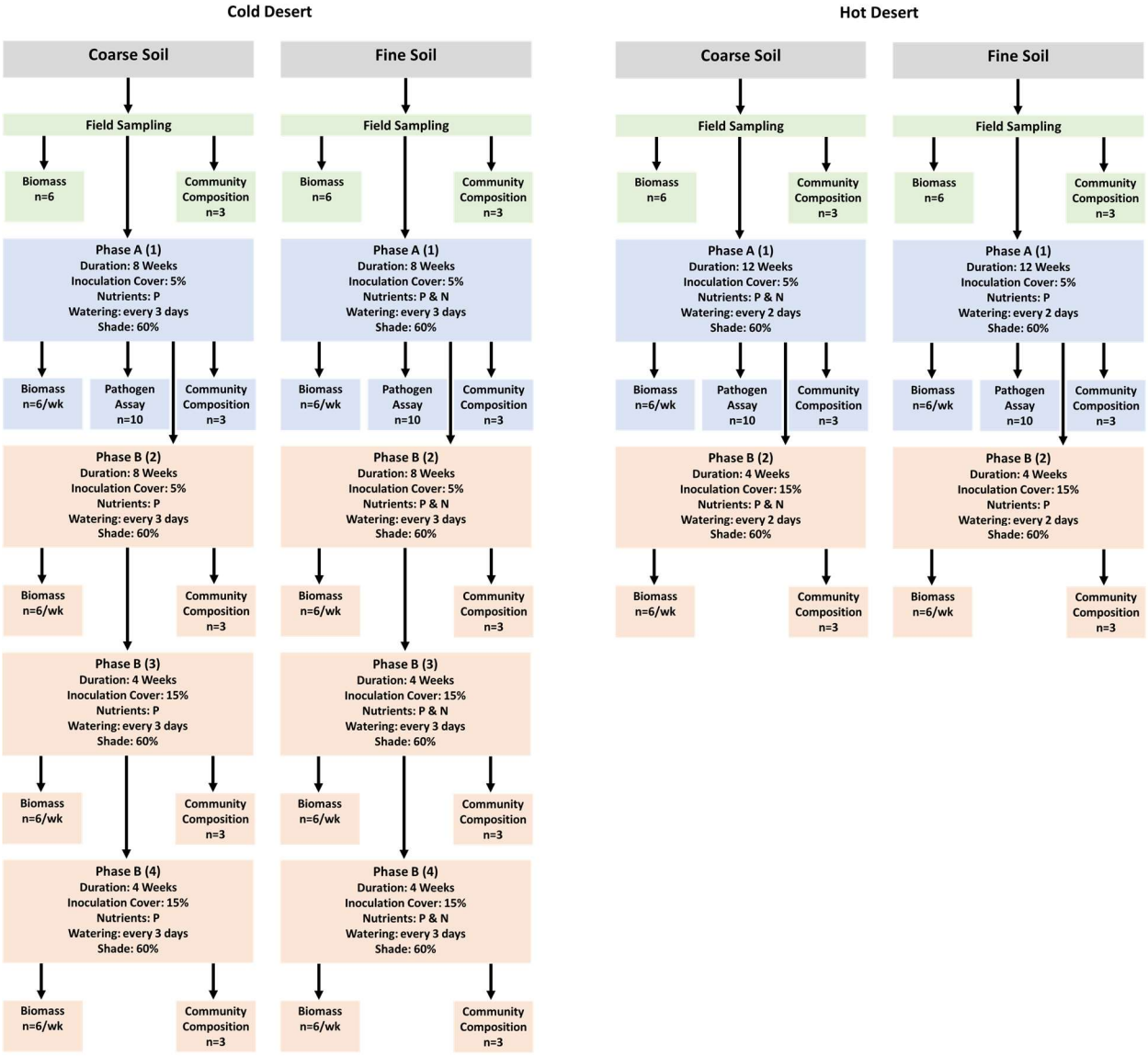


Figure 6. 2-D MDS of cyanobacterial community composition based on 16S rRNA amplicon sequencing, in biocrusts collected from the field (field), from the Phase A incubation (1) and those resulting from recurrent production according to round (2), and for cold desert biocrusts (3 and 4).

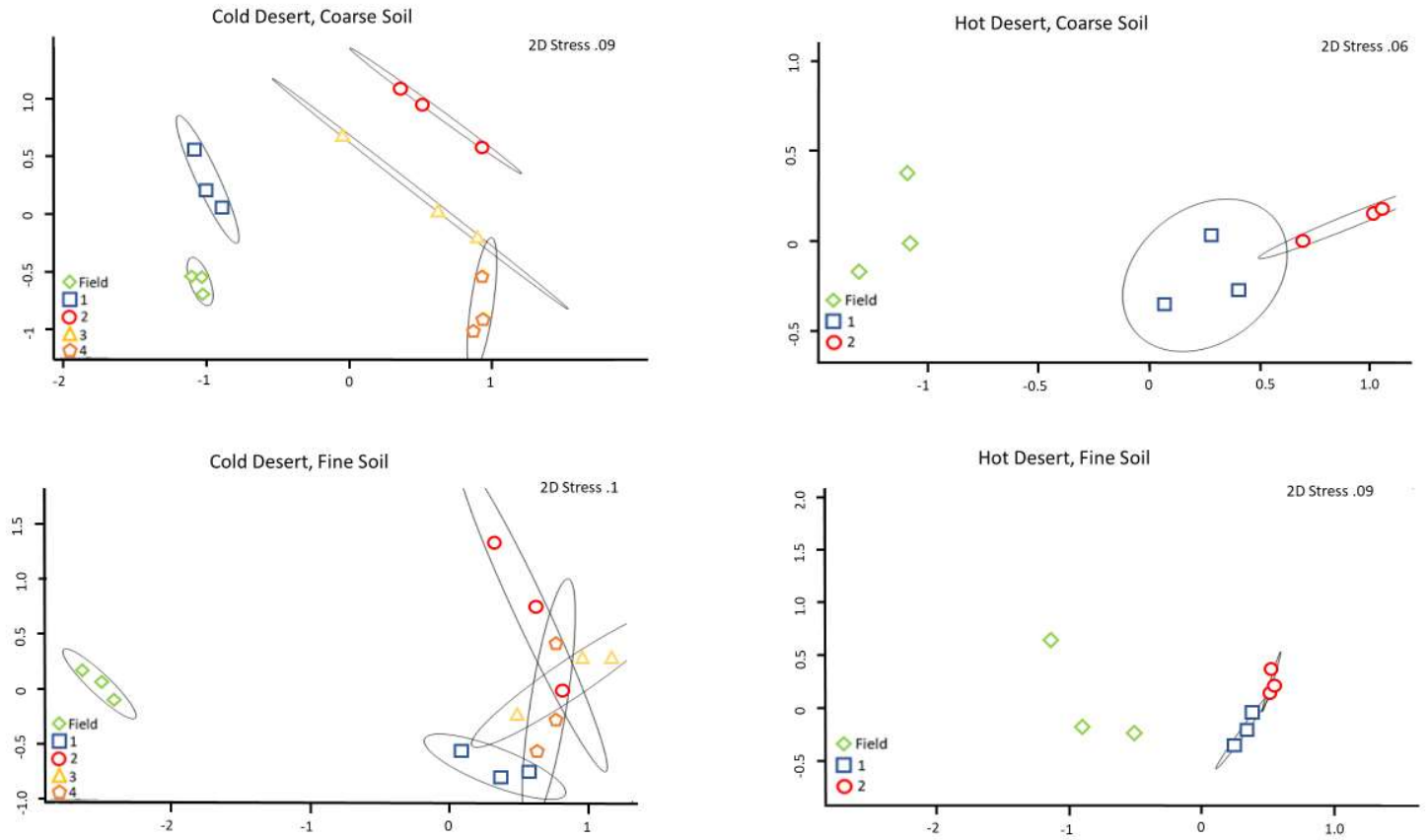
Figure 7. Bioassay determination of virulence towards *M. vaginatus* PCC9802. Liquid cultures were inoculated with 0.2 g of biocrust soils and the potential to kill *M. vaginatus* determined visually after 5 and 12 days of incubation. Top photograph is a positive for virulence, bottom is a negative for virulence.

Supplementary Information

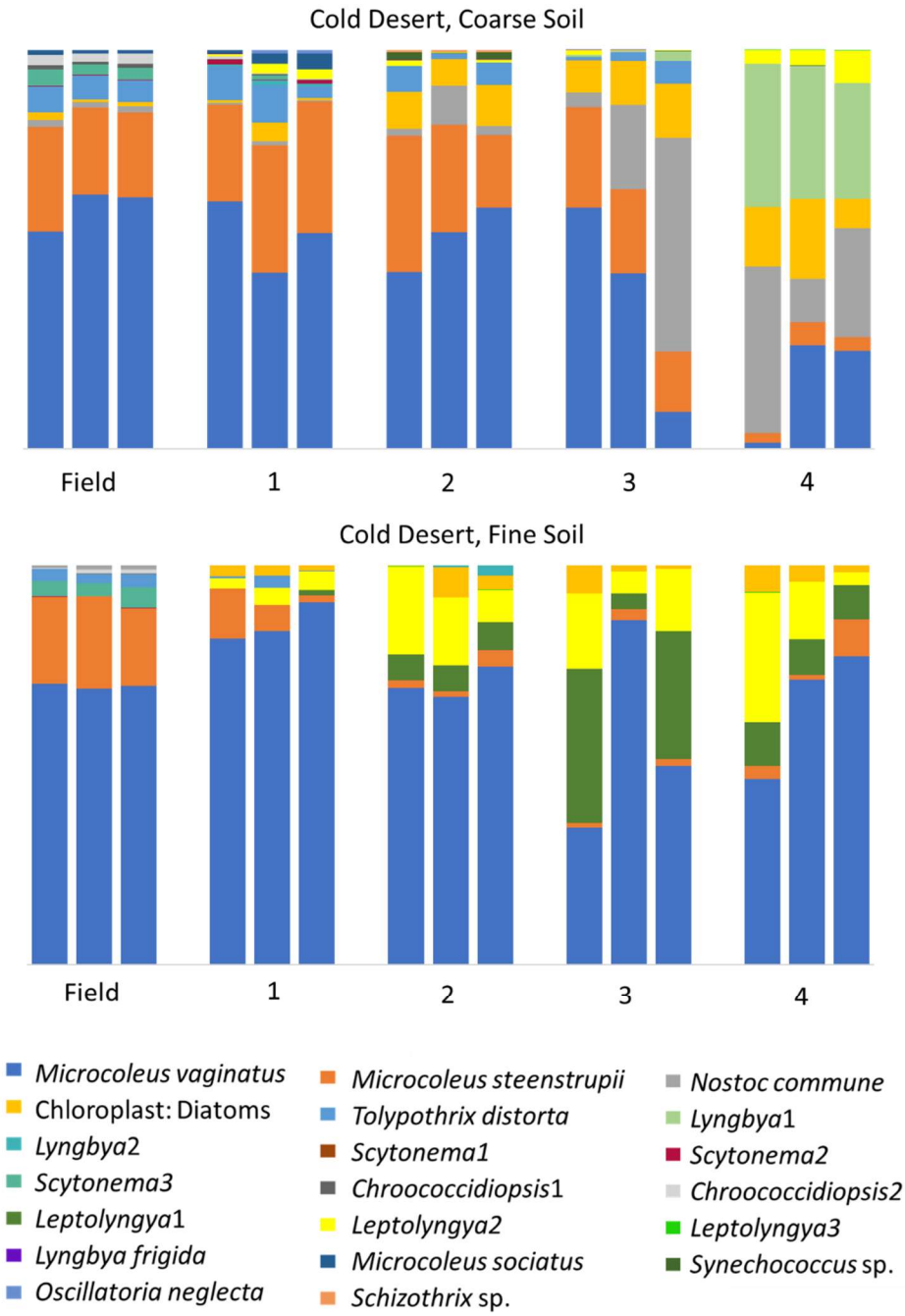
Supporting Figures



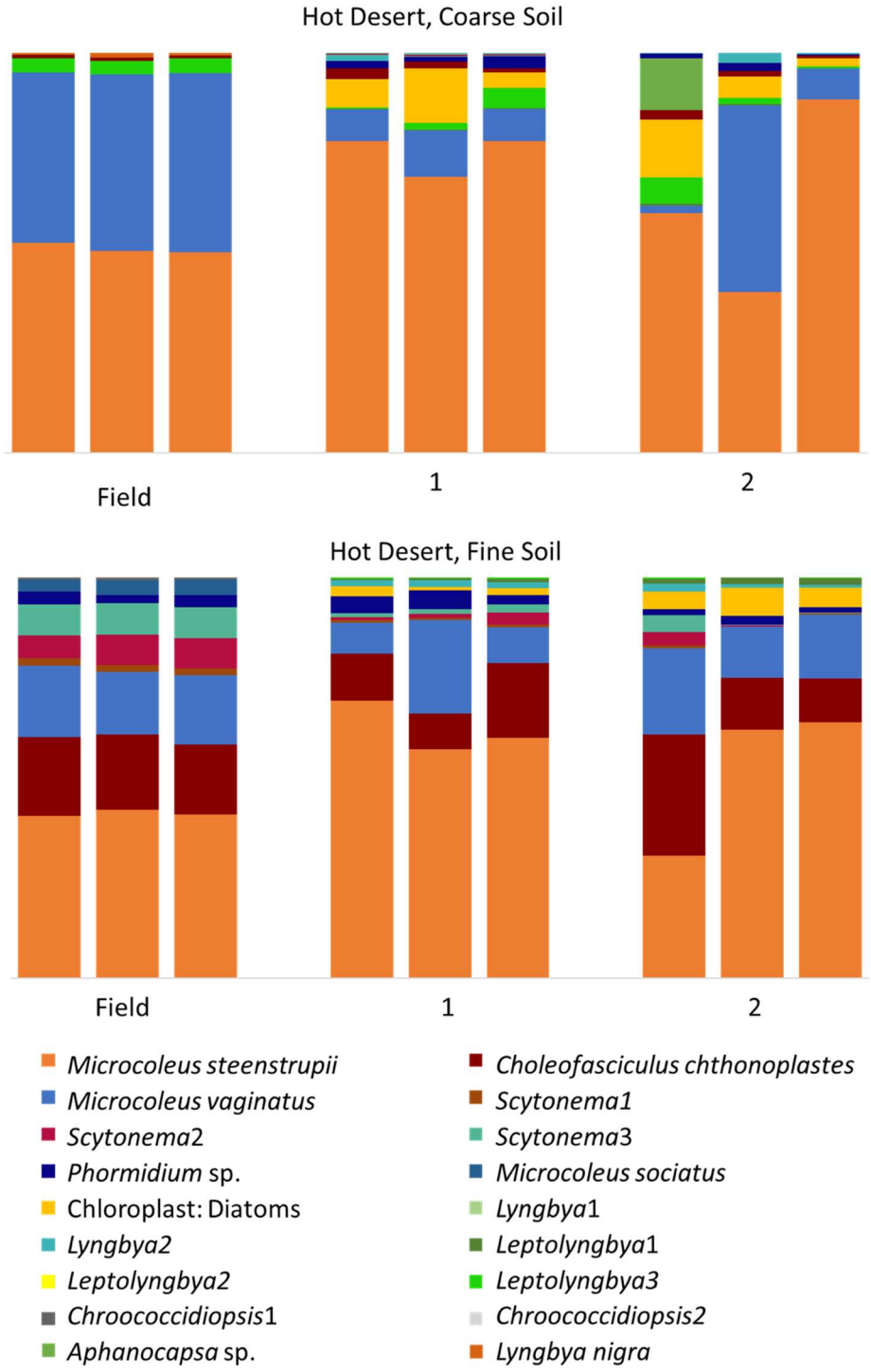
Supplementary Figure 1. Experimental flow chart including inoculum levels, growth rounds, sampling and time points. Field biocrusts are shown in green. Phase A is shown in blue and Phase B is shown in orange.



Supplementary Figure 2. 2-D MDS of cyanobacterial community composition, similar to Fig. 6, in which the taxonomic resolution for cyanobacteria has been increased to the maximum possible. Legend: biocrusts collected from the field (Field), from the end of Phase A incubation (1), and those resulting from recurrent production in Phase B, according to round (2, 3, and 4).



Supplementary Figure 3. Cyanobacterial community composition at the species, or phylotype level where species identification was not possible, in cold desert biocrusts, based on 16S rRNA amplicon sequencing. Communities shown include biocrusts collected from the field (Field), from the end of Phase A (1), and those resulting from recurrent production in Phase B according to round (2, 3 and 4).



Supplementary Figure 4. Cyanobacterial community composition at the species, or phylotype level where species identification was not possible, in hot desert biocrusts, based on 16S rRNA amplicon sequencing. Communities shown include biocrusts collected from the field (Field), from the end of Phase A (1), and those resulting from recurrent production in Phase B according to round (2).

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3 - CYANORAPTOR TOGATUS GEN. ET SP. NOV., A *BACTEROIDETES* BACTERIUM PREDATORY ON FILAMENTOUS CYANOBACTERIA THAT SUSTAINS EPIDEMICS IN BIOLOGICAL SOIL CRUSTS

Coauthors have acknowledged the use of this manuscript in my dissertation.

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Abstract

Predatory bacteria constitute a guild of heterotrophs specialized in obtaining resources for growth from the live bacteria they prey upon. They have been isolated from many environments and are phylogenetically diverse, each having evolved their mode of life independently. I report on a novel type of obligatory predatory bacterium, *Candidatus* Cyanoraptor togatus, belonging to the *Chitinophagaceae* family (*Bacteroidetes* phylum). Cyanoraptor attacks filamentous cyanobacteria in soils, including *Microcoleus vaginatus*, the most abundant terrestrial cyanobacterium, and can demonstrably bring about catastrophic mortality events to their populations in nature and in production facilities. A polyphasic study combining confocal and electron microscopy, cultivation in co-culture with its prey, genomic analyses and physiological assays reveal that this organism sustains a complex life cycle, with submicrometer, non-growing, non-motile, internally compartmentalized coccoid propagules that gain attachment and entry into the prey's cytoplasmatic space, where they hydrolyze its contents, growing into bacilloid and eventually pseudofilamentous forms, which then undergo multiple divisions and secretion of extracellular vesicles to form new propagule cells. The type strain, LGM-1^T, has a 3.3 Mbp genome that is rich in hydrolase-encoding genes endowed with signal peptides for secretion but deficient in pathways for amino acid biosynthesis enzymes. Physiologically, the propagule cells are quite vulnerable to environmental stressors, likely constraining its success in natural systems. While no close relatives exist in culture, 16S rRNA sequences with affinities to this organism are found in molecular surveys from a variety of environments.

Introduction

Predatory bacteria are a guild of heterotrophic bacteria specialized in obtaining carbon and energy for growth from living microbial cells of species other than their own[1]. This unusual *modus operandi* has apparently evolved multiple times independently among bacteria, as examples exist in phylogenetically distant groups, including the *Proteobacteria*[2], which host the majority of studies, but also the *Firmicutes*[3,4], *Bacteriodetes*[5], *Melainibacteria*[6], and *Chloroflexi*[7]. The range of prey species for all known predators is rather wide, often including bacteria from various phyla[2], and even yeasts[4]. Some are obligately predatory, and some resort to predation facultatively[1]. Predatory bacteria "hunt" using a variety of approaches. Some use swarms of predatory cells that release hydrolytic enzymes to target prey bacteria in their proximity[8]. This is known as wolf-pack predation and is typical of the delta-proteobacterial *Myxobacteria*[9] and the *Chloroflexi*'s *Herpetosiphon* spp[10]. In a more directed interaction akin to parasitism, epibiotic predators will target and attach directly to prey cells, injecting them with hydrolytic enzymes to eventually assimilate the prey's hydrolysate, as is the case of *Vampirococcus*, which preys upon populations of the purple sulfur bacterium *Chromatium* sp.[11]. Finally, endobiotic predators not only attach, but also gain direct entry into the prey cells after locally hydrolyzing their tegumentary polymers, accessing either the periplasmic or the cytoplasmic space[12], assimilate the prey's cellular hydrolysate, and growing and dividing inside the prey[13]. *Bdellovibrio bacteriovorus* was the first among such endobiotic predators to be isolated[1] and remains the best studied predatory bacterium. Many of the known predatory bacteria remain to be rendered into pure culture, perhaps because of their obligatory reliance on live prey[14]. Predatory bacteria have been

isolated from marine[15] and freshwater systems[16], sewage[17,18], soil [19–21] as well as host-associated microbiomes[22].

Predation and disease can be significant forces shaping microbiomes in nature. Phage can regulate population structure, dynamics and primary production in aquatic blooms of cyanobacteria[23–26], as can protozoan grazing[27]. However, because what is known about predatory bacteria has largely been derived from culture-based studies, the relevance of this phenomenon as an ecological loss factor in natural microbiomes remains to be ascertained rigorously[28]. That such a diversity of bacterial types can be predators, and the fact that predatory bacteria can be recurrently isolated from so many environments, suggest that they could well be relevant.

Biological soil crusts (biocrusts) are topsoil photosynthetic communities of cyanobacteria[29], heterotrophic bacteria[30], fungi[31], and archaea[32]. In some, lichens and mosses[33] are also present. They develop conspicuously in plant interspaces throughout arid and semiarid regions[34], where they provide various ecosystem services, including carbon and nitrogen fixation[35–37], soil stabilization against erosion[38,39] and hydrological control between percolation and runoff [40–42]. The cosmopolitan *Microcoleus vaginatus* is a foundational member, and the most common cyanobacterium within biocrusts, contributing crucially to early soil crust formation[38,39]. The value of biocrusts for the regeneration of degraded arid soils has been a recent focal point in restoration ecology[43] (see Giraldo-Silva *et al.* for a review of restoration approaches[44]). While disease agents have been documented in almost all natural settings, until very recently the biocrust literature was strikingly devoid of reports on the presence or incidence of disease. I could find a single report of bacteriophages targeting *Firmicutes*[45] and no cases impacting

primary producers at all. But recent findings on the devastating impact of non-viral disease agents during the production of cyanobacterial inoculum intended for arid land soil restoration[43] suggested that this may have been an important omission and spearheaded my current work. I report here on the discovery, enrichment and characterization of a novel type of endobiotic predatory bacterium that targets populations of filamentous cyanobacteria in biocrusts and that demonstrably exerts major control over their populations, and propose for it the epithet '*Candidatus* Cyanoraptor togatus', as a new generic and specific entity to accommodate it.

Materials and Methods

Sources of symptomatic biocrusts

I observed cyanobacterial mortality recurrently on biocrusts incubated or grown in greenhouses for experimental purposes. This took the form of unexpected, catastrophic mortality events[43], or of spatially restricted, macroscopically visible clearing zones reminiscent of viral plaques or fairy-rings[46]. These experimental soils, as well as their natural crusts of origin, were the source materials for the work presented here. Field locations were in the foothills of the Superstition Mountains (33.3923072, -111.3539764) of Arizona, USA. Clearings in cyanobacterial population cover resembling viral plaques were noted in areas of previously healthy cyanobacterial growth (see Fig. 8). Surface soil samples in and around these plaques were excised using sterile forceps and stored for no more than 24 hours in a dry state before further processing.

Detection of pathogens to cyanobacteria in biocrusts (*Microcoleus* mortality assay; MMA)

To determine the presence of agents pathogenic to cyanobacteria I developed a bioassay based on the fate of an axenic culture of *Microcoleus vaginatus* (PCC 9802)[29], a representative of the most common and widespread biocrust cyanobacterium. Liquid growth medium was inoculated with the test cyanobacterium at an initial concentration 0.235 mg (Chl *a*) l^{-1} as prey, and with 25 mg from a homogenized mixture of the soil to be tested. The medium was Jaworski's minimal medium[47] and assays were incubated under 10 μmol $m^{-2}s^{-1}$ of illumination from fluorescent bulbs with a 12 h photoperiod at room temperature in vented-cap, plastic 75 ml tissue-culture flasks kept upright. MAA were read after 5 days of incubation by simple inspection, wherein positives for *Microcoleus* mortality developed conspicuous chlorosis first, followed quickly by patent structural degradation of biomass. The assay invariably included a) uninoculated negative controls and b) controls for the possibility of mortality due to abiotic toxicants in which the soil was autoclaved before assay inoculation. All assays and controls were run (at least) in triplicate and simultaneously.

Test for the prokaryotic nature of pathogenic agents (Expanded *Microcoleus* Mortality Assay; EMMA)

This was developed as a complement to the MMA assay to determine the biological nature of the responsible pathogen. EMMA consists of two parts. In part one, 1 ml of liquid suspensions of positive MMA assays (containing both pathogen and dead *M. vaginatus* (PCC 9802)), were added to 9 ml of a healthy axenic *M. vaginatus* (PCC 9802)

culture prepared as in the MMA assay, but also containing the eukaryotic inhibitor, cycloheximide, at a final concentration of 12.5 µg/ml. Visual cyanobacterial death after 5 days signaled the presence of a non-eukaryotic agent. For EMMA's part two, four 1 ml aliquots of the end-point positive MMA suspension were vacuum filtered through polycarbonate filters with nominal pore diameters of 0.2, 0.45, 0.8 and 1 µm each. Each filtrate was then added individually to 9 ml of a healthy *M. vaginatus* (PCC 9802) culture as above. Cyanobacterial mortality indicated that the infectious agent's effective size was smaller than the filter pore size used; lack of mortality in the 0.2 µm pore size filtrate was taken as indicative of a non-viral pathogen. EMMA also included uninoculated controls, and incubation conditions were identical as in the MMA. See Table S1 for a condensed list of components of the EMMA assay.

Enrichment and isolation of predatory bacteria

In order to obtain an isolate of the infectious agent, I carried out a sequential battery of enrichment or isolation techniques, starting with direct plating of size-filtered contents of positive MMA assays. Plating was done on tryptic soil broth medium solidified with 1.5% agar. Colonies were examined after 3, 5 and 10 days of incubation at room temperature, and colonies of unique characteristic selected for further testing. Pathogenicity of the isolates was tested using the MMA inoculated with suspensions of the cultures instead of the soil slurry.

For enrichments, I first used size-fractionation (SF) of positive MMA assays, so as to enrich for bacteria with cell sizes between 0.2 µm and 1 µm. This was done using 1 ml of positive MMA assays vacuum filtered through sterile polycarbonate filters and added to 9

ml of *M. vaginatus* (PCC 9802). I also undertook a dilution-to-extinction (DTE) approach based on the SF preparations, which were diluted in standard medium in 1/10 steps up to a dilution of 10^{-7}. 1 ml of each dilution was inoculated into standard MMA, with ten replicates for each dilution. The highest dilution that still killed *M. vaginatus*, was then used for recurrent dilution attempts.

Because the SF/DTE approach did not produce a pure co-culture of predator and prey, flow cytometry/cell sorting (FCCS) was subsequently used to further purify the enrichment. SF/DTE preparations were filtered (<1 μm) and injected into a BD FACSAria Ilu cell sorter. Cells between 0.79 μm and 1.3 μm in diameter were sorted into 100 96-well plates containing healthy, axenic *M. vaginatus* (PCC 9802) cultures (Fig. S1) and incubated under standard MMA conditions. Individual wells were visually monitored for death of *M. vaginatus* (PCC 9802) under the dissecting scope (Nikon SMZ-U). Wells containing dead *M. vaginatus* (PCC 9802) were harvested and used to scale up biomass of the predatory bacterium by addition to consecutively larger quantities of *M. vaginatus* (PCC 9802), from 10 ml to 50 ml to 200 ml. This FCCS procedure was performed twice for progressively cleaner enrichments of the predatory bacterium. The final enrichment stage was used for downstream analyses under denomination strain LGM-1.

16S rRNA gene community analyses

DNA was extracted via the Qiagen DNeasy PowerSoil Kit (QIAGEN), following manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified using the barcoded primer set 515F and 806R[48]. Triplicate PCR reactions included the following: denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 64°C for 45

seconds, annealing at 50°C for 50 seconds, extension at 72°C for 90 seconds and a final extension at 72°C for 10 minutes. PCR amplifications for each composite sample were pooled and DNA yield quantified with Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, New York, USA). 240 ng of DNA per sample were used for library preparation after purification via QIA Quick PCR Purification kit (QIAGEN, Valencia, CA, USA). The DNA concentration of the PCR pooled library was quantified by the Illumina library quantification kit ABI Prism (Kapa Biosystems, Wilmington, MA, USA). The PCR pooled library was diluted to a final concentration of 4nM and denatured before being mixed with 30% (vol/vol) of 4 pM denatured Phi X viral DNA. Finally, the PCR pooled library and Phi X mixture was loaded in the MiSeq Illumina sequencer cartridge, and the run was performed using chemistry version 2 (2 x 250 paired-end) following the recommendations of the manufacturer (Illumina, San Diego, CA, USA). Sequencing was performed in the Microbiome Analysis Laboratory at Arizona State University (Tempe, AZ, USA), yielding raw FASTQ sequence files. The raw FASTQ files were de-multiplexed within the MiSeq Illumina workflow under default parameters. Paired sequences were de-multiplexed and analyzed via Qiime2 2019.7[49], using the DADA2 plugin[50] to create a feature table with representative sequences (features) and their frequency of occurrence. To remove highly variable positions, sequences were aligned with the MAFFT program[51]. FastTree[52] was used to generate a tree. Taxonomy was assigned with the Naive Bayes classifier trained on the Greengenes 13.8 release[53], where sequences were trimmed to include 250 bases from the V4 region, bound by 515F/806R primers[48].

Genome sequencing

Biomass from final enrichments of Cyanoraptor were extracted using the Monarch Genomic DNA Purification Kit (Thermo Fisher) following standard protocol. DNA was sheared with G-tube (Covaris) at 4,000 rpm using manufacturer's suggested methods. The sheared DNA, following bead cleanup, resulted in molecules ranging in size from approximately 8 to 15 kb. A PacBio sequencing library was constructed using the PacBio Express II protocol and reagents (SMRTbell Express), as specified by the manufacturer. The final library was bead cleaned, resulting in a 10.02 kb library, determined by FEMTO Pulse (Agilent). Sequencing was performed on a PacBio Sequl II instrument (PacBio) following manufacturer's protocols, with the following parameters: binding kit 2.0, primer V4, sequencing plate 2.0, 8 M v2 cell, loading concentration of 55 pM, sequencing time of 30 hours with CCS mode, yielding a FASTQ file. Following raw sequencing data collection, CCS analysis was performed using default settings on SMRT Link V8, with an average quality score of QV41.

Genome bioinformatic analysis

The raw FASTQ file was imported into a local instance of EDGE (Empowering the Development of Genomics Expertise)[54] for contig assembly using 10% of data. Assembly was performed with Irasm-wtdbg2 into 1,416 contigs at 22.97-fold coverage. Contig binning, identification and quality checks utilized CheckM[55]. The predatory bacterium was identified using partial 16S rRNA gene information and the resulting (single) bin was then curated with SEED[56] on the RAST-Server (Rapid Annotation using Subsystems Technology)[57]. Initial annotations were performed using Prokka[58] with transfers from the

closest relative, *Chitinophaga pinensis.* All putative genes were then cross-referenced using UniProt (Universal Protein Resource)[59]; hypothetical genes were not cross-referenced. Proteins of interest were then confirmed using BLASTp[60] on the NCBI database[61]. The KEGG database (Kyoto Encyclopedia of Genes and Genomes)[62–64] was used to detect the presence of metabolic pathways of interest; enzymes identified by KEGG in pathways of interest were then cross referenced with manual annotations of putative genes, to determine if a full pathway was present. In order to compare the genome size of organisms within the *Chitinophagaceae* family and that of my bacterium of interest, full genome sizes were gathered from NCBI[65].

Phylogenetic analysis

Sequence phylogenetic placements were resolved by constructing a reference tree using the maximum-likelihood + thorough bootstrap (1000 replicates) method and the GTRGAMMA model from sequences available in the SILVA rRNA database[66]. The tree was constructed using the 16S rRNA gene of my bacterium of interest and sequences (145) from all cultured organisms within the phylum *Bacteroidetes* and the family *Chitinophagaceae* with 1485+ bases. Sequences within the family *Saprospiraceae* (36) were used as an outgroup. Sequences were aligned with SSU-ALIGN, using a profile-based alignment strategy, in which each target sequence is aligned independently to a covariance model that uses the 16S rRNA gene secondary structure. Poorly aligned columns were removed from the alignment based on a 95% confidence profile calculated within SSU-ALIGN. Tree topology was inferred on the CIPRES[67] high-performance computing cluster, using the RAxML-HPC2 workflow on XSEDE with the ML +

Thorough bootstrap (1000 bootstraps) method and the GTRGAMMA model. The resulting tree was imported and visualized on the iTOL[68] 3 server. Further analysis of uncultured organisms that were the nearest relative of my organism of interest was carried out by a BLAST search within NCBI[60,65]

Transmission Electron Microscopy (TEM) and trait quantitation

Enrichments of the strain LGM-1, were used to follow two individual infection cycles with daily subsampling over the course of 5 d. Infected subsamples were pelleted and fixed with 2.5% glutaraldehyde and sodium cacodylate wash buffer (0.1 M, pH 7.4) at room temperature for 2 h and then suspended in agarose, fixed with 1% buffered OsO_4 at 4 °C for 2 h and block stained in 0.5% uranyl acetate at 4 °C, overnight. Co-cultures were rinsed with deionized water and dehydrated using successively increasing concentrations (20%, 40%, 80%, 100%) of acetone for 20 min each and rinsed twice with 100% propylene oxide at room temperature. Pellets were then infiltrated, at room temperature with rotation, with successive levels (10%, 25%, 75%, 100%) of epoxy resin for 1, 2, 8, and 12 h, respectively. Infiltrated pellets were embedded in fresh 100% resin and held at 60 °C for 24 h. Pellets in resin were thin sectioned (70 nm) and post-stained with 2.5% uranyl acetate/lead citrate. Sections were imaged using a JEOL 1200EX TEM (JEOL) and a Philips CM 12 TEM (Philips Microscopes) at 80 kV.

To calculate the percent volume the inner compartment, or nucleoid, occupied within the total cell, TEM micrographs were used. Micrographs were printed, cells cut out and weighed. Nucleoid regions were then cut out and weighed. The percent volume of the cell the inner compartment comprised was calculated by dividing the sum of the weights of

the nucleoid regions by the sum of the total cell weights, providing an estimate of average nucleoid percent volume. TEM micrographs were also used to visually count the number of extracellular dividing cells, determined as two connected cells but with individual nucleoid regions, of the predatory bacterium (n=256). Further interrogation of TEM micrographs was used to determine the presence or absence of vesicles in early (1 propagule) and late (2+ propagules) propagule formation (n=732), and the location of ribosomes (n=10).

Confocal Microscopy

Preparations of strain LGM-1 were observed during an infection cycle by confocal microscopy after fixation with 2.5% glutaraldehyde and staining with 0.1 mg/ml DAPI (4'-6-diamidino-2-phenylindole). DAPI stained Cyanoraptor cells were observed with excitation at 358 nm and emission at 461 nm. *M. vaginatus* was observed by its autofluorescence based on photosynthetic pigments (emission at 663 nm with excitation between 620 and 630 nm) in addition to DAPI fluorescence DIC (differential interference contrast) was used concurrently. Confocal microscopy was performed with a Zeiss SM 880 microscope at 63x magnification, with an oil immersion lens at Biodesign Imaging Facility of Arizona State University, Tempe, AZ.

Prey Range

Seventy strains of cyanobacteria were tested for susceptibility to attack by the predatory bacterium, all isolated from soil (Tables 2, S2). For this, 1 ml of a standard enrichment was added to 9 ml of an exponentially growing culture of each tested strain. Triplicate

cultures were used, and positive controls were concurrently run (using *M. vaginatus* PCC9802 as prey). All tests were incubated at 23 ℃ under standard lighting. Activity of the predator was scored by visually monitoring for death of the prey species, as determined by loss of pigmentation.

Predator physiology

To determine the effect of temperature on viability of the predatory bacterium propagules (without prey), 10 ml of liquid suspensions containing the predatory bacterium were incubated in the dark for five days at nine different temperatures: 0, 4, 10, 15, 23, 30, 35, 40, and 45 °C, a range typical found in desert environments[69], using standard laboratory incubators. After incubation, viability/infectivity was scored in triplicate with standard MMAs. To determine desiccation tolerance, predator propagule preparations (1 ml from a standard MMA) were vacuum-filtered onto 0.2 µm polycarbonate filters, allowed to completely dry and then incubated in the dark for 7 days. 3 such filters were then used to inoculate triplicate standard MMA assays to test for predator viability/infectivity, but monitoring was extended to 20 days. Remaining filters were submerged in 10 ml of double distilled water for 7 days, then desiccated and kept dry again for 7 days, after which 3 treatment filters were used to inoculate MMA assays. This was repeated for a total of 5 desiccation cycles, checking for infectivity at each cycle. Controls consisted of predator preparations that had been filtered but not desiccated. Similarly, prepared predator propagule filters were used to gauge sensitivity to light in the dry state. Light was provided by fluorescent tubes in a 12 h photoperiod for 3, 6, or 9 days under light intensities of either 25, 80 and 100 µmol photon m^{-2} s^{-1}. Filters were then used to

inoculate MMAs, visually monitoring for prey death during 20-day long incubations. Controls consisted of filtered but neither desiccated nor illuminated preparations. To determine whether extracellular propagules were in a spore-like quiescent state or were metabolically active, 3 ml of propagule preparations from an MMA assay were incubated with either ampicillin (16 µg/L), chloramphenicol (50 mg/L), or rifamycin (250 µg/L) in the dark for 7 days. Controls were incubated under the same conditions minus antibiotics (n=4). All treated preparations and controls (n=4 for each) were centrifuged, discarding the supernatant. Pelleted cells were resuspended in 2 ml of JM medium, 1 ml of which was added to 9 ml of axenic *M. vaginatus* and incubated for 7 days under standard MMA conditions to test for infectivity.

Data availability

Raw sequence data have been submitted to NCBI and are publicly available under accession number MT664819 and BioProjectID PRJNA730549. The annotated genome has been deposited under BioProjectID PRJNA730811.

Results

During the process of growing biocrust cyanobacteria from the Sonoran desert on soil substrates in a greenhouse I observed the development of macroscopic circular patches devoid of cyanobacteria resembling viral plaques or small fairy rings in areas that had previously sustained healthy cyanobacterial populations[46]. Independently, I also detected recurrent episodes of mass mortality, and the presence of pathogenic agents to cyanobacteria, during greenhouse production of biocrust from the Great Basin and

Chihuahuan[43] deserts. Subsequent careful inspection of naturally occurring biocrusts in several of the respective sites of origin of the diseased greenhouse biocrusts revealed that the presence of similar clearing plaques in these sites was common. Samples from both field and greenhouse plaques were then tested for the presence of pathogens to cyanobacteria using the MMA assays (Fig. 9). In all cases, soil from plaques showed pathogenicity, whereas soil from visually healthy sections of the biocrusts surrounding plaques did not (Table S3). Sterilization of the soils prior to MMA inoculation resulted in a loss of pathogenicity, indicating that cyanobacterial death was due to a biological agent (rather than toxicity of the soil). Further, inoculation of healthy field crusts with end-point suspensions from positive MAA resulted in the local development of plaques. Completion of extended assays (EMMA) indicated that, in all cases, the pathogen was filterable above 0.8 μm pore sizes (i.e., it was not a virus) and was not sensitive to eukaryotic inhibitors (cycloheximide), pointing to its prokaryotic nature. Differential filtration recurrently yielded results consistent with a pathogen with an effective size between 0.8 and 1 μm.

Enrichment cultivation and molecular identification

The MMA assay provided the basis for long term cultivation of the pathogenic agent, whereby re-inoculation of positive assays could be used to maintain enrichments indefinitely. Original positive MMAs maintained pathogenicity when stored in liquid suspension in the dark for at least 3 years. However, because assays were started by inoculation of natural soil, the enrichments typically contained not just the pathogen and the prey cyanobacterium, but also a very large array of adventitious soil bacteria that

proliferated on the organics liberated by the dying cyanobacteria. In this sense, they were only very rough enrichments. Repeated attempts to culture the predators by plating on complex heterotrophic media failed; in excess of 30 different morphotypes of colonies isolated by streaking on agar plates end-point solutions of positive MMAs were tested, but none showed a capacity to kill *Microcoleus*. This suggested that the pathogen was obligate, and I switched my efforts to purifying the enrichments by a combination of techniques, monitoring the successive purification steps using 16S rRNA-based community analyses. Filtration-based sized fractionation (SF) combined with a dilution to extinction (DTE) approach were used first. The fractionated, diluted samples were used then to inoculate MMAs. After four consecutive rounds of SF/DTE, the enrichments were purified to the point where the total number of ASVs was cut down from 116 to 17 (Fig. 10, Table S4) and a particular ASV was enriched to the highest abundance (82.6%), making it a good candidate for the predator sequence. BLAST[60,65] analyses of this short ASV showed that it was most similar to a little-known group of members of the family *Chitinophagaceae* in the phylum *Bacteroidetes*, and its closest cultivated (but uncharacterized) bacteria were only 90% similar to it. However, there were thousands of matches in the public databases to uncultured environmental sequences with similarities ranging from 96% to 100% stemming from a variety of soil, freshwater and anthropogenic environments, indicating that similar organisms are not so rare in nature. Most of the other common ASVs found in this enrichment matched quite closely to those of non-predatory, cultivated isolates of bacteria, further supporting the initial assignment. More robust analyses of the predator based on its complete16S rRNA gene, as obtained from whole genome sequencing (see below), indicated that it was less than 90% similar

to its closest cultivated, characterized species (*Taibaiella yonginensis*; Fig. 11), indicating that I am likely dealing with a new taxonomic entity differentiated from known bacteria at the supra-generic level. The final enrichment stage constituted the basis for all further experimentation presented here, and the enriched pathogen contained therein, likely to have been derived from single cells twice, given the strain designation LGM-1.

Morphology and life cycle

I studied the life cycle by following the infection dynamics within a typical MMA through transmission electron microscopy (TEM) and confocal microscopy. End point MMAs revealed the presence of ghost filaments of *Microcoleus* (Fig. 12f, S2b) and a large number of small, morphologically unusual Gram-negative cocci (Fig. 12a, 13a), often distributed in groups and in proximity to *M. vaginatus* filaments (Fig. 12a, b). No evidence for dividing cells was detected among them, even after surveying in excess of 250 single cells. These bacteria ranged between 0.8 µm and 1 µm in diameter, lacked flagella, and were made up of two conspicuous cellular compartments (Fig. 12a, b, 13a). An inner compartment, some 0.5-0.6 µm in diameter, and comprising on average 55% of the cell volume, was electron dense, contained a typical fibrillar nucleoid region and putative ribosomes (Fig. 13b) and was circumscribed by 2 membranes separated by an electron transparent region typical of the tegumentary envelope of gram-negative bacteria (Fig. 13b, see Fig. 13c for a schematic of the cell). This compartment often presented quasi polyhedral shapes. An outer compartment, typically 0.1-0.4 µm thick, comprising some 45% of the cell volume was much more electron lucent and separated from the extracellular space also by a double membrane with a clear interspace. Within one day of

the addition of propagules to healthy *Microcoleus* cultures, these cocci appeared to localize preferentially in close proximity to *M. vaginatus* filaments, developing tegumentary contact zones between them and *Microcoleus* (Fig. 12b). As infection proceeded, clear localization of predatory cells to the cytoplasm of *M. vaginatus* was evident, but when intracellular, they lost all trace of cell compartmentalization, and started to grow into bacilli and on to pseudo-filamentous forms (Fig. 12c, d), developing large numbers of cytoplasmatic inclusions (reserve polymers; Fig. 12c), that eventually separated into individual propagules (Fig. S3e, f, h). The cytoplasm and tegumentary structures of *M. vaginatus* (Fig. 12f, S2b) were patently degraded in the process. Massive degradation of *M. vaginatus* filaments (Fig. 12f) was widespread by 4-5 days after infection (healthy *M. vaginatus* is shown for comparison in Fig. S2a), with peptidoglycan damage evidenced by the outward bulging of cells with weakened sacculus under turgor pressure (Fig. S3e-h; healthy *M. vaginatus* filaments are shown for comparison in Fig. S3a-d). Infected prey cells detectable by a significant loss of photopigments, contain single large DNA bodies (Fig. S3e, f), which I attribute to the formation of predator cell nucleoids (Fig. S3g), since cyanobacterial DNA in healthy cells shows a reticulated structure (Fig. S3a-d). As pseudofilaments occupied most of the *Microcoleus* intracellular space, the infection often spread to multiple adjacent prey cells (Fig. S3e-h). TEM revealed that multiple cell division occurred simultaneously in the pseudo-filamentous forms and was characteristically accompanied by a loss of reserve polymers (Fig. 12d), and by the secretion of large numbers of membrane-bound extracellular vesicles (Fig. 12e). The presence of extracellular vesicles within the internally replicating cells was only noted in “late intracellular stage” (more than 1 cell), whereas early stage (1 cell)

intracellular pathogen did not produce vesicles. After full degradation of *M. vaginatus,* propagules were released into the environment (Fig. S4a, b, d early-stage release and Fig. S4e, f, h late-stage release), evidenced by DAPI stained propagules (Fig. S4b, d, f, h), leaving behind ghost, pigment-free filament sections (Fig. S4c, g).

Genome

Pac-Bio Genome sequencing of preparations from Cyanoraptor, after subtraction of bona fide *Microcoleus* sequences, could be bioinformatically assembled into a single contig of 3.3 Mb, which could be unambiguously assigned to the predatory bacterium by comparison of the 16S rRNA gene sequences obtained before. This genome contained 1,781 putative and 1,328 hypothetical genes and was three times smaller than that of its nearest relative with a fully sequenced genome, *Chitinophaga pinensis* (9.1 Mb)[70], though only slightly smaller than the average genome size of sequenced organisms within the family *Chitinophagaceae* (4.5 Mb). However, it showed only little homology to the *C. pinensis* genome, with most homologous proteins having sequence identities less than 70%. GC content was 42%. Two identical copies of the 16S rRNA gene were present. No motility or chemotaxis genes were detected by automated or manual annotation, and there was a widespread lack of amino acid biosynthesis genes, missing full pathways for all but glutamine and asparagine. The genome contained a variety of hydrolase genes (some 3% of its genes), some of which were characterized by sec export pathway signal peptides.

Prey Range

The prey range of Cyanoraptor was tested by substituting *M. vaginatus* (PCC9802) for different cyanobacterial strains isolated from biocrusts in the MMA assay. Out of 70 strains belonging to 14 genera tested, only 14 strains (belonging to only 4 genera) were susceptible to attack (Table 2). Highest susceptibility was show by *M. vaginatus* strains (55%), *Crassifilum* sp. (33%) and *Xeronema* sp. (25%) (Table 2). However, susceptible cyanobacterial strains varied in their resistance to the predator, as determined by days until death of the prey. *M. vaginatus* strains died between 5 and 14 days, *Schizothrix* spp. after 21 days, *Crassifilum* sp. after 7 days and *Xeronema* sp. after 14 days.

Propagule metabolic activity and sensitivity to environmental stress

The range of temperatures at which propagules from strain LGM-1 remained viable was tested at 5 °C intervals between 45 and 0 °C, in independent triplicates at each temperature. Infectivity was abolished by incubation at 45 or 40 °C for seven days, but not by incubation at lower temperatures down to 0 °C (Fig. S5). I note, however, that freezing at -20 °C abolished viability completely. Desiccation tolerance was tested by submitting preparations of propagules to successive air drying and wetting cycles. All replicates (n=4) submitted to one or two drying/wetting cycles remained infective, even though some loss of viability could be detected in the second cycle, as judged by the time needed for assays to attain complete cyanobacterial death (5 ± 1 days with one cycle but 12 ± 1.5 with two cycles). Three or more consecutive cycles resulted in complete loss of infectivity (Table S5). Exposure to light also affected infectivity significantly. Tests subjecting predator propagules in the dried state to increasing doses resulted in

progressively more intense loss of infectivity, as judged by the increasingly long times until death in MMA assays. Based on calculations of percent infectivity remaining (relative number of infective particles divided by the initial relative number of infective particles), the data fit well a model of exponential decay (typical for processes of radiative inactivation), with a steep decay constant (Fig. S6). Exposure of propagule preparations to various broad-spectrum antibiotics, including ampicillin (peptidoglycan synthesis inhibitor), chloramphenicol (protein synthesis inhibitor) and rifamycin (inhibitor of DNA-dependent RNA synthesis), did not result in any measurable loss of subsequent infectivity, indicating that the propagules are likely to sustain very little metabolic activity, at least as it involves basic cell-growth processes and acclimation to environmental stressors, like cell wall or protein synthesis and gene expression.

Discussion

In this study I describe, with a polyphasic approach based on co-culture experiments, genomics and microscopy, the basic biology of a novel genus of predatory bacterium in the *Bacteroidetes* phylum that specializes in cyanobacteria as prey and presents an intracellular phase in its life cycle. Strain LGM-1 was enriched from plaques in greenhouse-grown biocrusts[46]. It was capable of producing stochastic failures in mass production there[43] and subsequently detected in various natural settings. While predatory bacteria have been previously isolated from soil environments[19–21,71,72], this is the first documented instance of their presence in and impact on biocrust communities. Because of the affinity of Cyanoraptor for *Microcoleus vaginatus* as prey, the pioneer biocrust former, one of the foundational species of biocrusts[38,39], which presents a global

distribution[34] and is considered to be the most abundant terrestrial cyanobacterium[73], the potential clearly exists for the ecological impact of this bacterium to be significant. While a pure culture could not be obtained, I based my descriptions on a highly enriched co-culture with *M. vaginatus* PCC9802, in which ultimately strain LGM-1 was the dominant non-phototroph. This is not a rare occurrence among predatory bacteria, as many seem to be obligately parasitic on live prey[1], and the release of prey debris tends to create a habitat for adventitious heterotrophic contaminants. Consistent with the notion of Cyanoraptor being an obligate pathogen, it displayed a conspicuous lack of full biosynthetic pathways for most amino acids, likely as a result on an evolutionary loss enabled by a reliance on prey-derived sources.

Extracellular phase

A reconstruction of strain LGM-1's life cycle as deduced from the available evidence is presented in Fig. 14. The extracellular phase consists of small-sized cocci. Like other predatory bacteria, including the model organism *Bdellovibrio bacteriovorus*, the size of the cyanobacterial predator propagules is, between 0.8 and 1 μm, much smaller than that of the prey's cells (2-3 by 6 μm), which is likely necessary in order to acquire enough nutrients and replication space within its prey to complete at least one cycle of replication[2]. Similarly to *Bdellovibrio*[74], Cyanoraptor undergoes differentiation into distinct dispersal and replication phases (Fig. 12c, d). However, the similarities may end there. While *Bdellovibrio* spp. divides within the periplasmic space of its prey[74], Cyanoraptor instead lodges within the cytoplasm. While *Bdellovibrio* finds its prey by actively swimming towards it, Cyanoraptor is apparently non-motile in any of its life

stages, having no genes related to motility or taxes in its genome, and apparently uses a strategy of ambush predation, lurking and waiting for motile cyanobacteria to find it. Perhaps not coincidentally, all of the strains within its prey range (Table 2) are motile filamentous cyanobacteria, and none of the sessile species tested were susceptible to attack. Contact with the prey seems to elicit specific docking structures as seen in TEM images (Fig. 12b). This docking may well result in a local infection, but it may also serve in the dispersal of infective cells "hitchhiking" on the motile cyanobacteria, a strategy similar to that of nonmotile actinobacterial spores within the rhizosphere[75]. In nature, *M. vaginatus* and other filamentous cyanobacteria are known to move vertically within the soil to gain optimal light exposure or to avoid desiccation fronts[76] and horizontally to colonize bare soil [46,77]. As they do, they are likely to spread the predator to other cyanobacterial filaments. *M. vaginatus* conveniently assists in predatory efficiency by being densely packed in bundles of filaments within a common sheath[39] making transfer between prey filaments more efficient. The choice of an ambulant prey that likes crowds is consistent, from the point of view of a pathogen, with basic principles of epidemiology[78]. All strains sensitive to predation in Table 2 were motile and crowded into bundles of filaments. Those forming the bigger bundles (*M. vaginatus*) were the most sensitive, those characterized by bundles of only a few filaments (*Schizothrix* sp.) the least.

Cyanoraptor's extracellular propagules present a very unusual ultrastructure. Its inner compartment, reminiscent of a eukaryotic nucleus, is in fact typical of a Gram-negative cell, with a double membrane surrounding it, and with a loosely demarcated nucleoid. The outer compartment, by contrast is unusually electron-light, and does not contain

visible structures or ribosomes (Fig. 13b), but it is also constrained by a Gram-negative double membrane. Double-membrane internal compartmentalization similar to Cyanoraptor's is known in the planctomycete *Gemmata obscuriglobus*[79] (single-membrane compartmentalization is also known from other *Planktomyces*[80] and *Poribacteria*[81]). I posit that the outer compartment acts as a repository for hydrolytic enzymes, a common strategy in endobiotic as well as epibiotic predatory bacteria to lyse the prey's outer membrane and peptidoglycan before hydrolyzing its cellular components[82,83]. Cyanoraptor genomic abundance of genes annotated as polymer hydrolases endowed with signal peptides for secretion supports this contention.

Intracellular phase

Although I could not observe specific structures relating to penetration of propagules into the cyanobacterial filaments, as soon as the infective phase starts, symptoms of degradation of cyanobacterial peptidoglycan in the form of cellular bulging (Fig S3e-h) become apparent in the cyanobacteria, implicating this capacity as an important part of the process. Upon entry, there was also a conspicuous disappearance of the outer compartment in intracellular pathogen cells, implying that a fusion of the outer compartment with the cyanobacterial membranes was possibly at play. As the infected cells became more and more patently degraded, the pathogens grew into bacillar and eventually pseudo-filamentous forms characterized by large numbers of intracellular reserve inclusions (growth stages in Fig. 14), to eventually undergo coordinated and multiple fission (cell division phase in Fig. 14). Concurrent with these cell division stages I observed the massive production of extracellular vesicles (EVs; Fig. 12e). Lateral

infection without an extracellular phase likely takes place as well, as typical infections involved foci of a few adjacent cyanobacterial cells (Fig. S3e-h). Because the cyanobacterial cytoplasm is at this stage severely degraded, I see it as unlikely that the secretion of MVs serves as a means to excrete more hydrolytic enzymes, as is does during predation in *Micrococcus xanthus*[84] and *Lysobacter* sp.[85] using outer membrane vesicles (OMV). Rather, I posit that these EVs will coalesce and fuse around the single cells of Cyanoraptor to form the outer compartment in preparation for their release as fully formed propagules. If this is a correct interpretation, the extracellular vesicles formed by Cyanoraptor must be surrounded by a typical double membrane, to yield the external double membrane of the propagules. This is unlike the typical single-membrane vesicles reported from Gram-negative bacteria, which are formed from the outer membrane and typically pack periplasmatic contents[85]. The TEM resolution in my study does not allow me to clarify this contention. Presumably, hydrolytic enzymes are secreted in an anticipatory way into the EVs, so as to endow the forming propagules with the necessary enzymatic capacity to enter into the next infection before release into the environment. This is consistent with the physiological data pointing to the relatively low metabolic activity displayed by cells in the propagule stage with respect to peptidoglycan and protein synthesis, as well as gene transcription and lack of visible cell division. Finally, and to complete the cycle, the release of fully formed groups of propagules (Fig. S4 a, b, d, e, f, h) is enabled by advanced degradation of the sacculus of *M. vaginatus* (Fig. 12f, S2b, S4c, d) and these propagules tend to remain in batches held by extracellular polymeric substances.

Keeping natural epidemics in check

While in culture and in biocrust production settings predation by this organism did have devastating effects on cyanobacterial populations with high morbidity, both in terms of prevalence and incidence, and with mortality affecting the whole population, the situation in natural biocrusts seems to be more constrained, affected areas (as defined by visible plaques - Fig. 8) appearing as distinct foci of limited extent (in the cm scale). I attribute this difference to two factors. On the one hand the dispersal of infectious propagules may well be restricted by the expansion range of their prey, which will diminish in proportion to the fraction of the population affected. On the other hand, the low levels of activity of propagules probably are the reason for their high sensitivity to environmental stressors typical of the desert soil environment like high temperatures, recurrent wetting/desiccation cycles and high UV radiation. This may well restrict the viability of the propagules significantly. For example, the decay rates in infectivity with light exposure presented in Fig S6 can be used to calculate that an L_{50} dose of natural sunlight would be reached by a mere 15 minutes of exposure to noontime Arizona sunlight on a sunny day.

Description of '*Candidatus* Cyanoraptor togatus' gen. nov. et sp. nova

Candidatus Cyanoraptor. Cy.a.no.rap'.tor, Latinized Gr. m.n. Cyanos, blue-green and L. raptor m.n, plunderer, M.L. Cyanoraptor m. n a plunderer of the blue-green, attacker of cyanobacteria.

Prey-dependent intracellular Gram-negative bacteria in the family *Chitinophagaceae* (*Bacteroidetes* phylum), predatory of cyanobacteria with conspicuous cell differentiation into distinct intra and extracellular stages in its life cycle. Extracellular propagules are non-growing or dividing, coccoid to oval, and internally compartmentalized by a double membrane. The inner compartment holds the nucleoid. Intracellular stages do not have an outer compartment, grow into rods and eventually pseudo-filaments, then undergo simultaneous, multiple cell division. No motile phases exist. Flagella are absent. Likely auxotrophic for various amino acids according to genomic data.

Candidatus Cyanoraptor togatus. To.ga´.tus, L . togatus m. adj., dressed in a robe or toga, in reference to the outer compartment surrounding the core of the propagules. Extracellular cocci are 0.93 ± 0.15 μm, intracellular cells 1.61 ± .96 μm in the long axis and 1.02 ± 0.41 μm in the short axis. Preys on *Microcoleus vaginatus* and other non-heterocystous, motile filamentous terrestrial cyanobacteria. Strain LGM-1^{T}, in enrichment form, and its genome, is the type material. Its genome is 3.3 Mb with 42% G+C content. Isolated from biological soil crust from Arizona, USA. It is maintained as a co-culture enrichment with its prey *M. vaginatus* PCC 9802.

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Conflict of Interest

The authors declare no conflict of interest.

Tables

Taxa	Number of strains tested	Number of strains susceptible	% of strains susceptible
Oscillatoriaceae			
M. vaginatus	18	10	55%
Lyngbya sp.	1	0	0%
Schizotrichaceae			
Schizothrix sp.	14	2	12%
Coleofasciculaceae			
("Microcoleus steenstrupii complex")			
Funiculus sp.	2	0	0%
Allocoleopsis sp.	2	0	0%
Crassifilum sp.	3	1	33%
Parifilum sp.	1	0	0%
Xeronema sp.	4	1	25%
Chroococcidiopsidaceae			
Chroococcidiopsis sp.	1	0	0%
Leptolyngbyaceae			
Leptolyngbya sp.	3	0	0%
Phormidiaceae			
Phormidium sp.	1	0	0%
Nostocaceae			
Nostoc sp.	8	0	0%
Scytonemataceae			
Scytonema sp.	7	0	0%
Tolypothrix sp.	5	0	0%

Table 2. Determination of prey range of strain LGM-1 using EMMA with PCC9802 as the positive control. *Identity and source of strains tested can be found in Table S2.

Figures

Figure 8. Top view of a sample of cyanobacterial soil biocrust taken showing multiple plaque-like or fairy-ring clearings, visible symptoms of bacterial predation. Scale bar 1 cm.

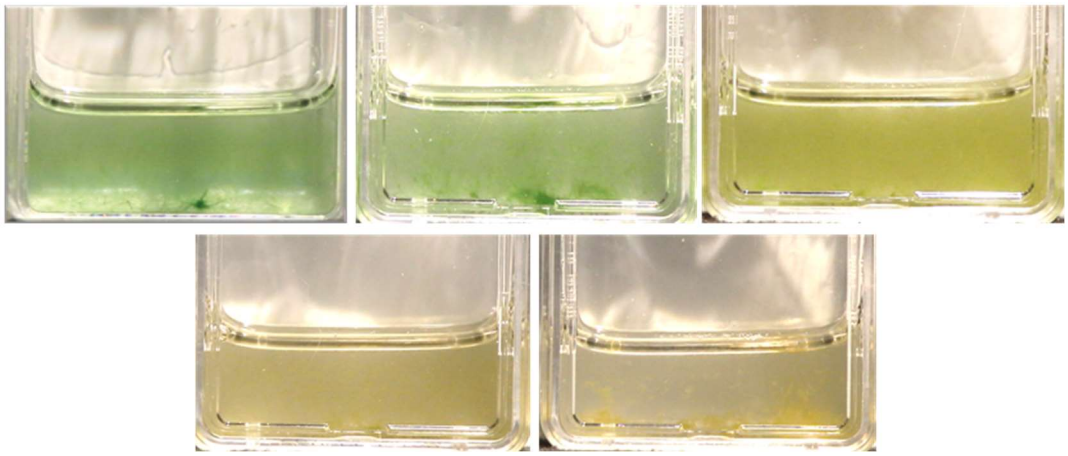


Figure 9. Typical aspect in the progression of an MMA using *M. vaginatus* PCC 9802 as prey.

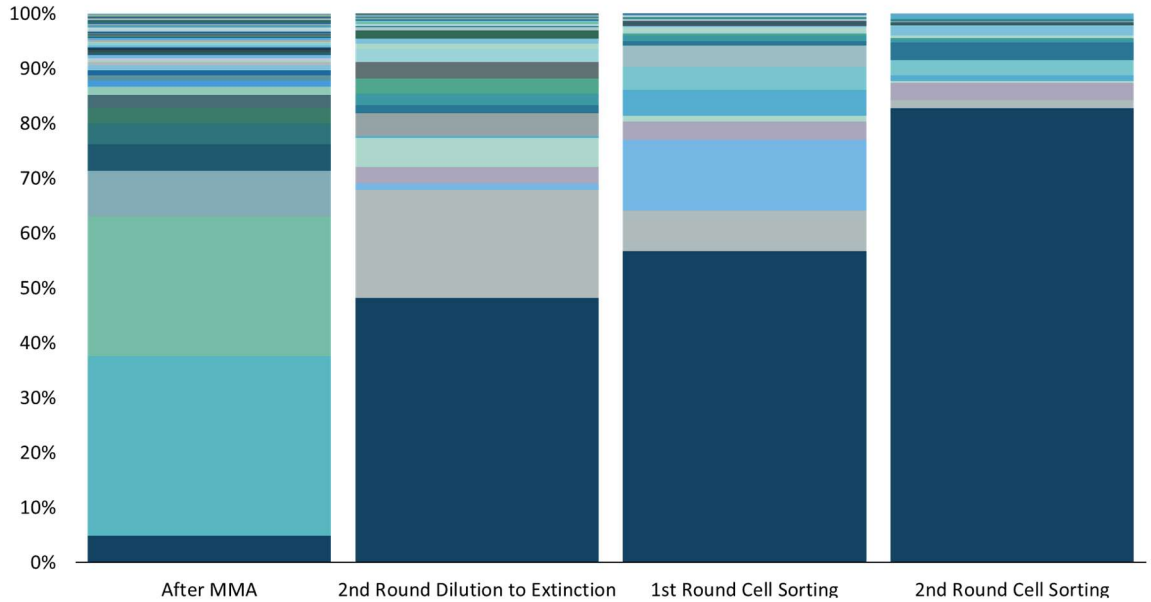


Figure 10. Community composition analyses based on 16S rRNA gene ASVs of progressively enriched final MMA suspensions of the predatory bacterium *Candidatus* Cyanoraptor togatus LGM-1. Quantitative data are in Table S4.

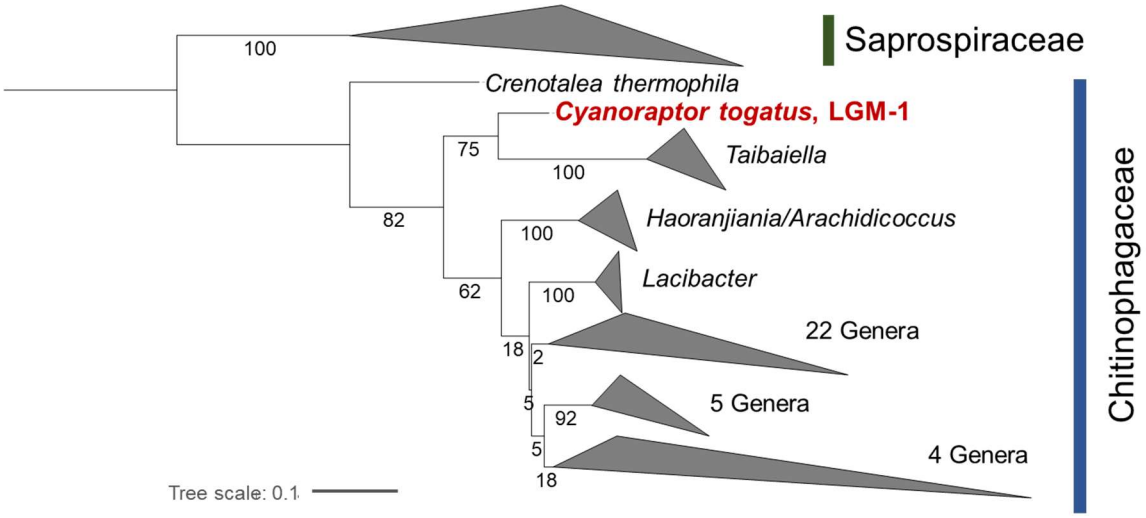


Figure 11. Maximum likelihood phylogeny of *Chitinophagaceae* with outgroup *Saprospiraceae* based on 16S rRNA gene sequences. The full-length 16S from *Candidatus* Cyanoraptor togatus LGM-1 (1485 bases) determined in this study is indicated by red text. Support at nodes comes from 1000 bootstrap replicates. The tree encompasses 181 individual sequences, collapsed for clarity.

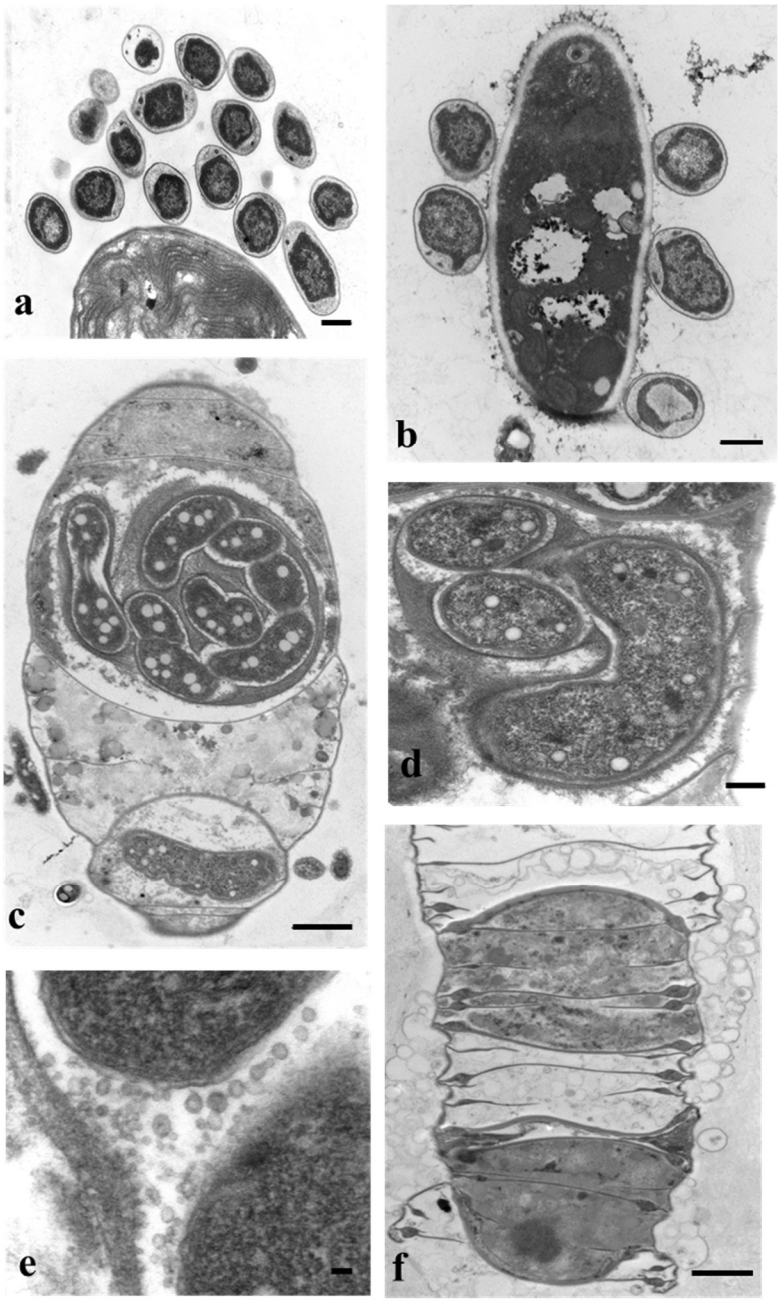


Figure 12. Transmission electron microscopy imaging of Cyanoraptor, strain LGM-1 and *M. vaginatus* during an infection cycle in co-culture. (a) Morphologically unusual Gram-negative bacterial propagules associate with *M. vaginatus* filaments and (b) form tegumentary contact zones. (c) Strain LGM-1 lodged in the cytoplasm of *M. vaginatus* in a pseudofilamentous form, undergoing multiple cell division. (d) Strain LGM-1 accumulating copious reserve polymers (as inclusions) prior to cell division. (e) Release of extracellular vesicles in late intracellular phase cells. (f) Aspect of *M. vaginatus* is structurally degraded after infection cycle. Scale bars: (a) 1 µm; (b) 0.5 µm; (c) 1 µm; (d,e) 0.125 µm; (f) 1 µm.

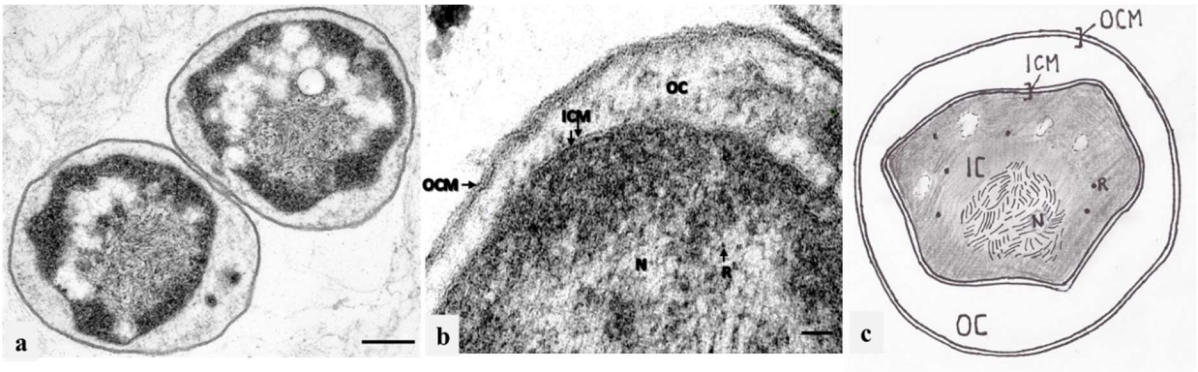


Figure 13. Ultrastructural details of the extracellular propagule phase in *Candidatus* Cyanoraptor togatus LGM-1. (a) A pair of extracellular propagules, showing internal compartmentalization. (b) Detail of the double Gram-negative like membranes surrounding the inner and outer compartments, nucleoid (N) and ribosomes (R). (c) Schematic interpretation of ultrastructural features: IC (inner compartment), OC (outer compartment), ICM (intracellular compartment double membrane), OCM *(*outer compartment double membrane), N (nucleoid region with DNA fibrils) and R (ribosome). Scale bars: (a) 0.2 μm; (b) 50 nm.

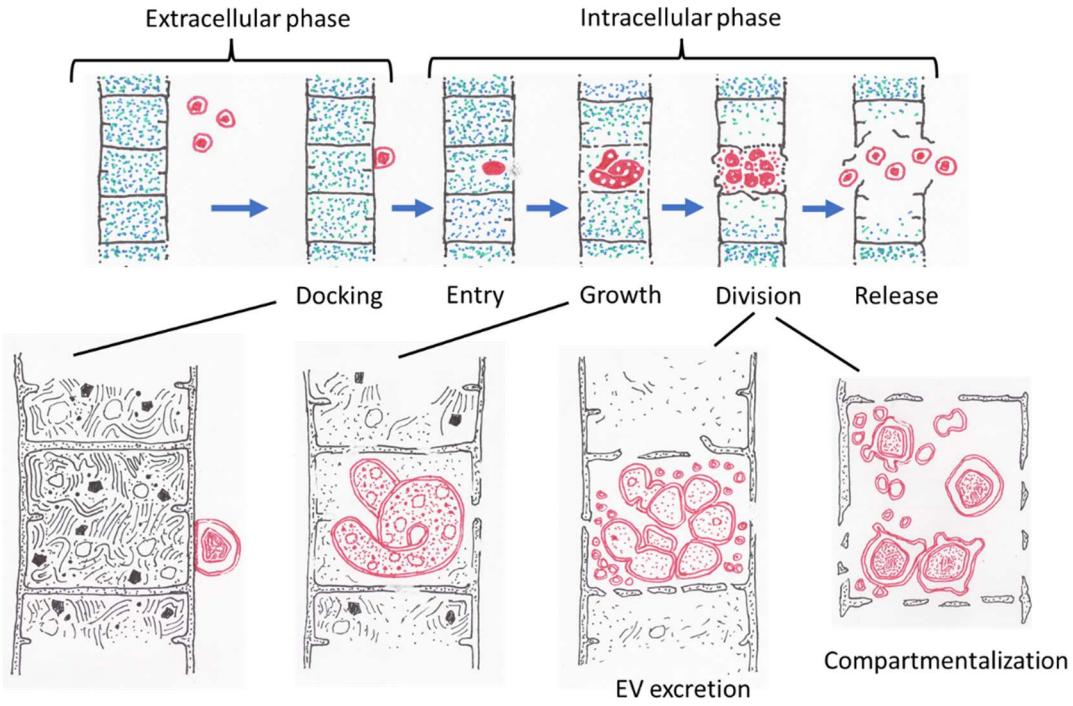


Figure 14. Reconstructed life cycle of *Candidatus* Cyanoraptor togatus LGM-1. Free compartmentalized propagules lurk in the environment waiting for a motile cyanobacterium to make contact, upon which docking structures develop. After gaining entry into the prey's cytoplasm, the predator loses compartmentalization and the growth phase begins, with cellular elongation and the accumulation of reserves. In the later intracellular phase, copious production of extracellular vesicles begins, along with concurrent multiple cell division. Daughter cells transform into mature propagules, which are released in groups as the cyanobacterial teguments yield.

Supplementary Information

Supporting Tables

Requirement
Biocrust inoculum infective on *M. vaginatus* (PCC 9802) cultures
Lack of infection with sterilized inoculum
Infection independent of cycloheximide presence
Agent larger than typical virus, 0.8 μm
Agent smaller than 1 μm

Supplementary Table 1. Required outcomes for a positive EMMA.

Taxa	Strains	Source
Oscillatoriaceae		
M. vaginatus	FB020, JS001, JS002, JS027, JS010	Chihuahuan Desert, USA
M. vaginatus	PCC9802	Colorado Plateau Desert, USA
M. vaginatus	HSN003, HSN013, HSN014, HSN015, HS016	Great Basin Desert, USA
M. vaginatus	IL9, IL10, IL13, IL20, IL22, IL23	Negev Desert, IL
M. vaginatus	BN15	Sonoran Desert, USA
Lyngbya sp.	DC007	Chihuahuan Desert, USA
Schizotrichaceae		
Schizothrix sp.	FB2	Chihuahuan Desert, USA
Schizothrix sp.	DW19, DW023, DW024, DW028, DW031, DW032, DW033, DW034	Great Salt Lake Desert, USA
Schizothrix sp.	HS001, HS008, HS011, HS036, HS038	Great Basin Desert, USA
Porphyrosyphonaceae		
Allocoleopsis sp.	PCC7113	San Francisco, USA
Allocoleopsis sp.	DW001	Great Salt Lake Desert, USA
Crassifilum sp.	BN027, SON60, SON62	Sonoran Desert, USA
Funiculus sp.	HSN011, HSN023	Great Basin Desert, USA
Parifilum sp.	SON57	Sonoran Desert, USA
Xeronema sp.	HS024, HS035, HS041, HS003	Great Basin Desert, USA
Chroococcidiopsidaceae		
Chroococcidiopsis	DW037	Great Salt Lake Desert, USA
Leptolyngbyaceae		
Leptolyngbya sp.	FB001, FB005	Chihuahuan Desert, USA
Leptolyngbya sp.	HSN024	Great Basin Desert
Phormidiaceae		
Phormidium sp.	HSN025	Great Basin Desert, USA
Nostocaceae		
Nostoc sp.	FB021, FB022, FB025, FB026, MRMEN2, P1N2	Chihuahuan Desert, USA
Nostoc sp.	DW004, DW2.2	Great Salt Lake Desert, USA
Scytonemataceae		
Scytonema sp.	FB005, T007, T8S, T10	Chihuahuan Desert, USA
Scytonema sp.	HS005, HS006, HSN006	Great Basin Desert, USA
Tolypothrix sp.	FB100	Chihuahuan Desert, USA
Tolypothrix sp.	HSN030, HSN031, HSN032, HSN033	Great Basin Desert, USA

Supplementary Table 2. Strains for prey range tests and source location of each strain. All strains kept at the culture collection in the Garcia-Pichel lab.

Desert	Location	Coordinates	Plaque	MMA	Number of positives: Inside	Number of positives: Outside	Replicates
Sonoran	Gold Canyon, AZ	33.39, -111.35	+	+	6		n=6
Sonoran	Gold Canyon, AZ	33.39, -111.35	-	-		0	n=6
Sonoran	Casa Grande, AZ	32.99, -111.76	+	+	6		n=6
Sonoran	Casa Grande, AZ	32.99, -111.76	-	-		0	n=6
Chihuahuan	Las Cruces, NM	32.59, -106.85	+	+	6		n=6
Chihuahuan	Las Cruces, NM	32.59,-106.85	-	-		0	n=6
Chihuahuan	Las Cruces, NM	32.50, -106.74	+	+	6		n=6
Chihuahuan	Las Cruces, NM	32.50, -106.74	-	-		0	n=6
Chihuahuan	Albuquerque, NM	34.33, -106.33	+	+	6		n=6
Chihuahuan	Albuquerque, NM	34.33, -106.33	-	-		0	n=6

Supplementary Table 3. Correlation between plaque symptomology and the presence of pathogenic bacteria.

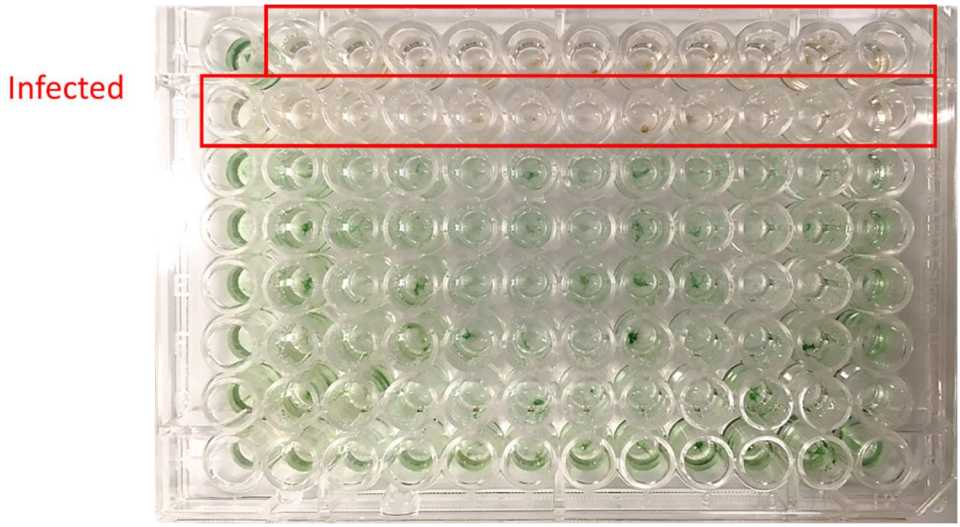
Step	Number of ASVs* detected	Relative abundance of presumptive infectious agent	Abundance rank of presumptive infectious agent
Direct extraction from plaque	116	4.8%	4
Serial dilution	63	48.2%	1
Flow cytometry cell sort, 1st round	29	56.7%	1
Flow cytometry cell sort, 2nd round	17	82.6%	1

Supplementary Table 4. Characteristics of sequencing progressively enriched suspension of presumptive infectious agent, with respect to bacterial diversity and abundance. * Amplicon Sequence Variant

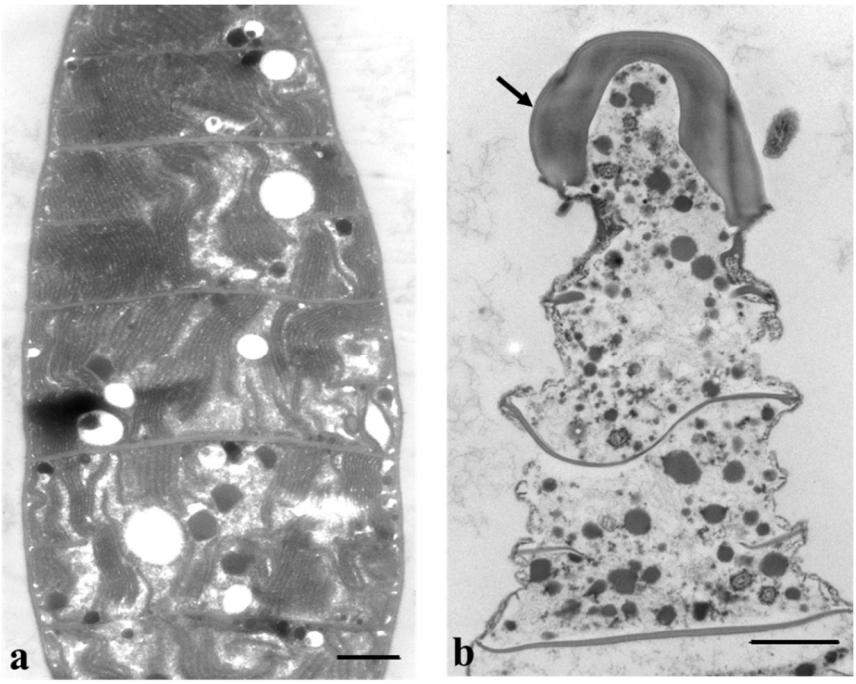
	Desiccation Cycles				
	1	2	3	4	5
Days until death	5 ± 1	12 ± 1.5	>20	>20	>20

Supplementary Table 5. Effects of desiccation on infectivity of propagules of strain LGM-1 under dry suspension (n=10).

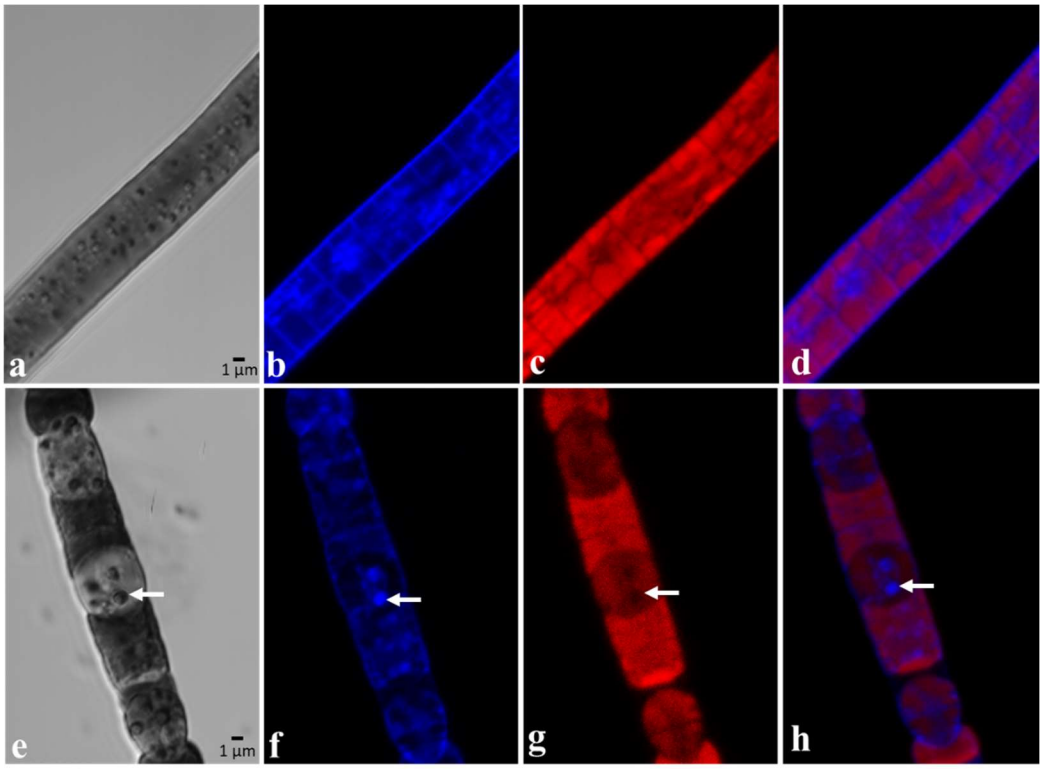
Supporting Figures



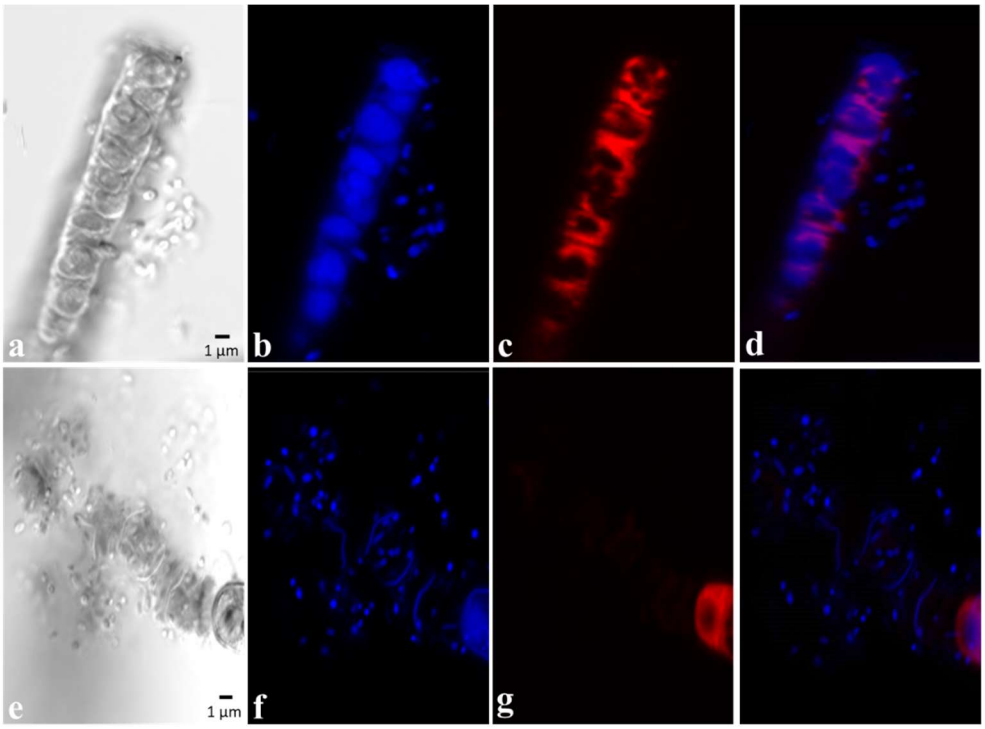
Supplementary Figure 1. Representative results of flow cytometric cell sorting enrichment on multi-well plates. Infected wells were assumed to contain the predatory agent and used for downstream applications.



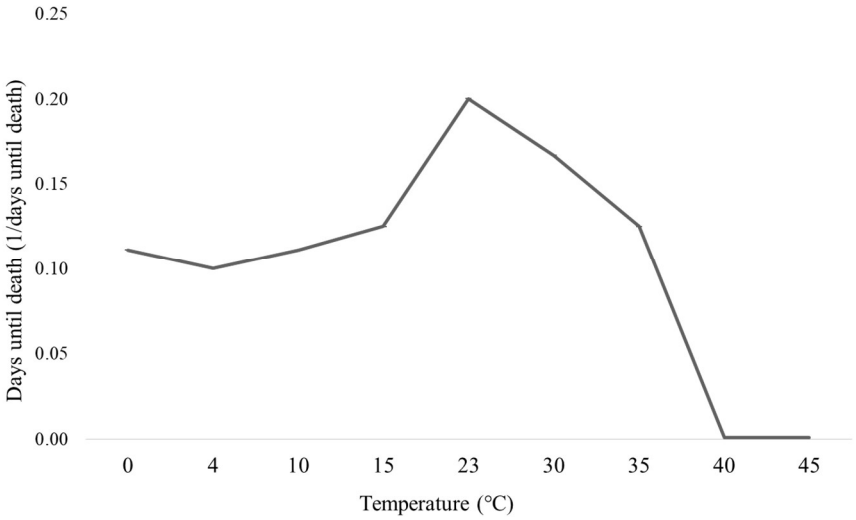
Supplementary Figure 2. TEM of healthy vs. infected *M. vaginatus*. (a) Axenic, uninfected *M. vaginatus*, (b) infected culture 4 days post infection. Arrow indicates typical apical cell with extended peptidoglycan "bumper". Scale bars 1 µm.



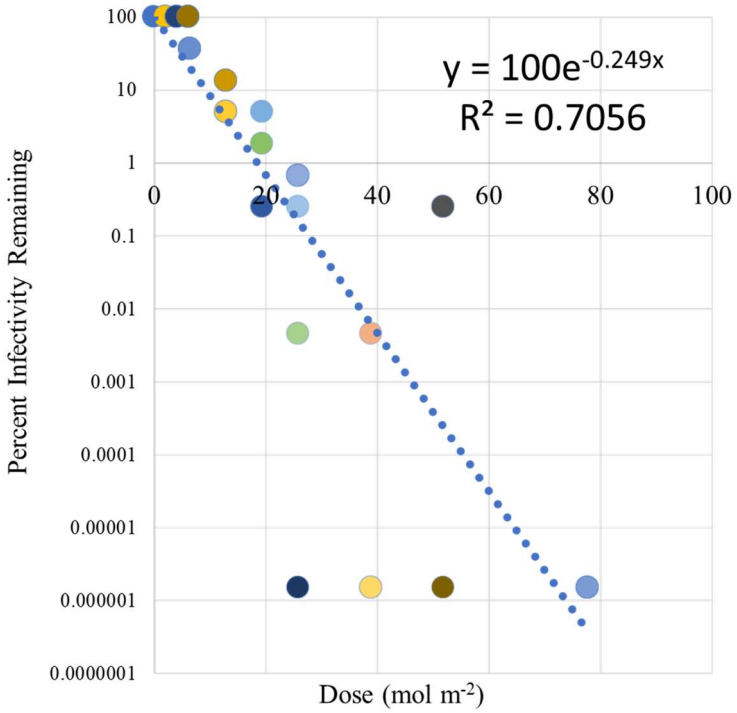
Supplementary Figure 3. Visualization of infection dynamics through confocal microscopy. Healthy *M. vaginatus* filaments (a-d), showing reticulated nucleoids, and infected *M. vaginatus* filaments showing predatory bacteria (arrows) with circular nucleoids (e-h). (a,e) DIC (b,f) DAPI stained (c,g) chlorophyll fluorescence of *M. vaginatus* (d,h) overlay. Scale bar 1 µm.



Supplementary Figure 4. Visualization of late-stage infection dynamics through confocal microscopy. Infected *M. vaginatus* filaments releasing propagules in, (a-h). (a,e) DIC (b,f) DAPI stained (c,g) chlorophyll autofluorescence of *M. vaginatus,* overlay (d,h). Scale bar 1 µm.



Supplementary Figure 5. Effects of temperature of pre-incubation on pathogenicity of strain LGM-1 (n=6).



Supplementary Figure 6. Effects of light exposure (dose) on the infectivity of Cyanoraptor, strain LGM-1 propagules, plotted and fitted to a dose-dependent exponential decay.

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4 – PREVALENCE AND ECOLOGICAL IMPACT OF A BIOCRUST PREDATORY BACTERIUM

Coauthors have acknowledged the use of this manuscript in my dissertation.

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Abstract

Predatory bacteria are a guild of heterotrophic bacteria that prey on other living bacteria to obtain nutrients for growth and replication. They are ubiquitous in the environment and are phylogenetically diverse. I report on the prevalence of a previously identified predator, *Candidatus* Cyanoraptor togatus, that specifically targets filamentous, bundle forming cyanobacteria within biocrusts. I also describe the symptoms of disease and assess quantitatively the ecological impacts of bacterial predation. Field surveys within the Southwestern US identified the symptomology of disease: plaques, or circular areas of clearing within otherwise healthy biocrusts. Plaques showed aggregated distribution at the local and landscape scales, at densities reaching up to 22% of biocrust coverage. A meta-analysis of biocrusts indicates that this organism is present in eight of nine deserts investigated. Based on 16S rRNA sequencing of plaques and healthy biocrusts, non heterocystous filamentous bundle forming cyanobacteria were specifically targeted, and the most abundant cyanobacterial species suffered the greatest losses. Ecosystem properties of biocrust suffered significantly under Cyanoraptor, with a loss in primary productivity, a complete loss of N pools and losses of exopolysaccharides that resulted in decreases in moisture retention and decreased dust trapping capacity of biocrust. At the landscape scale, losses of biocrust productivity approached 10%.

Introduction

Predatory bacteria are a guild of heterotrophic bacteria of diverse phylogenetic origins that prey upon other living bacteria for nutrient acquisition[1]. These pathogens have been documented in a wide variety of settings, including soils[2–4], marine[5] and freshwater[6] systems, wastewater[7,8] and host-associated microbiomes[9] through isolation or enrichment in culture[10 11], although many are obligate predators and not culturable[12]. While their presence[13–17], diversity[15,16], and abundance[18] have been documented in various natural systems, their impact as an ecological factor remains to be assessed. In Chapter 2, I reported the presence of a novel type of predatory bacterium, *Candidatus* Cyanoraptor togatus, that preys on filamentous cyanobacteria from biological soil crusts (biocrusts). Biocrusts are topsoil photosynthetic communities containing cyanobacteria[19], heterotrophic bacteria[20], fungi[21], archaea[22], and in some cases lichens and mosses[23]. Biocrusts cover an estimated 12% of terrestrial surfaces[24] and provide various ecosystem services, including soil stabilization[25,26] , as well as soil fertilization through carbon[27,28] and nitrogen fixation[29] and through trapping and binding of nutrient-containing dust particles[30]. Due to the extent of their coverage in terrestrial ecosystems, their contribution to global biogeochemical cycles is sizable: 15% of terrestrial net primary productivity and nearly 50% of biological nitrogen fixation[27].

In Chapter 3, I documented how local epidemics by *Candidatus* Cyanoraptor togatus result in conspicuous, cm-sized areas of biocrust clearing, or plaques, visible to the naked eye because of the widespread loss of dominant cyanobacteria, in otherwise healthy biocrusts[31]. This organism is a small (< 1 μm), Gram-negative bacterium belonging to the

phylum *Bacteroidetes*, and unrelated at the family level to any other known bacteria. I have characterized it genetically and physiologically using enrichment co-cultures with its prey. It lurks, immotile, until a motile cyanobacterium stumbles upon it, to then attach to it, degrading its teguments locally, and gaining entry into the prey's cytoplasm. Once inside, it begins its reproductive cycle, growing on hydrolyzed prey cytoplasm, with the eventual release of multiple infective propagules from the dead cyanobacterial cells. It preferentially targets filamentous, bundle-forming, non-heterocystous cyanobacteria, which are the pioneer biocrusts formers[25,26] in arid lands, and specifically *M. vaginatus*, possibly the most abundant terrestrial cyanobacterium globally[32].

This choice of prey logically leads to the suspicion that the predator can be a significant factor in biocrust ecology. If this were so, the global importance of biocrust would imply that its actions may have global relevance as well. In nursery facilities designed to produce biocrust inoculum for soil restoration, the predator can have demonstrably catastrophic effects, as described in Chapter 2.

In this contribution, I carried out an assessment of the ecological consequences and relevance of this cyanobacterial pathogen, aided by the relative ease with which diseased areas can be spotted in nature. For this I combined a quantitative survey of disease extent in various sites in the Sonoran and Chihuahuan deserts of North America, with a concurrent molecular and culture-based detection of the organism, so as to establish its landscape-scale incidence. A molecular survey for the presence of Cyanoraptor-like organisms in biocrust molecular tallies existing in public databases was then carried out to assess its global reach. These surveys were complemented with functional assays

conducted at the scale of plaques, comparing relevant ecological parameters such as primary productivity, water holding capacity and dust entrainment within and outside plaques to ascertain the impact of Cyanoraptor's activity. The combined picture that emerges from my efforts is that Cyanoraptor and its lifestyle constitute a significant loss factor for the ecology of biocrusts worldwide.

Materials and Methods

Field surveys and sampling

I conducted 9 linear transects to document the presence, spatial distribution and extent of plaques, three each at three geographical locations, one in the Chihuahuan (Site 4 in Table 3) and two in the Sonoran deserts (Sites 6 and 9 in Table 3). The locations were chosen for the presence of widespread biocrust cover, and the transects were documented during or immediately after rain events in all cases, as this makes the cyanobacteria come to the surface[33], rendering plaques conspicuous and easily detectable by eye while the soil is wet. Transects were 45 m long with quadrats 1 m apart in Site 9, and 85 m with quadrats 8.5 m apart elsewhere. Quadrats were documented photographically (Fig. 15). Photographs were then analyzed using ImageJ Fiji[34] by manually outlining the areas of macroscopically visible plaques, as well as the area covered by healthy biocrust, thus excluding bare soil. Derived parameters obtained were a) the percent of biocrust area impacted by plaques, b) the areal density of plaques and c) the distance from a plaque to its nearest neighbor to characterize spatial plaque distribution.

Physical samples of plaques and biocrusts were also collected for downstream laboratory determinations using 10 cm diameter Petri dishes, so as to encompass at least one well developed plaque and some unaffected adjacent biocrusts. These samples were air dried and stored dry until analyses.

Expanded *Microcoleus* mortality assay (EMMA)

To screen for the presence of bacterial agents pathogenic to cyanobacteria within plaques and in healthy crusts adjacent to them I used a three-step bioassay, EMMA (Expanded *Microcoleus* Mortality Assay)[35], based on the fate of an axenic culture of *Microcoleus vaginatus* (PCC 9802)[19] upon inoculation with a small amount of appropriate soil. Triplicate assays were run in liquid minimal medium containing the test cyanobacterium as prey. Bioassays were read after 5 days of incubation by simple inspection, wherein positives resulted in *Microcoleus* mortality and loss of color. Aliquots from those positives were then added to healthy axenic *M. vaginatus* containing the eukaryotic inhibitor cycloheximide, death indicating a prokaryotic nature of the pathogen. Aliquots of these second positives were vacuum filtered through polycarbonate filters with nominal pore diameters of 0.2, 0.45, 0.8 and 1 μm each. Each filtrate was then added individually to healthy *M. vaginatus* cultures as described above. Lack of mortality in the 0.45 μm pore size filtrate was taken as indicative of a non-viral pathogen, and lack of mortality in the respective size cut-off indicated the rough cell size of the infective particles. The assay invariably included a) uninoculated negative controls and b) controls

for the possibility of mortality due to abiotic toxicants in which the soil was autoclaved before assay inoculation.

16S rRNA gene analyses

In order to determine if *Candidatus* Cyanoraptor togatus was present in my samples, and to assess its effects on overall community composition, I used amplicon sequencing analyses of the V4 region in the 16S rRNA gene. Plaques were sampled and cored in triplicate at a depth and diameter of 0.5 cm and homogenized. 0.25 g of the homogenate was used for DNA extraction. In addition, healthy biocrusts from sites 1, 4, 5 and 6 were cored and processed as above from healthy areas just outside plaques. DNA was extracted with a Qiagen DNeasy PowerSoil Kit (QIAGEN), following manufacturer's instructions. 16S rRNA gene PCR amplicons (V4 region) were obtained using the barcoded primer set 515F and 806R[36] and sequenced with high throughput Illumina sequencing. PCR reaction and sequencing details are given in[31]. Sequencing was performed in the Microbiome Analysis Laboratory at Arizona State University (Tempe, AZ, USA), yielding raw FASTQ sequence files. The raw FASTQ files were de-multiplexed within the MiSeq Illumina workflow under default parameters. Paired sequences were de-multiplexed and analyzed via Qiime2 2019.7[37], using the DADA2[38] plugin to create a feature table with representative sequences (ASVs) and their frequency of occurrence. To remove highly variable positions, sequences were aligned with the MAFFT[39] program. FastTree[40] was used to generate a tree. Taxonomy was assigned with the Naive Bayes classifier trained on the Greengenes 13.8 release[41], where sequences

were trimmed to include 250 bases from the V4 region, bound by 515F/806R primers[36]. Given the poor taxonomic resolution obtained with Greengenes[41] in the case of cyanobacteria, cyanobacterial (=oxyphotobacterial) sequences from the master feature table were selected using the filter_taxa_from_otu_table.py function in Qiime1[36] and phylogenetically assigned using Cydrasil[42]. To assess and quantify the presence of Cyanoraptor-like sequences, matches were sought against the 16S rRNA sequence of *Candidatus* C. togatus by BLAST[43,44].

Populational 16S rRNA gene copy number quantification

To determine the absolute abundance of bacteria between healthy and diseased biocrusts I used qPCR (quantitative real-time PCR) using aliquoted DNA extracted from homogenized cores. After fluorometric determination of DNA concentration in the extract (Qubit, Life Technologies, NY, USA), I used universal (bacteria+ archaeal) 16S rRNA gene primer set (338F 5′-ACTCCTACGGGAGGCAGCAG-3′, 518R 5′-GTATTACCGCGGCTGCTGG-3′), which produces amplicons of ideal size for qPCR, to determine the number of 16S rRNA gene copies present in each extract. The PCR reaction was performed in triplicate using the Sso Fast mix (Bio-Rad, Hercules, CA, USA) under conditions previously published[45]. The final 16S rRNA areal gene copy number of biocrust were back calculated from copies per gram of soil and the volume of the cores. Taxon-specific areal 16S rRNA gene numbers were arrived at by apportioning the total number of genes by the relative abundance of the taxon in question, as determined by Illumina sequencing and bioinformatic analyses above[46].

Photosynthetic biomass determination

Chlorophyll *a* areal concentration was used as a proxy for photosynthetic biomass. Biocrusts containing plaques were sampled in triplicate within the plaque, and again in triplicate within 5 cm from the edge of the plaque using soil 5 mm diameter cores to depth and diameter of 1 cm. From each core, 1.5 g was used for chlorophyll *a* extraction. Chlorophyll *a* was extracted in the dark at 4°C for 24 hours following the Giraldo-Silva method[47], after sample grinding by mortar and pestle in 90% acetone. The centrifuge-clarified (15° C, 10 minutes at 8437 *g*) extracts were analyzed spectrophotometrically in a Shimadzu UV1601 spectrophotometer, following the protocol of Garcia-Pichel and Castenholz [48], which corrects for scytonemin and carotenoid interference.

Primary productivity

Primary productivity was assessed using oxygen exchange rate determinations over the crust surface[47]. For this I used benthic flux chambers as previously described[49], but miniaturized to a measuring area of 12.6 mm^2 (4 mm diameter circular opening) to a total volume of 26 µL. This miniaturization ensured internal mixing by convection. The chambers were provided with an internal O_2- measuring microoptode (50 µm) tip diameter, connected to a Fire-Sting-O_2 oxygen meter, both from Pyroscience GmbH. Chambers were set in place over the crust using a micromanipulator. The optode was calibrated at 100% saturation using the water surface meniscus away from the crusts, and was temperature corrected in real time (19-20 °C). For incubations, biocrust held in 15 cm Petri dish bottoms were wetted to saturation with deionized water, let stand at 20

μmol photons $m^{-2}s^{-1}$ of illumination from fluorescent bulbs during 4 hours for acclimation and full activation, then placed inside a larger glass circular container (17 cm diameter and 6.5 cm tall), submerged in deionized water to about 2.5 mm above the surface of the crust, under an air-current driven flow and illuminated at saturating 350 μmol $m^{-2}s^{-1}$ of white light (Fiber-Lite Illuminator model 190), as measured with a quantum meter (Li-Cor model LI-250). The benthic chamber was then brought down onto the desired area to seal with the crust surface using the micromanipulator. Measurements (rate of change in oxygen concentration within the chamber) were taken along a transect crossing from healthy biocrust areas into a plaque. Measurements at each spot were carried out in triplicate. The O_2 exchange rates were back calculated, using the measuring area, the chamber capacity and an O_2 saturation of 8.79 mg/L (19.5 °C, 0% salinity, 335 m elevation). By convention, exchange rates bear a positive sign when they signify a net export of O_2 from the crust whereas O_2 consumption rates bear a negative sign.

C and N content

Total organic nitrogen (TON), total organic carbon (TOC), total inorganic nitrogen (TIN) and total inorganic carbon (TIC) content were determined, $n \geq 14$, within a plaque and in healthy biocrust within 5 cm from the edge of the plaque, from cores with depth and diameter of 0.5 cm. Cores were ground to a fine powder in a SPEX Certiprep 8000D mill and milled for 5 minutes. For TOC/TIC acid fumigation was used on a Perkin Elmer series II CHNS/O analyzer. For TON and TIN content, milled cores as above were used for the potassium chloride (KCl) extraction of nitrate and ammonium or for total nitrogen

(TN) on a combustion analyzer. The former were analyzed in a Lachat FIA Quikchem 8000 or Seal AQ2 Discrete analyzer. Standard protocols[50,51] for processing and analyses were followed, which took place in the METAL (Metals Environmental and Terrestrial Analytical Laboratory) Core Lab at Arizona State University.

Soil desiccation dynamics

Loss of soil water content during desiccation was determined using plaques and healthy adjacent biocrusts from six locations, sites 1 (n=3) , 4 (n=2) and 6 (n=1) using a commercial (UP Umweltanalytische Produkte GmbH) conductivity-based water content miniprobes[52]. Probes were inserted at a depth of 2 mm at locations with little to no slope. Water content (as conductivity voltage) and temperature, a consistent 23°C, were measured concurrently inside and outside of a plaque every 10 min during desiccation. Soil was wetted with distilled water to saturation, as determined by a glossy sheen across the soil surface and stable voltage readings. A fan mounted above the biocrust and soil was used to speed the drying process. The fan was briefly stopped 5 s prior to taking measurements. Measurements proceeded until voltage reading remained unchanged and the soil surface was patently dry. To quantify the delay in water loss in healthy biocrust, I reported the times required to reach 80% soil water content.

Exopolysaccharide (EPS) content

Exopolysaccharide (EPS) content was measured in 16 plaques from sites 1, 4, 6 and 9 (four each) and in the respective healthy biocrusts within 5 cm of the edge of the plaque. Soil was cored, at a depth and diameter of 1 cm, individually homogenized and 100 mg subsampled. EPS was extracted using 0.1 M EDTA, and precipitated in 100% ethanol (see Rossi et. al[53] for full protocol). After acid-hydrolysis, total sugars in the pellet were determined using the phenol-sulfuric acid method[54] based on commercial assay kits (Cell Biolabs, Inc.) following manufacturer's protocols.

Differential dust trapping and binding assay

Biocrust samples in 15 cm Petri dishes were wetted with distilled water slowly from the rim, photographed from above, and placed inside of a larger (17 cm diameter and 6.5 cm tall) glass circular containers. The containers were then slowly filled with a 50 g L^{-1} suspension of diatomaceous earth to a level 4-5 cm above the crust surface and let stand for several hours so that particles would sediment homogenously above the crust. When a noticeable layer had visibly accumulated (seen most clearly at the bottom of the large container), the sample was carefully removed and let stand subaerially in the light in a chemical hood under a gentle stream of air until the surface was completely dry. In the dry state, the dry diatomaceous earth layer imparted a strong surface reflectance to the crust. The sample was then photographed again to ensure that the layer was homogenous, wetted by pipetting above the fringe, and placed again in the large dish on a plinth of modeler's clay so that it was elevated from the bottom by about 1-2 cm. The container

was then filled with distilled water to about 1 cm above the crust and placed under a stream of air to create a circular motion (some 3 rpm) in the liquid. A single-point flow of air (5 mm) from a flexible hose was applied from above, sufficient to cause moderate turbulence over the crust, in a scanning motion over it. This scoured and entrained preferentially the dust particles that were not trapped and bound by the microbes, which eventually settled on the bottom of the large dish. After this, the sample was retrieved from the dish, placed under a stream of air to slowly dry out, and photographed sequentially to "develop" qualitatively spatial patterns of differential trapping. For quantification, RGB image analyses (Fiji) of areas of interest (either plaques or healthy biocrust around them) the blue channel was used, which gave the least divergence among different bare soils. The average pixel intensity was calculated for a given area after the test and normalized by the average pixel intensity of the same area before the test. The normalized average pixel intensities of plaque over biocrusts areas, pairwise, were obtained as a measure of differential dust trapping.

Meta-analysis of biocrust molecular surveys for the presence of *Candidatus* Cyanoraptor togatus

I performed a literature search and downloaded from public databases raw sequence data from studies focused on biocrust surveys based on general bacterial primers and EMP (Earth Microbiome Primers)[55] as previously described [56]. For all but the dataset from Garcia-Pichel 2013[57], forward reads obtained with pyrosequencing and paired-end reads obtained with Illumina were demultiplexed, and quality controlled using the DADA2[38]

plugin available in Qiime2 2018.6[37], creating a feature table containing representative sequences (features) and their frequency of occurrence. Highly variable positions were removed using MAFFT[39], and phylogenetic trees were generated using FastTree[40]. Preliminary taxonomic assignment was done using the Naïve Bayes classifier[58] trained on the Greengenes 13.8 release database[41]. For the Garcia-Pichel 2013 dataset[57], because quality files (.fastq) were not available, and in an effort to control for sequence quality before preforming any downstream analysis, raw sequences were first filtered using USEARCH 7[59] to remove all sequences with less than 210 bp. Overall this step filtered out up to 5% of the total sequences in some samples. Additionally, the first and last 10 bp of each sequence were trimmed using Fastx (http://hannonlab.cshl.edu/fastx_toolkit/). Quality controlled sequences were assigned to individual samples and barcodes were removed using Qiime 1.8[37] using the *multiple_split_librairies_fastq.py* script. Operational taxonomic units (OTUs) were defined with a threshold of 97% similarity and clustered using UCLUST[59] using the *pick_open_reference_otus.py* script in Qiime. Potential chimeras, and singleton OTUs were removed from further consideration. Preliminary taxonomic assignments were done with the RDP (Ribosomal Database Project) classifier[58], and representative sequences were then aligned against the Greengenes database[41] core reference alignment. I used nBLAST[43] on the NCBI database[60], to find matches against the full 16S of the rRNA gene of *Candidatus* Cyanoraptor togatus. Any matches at the species (≥ 97% similarity), genus (≥ 94.5%) or family level (≥ 86.5%) were scrutinized to ensure they did not more closely match any other genera, and specifically *Taibaiella yonginensis,* its nearest cultured relative.

Statistics

To assess general patterns among all plaques, ratios of the paired values for each parameter (inside vs. outside) were calculated, and the probability that the median of the collection of ratios was significantly different from 1 assessed with a Wilcoxon test. Statistical significance between inside and outside of a plaque in averages of biomass, productivity, TOC, TIC, TON, TIN, soil moisture content, absolute abundances of cyanobacteria and heterotrophic bacteria by site, were assessed using Welch's t-tests. For microbial community analyses, significance in composition shifts at the ASV (amplicon sequence variant) level for heterotrophic bacteria and species level for cyanobacteria were tested with pairwise PERMANOVAs calculated on Bray Curtis similarity matrices of relative abundances derived from sequencing with 9999 permutations. To determine potential drivers of community composition shifts at the ASV level the maaslin2 package[61] was used. All calculations were performed using R[62].

Data Availability Raw sequence data have been submitted to NCBI and are publicly available under BioProjectID PRJNA786587.

Results

Agency of plaque symptomology

All sites surveyed contained plaques. In all plaques sampled, EMMA assays revealed the presence of a 1 µm or smaller prokaryote pathogenic to *M. vaginatus* within them, whereas no pathogenic activity was detected in any of the apparently healthy areas

adjacent to plaques (Table S1), implicating the agency of small-sized predatory prokaryotes in plaque formation. No plaques could be attributed to cyanophages.

Subsequent 16S rRNA gene sequencing of these plaques revealed the presence of various ASVs assignable to Cyanoraptor or closely related to it; more so than to any other known bacterium, including *Taibaiella yonginensis,* its closest cultured relative (Table S3). Within plaques I could detect anywhere from 15 to 68 such ASVs. However, even within plaques, the relative abundance of this clade of bacteria in the soil microbiome was moderate, typically reaching a few percentage points of all bacteria. While ASVs fully matching, or closely matching Cyanoraptor (87% and greater identity) were also found in healthy portions of the biocrust, ASVs were on average relatively more abundant in plaques (Fig. 16), although in absolute terms this was not significant (Table S6). Apparently, molecular detection of non-viable Cyanoraptor is common in biocrusts.

In my survey of the eight existing 16S rRNA datasets from biocrusts worldwide I also detected the presence of Cyanoraptor and Cyanoraptor-like organisms in settings from Australia, Africa North America and East Asia, and ranging from 0 to 11% of all reads (Table 4). Only in one site in the Middle East (Oman) was the closest ASV below my cut-off of 87% identity indicating that ASVs at this location do not belong to this genus. I note that these were not datasets obtained with the characterization of disease in mind, but simply surveys of biocrust microbiomes.

Plaque distribution and prevalence

Having established the agency of the plaque symptomology, I turned to the quantification of its prevalence and spatial distribution. Clearly the spatial distribution of Cyanoraptor plaques was not homogenous at various scales of sampling. At the m scale, they tended to be aggregated, regardless of site. In Clark and Evans (1954) Nearest Neighbor tests conducted on image documentation of quadrats from Sites 4, 6 and 9, (i.e., see Fig.15) I obtained aggregation ratios of 0.83, 0.68 and 0.72, respectively (all below 1 and $p = 0.01$ testing for a random distribution).

At the landscape scale, the standard deviations in plaque density among transects 10-100 m apart, was always higher than the mean (Tables S2, S8, S9). At larger scales, the average density of plaques varied also widely, but the standard deviation (9.75; n =3) was within the range seen between transects in a site. In terms of percentage area of biocrusts affected, image analyses indicated that impact could be very significant, although it was very heterogenous as well, with coverage varying between 0.1 and 22%, by site (Table S2). I note that my limit of detection for plaques was approximately 3 mm in diameter and this could have impacted my estimate by defect. Spot checks in a few quadrats photographed at higher resolution, however, showed that plaques below 3 mm accounted for 0.02% of biocrust cover, or, maximally, an underestimation by 20% of the reported percent area impacted by plaques (in site 4), less than 2% in Site 6, and less than 0.1% in Site 9.

Ecological Impacts

Because plaques are easily identifiable signs of disease (Fig. 15), I was able to experimentally determine and describe the ecological processes and parameters typical of biocrusts within and immediately outside of plaques. A results compilation of the tests carried out is in Fig. 16 and the full set of data and statistics is in Table S4 and S5. Based on visual assessment, plaques experience a notable loss in cyanobacterial biomass compared to healthy biocrusts, evidenced by a decrease in green pigmentation. Drops in Chl *a,* were thus expectedly significant in paired inside-outside samples in all sites, ranging from 52 to 11% of the healthy levels remaining within plaques. When the proportion was assessed as a whole set, the p values for a significant drop in Chl *a* areal concentration were < 0.001 according to a Wilcoxon test (Fig. 16, Table S4). This decrease in cyanobacteria as measured with Chl *a* was mirrored by a decrease in the relative and absolute size of the cyanobacterial populations measured by the areal content per cm^2 of cyanobacterial 16S rRNA gene copies, which resulted in anywhere from 54 to 17% (on average to 30%) of healthy levels (Fig. 16 and S1, Table S6). Considering the median drop among sites, the effect was significant to a p of 0.002. By contrast, the effect of the predator on numbers of non-cyanobacterial 16S rRNA genes (which in biocrusts are largely due to heterotrophs) did not show a consistent trend among sites (Fig. 16), and although the ratios showed a tendency for heterotrophs to increase within plaques, this was far from significant (Fig. 16 and S2, Table S6). TOCs showed a consistent trend among sites: lower within plaques by about 10%, and while this decrease was not significant by Welch's t-tests in any one site, the decrease in ratios for all sites considered

together did reach a significance of $p = 0.02$. The epidemic's effect on TON was similar to that of TOC, with consistent decreases in all sites (levels in plaques averaged 62% of those in healthy crusts) but only on the verge of significance at each site. The significance of the decrease in TON when considering the whole set was high ($p < 0.001$). The sample parameters increased significantly in diseased areas as TIN (by % on average); again here, while not significantly in each plaque, the overall effect was highly significant for TIN. The absolute increases in TIN, on average 0.2 g N kg (soil)$^{-1}$, were almost commensurate with the absolute losses of TON, on average 0.4 g N kg (soil)$^{-1}$, speaking for a transfer into TIN from the much larger pool of TON. TIC did not vary across plaques significantly either by site or as a whole (Table S5).

Perhaps the most severe effect of the Cyanoraptor epidemics was seen on primary productivity, where small-scale determinations along transects crossing the boundaries of plaques invariably demonstrated that net export of oxygen was completely obliterated right at the plaque's advancing front (Fig. 17, Table S4). Primary productivity in the healthy crusts was in line with previous biocrust determinations[33], and thus not unusually low. The plaques turned into net-respiration systems, and typically more strongly so the closer to the front. This implies that Cyanoraptor targeted the cyanobacterial components that contributed the most to primary productivity, given that productivity rates were affected more profoundly than the cyanobacterial standing stock (see above). It is also likely that respiration rates by heterotrophs in the plaques were enhanced based on the liberation of free organics from the dead cells (as it is known from culture experiments, Cyanoraptor's feeding is rather sloppy). In support of this, I can also look at rough

estimates of efficiency of biomass transfer during the process of predation. This can be calculated by examining TEM micrographs, from Chapter 3, of the number of cyanobacterial cells affected by a single infection, 5, and the number of Cyanoraptor propagules per infection, 6. Combined with the volume of Cyanoraptor propagules and *M. vaginatus* cells, the efficiency of biomass transfer is 0.35%.

Clear effects were seen in the ability of diseased areas to trap and bind allochthonous dust at $p = 0.02$ (Fig. 16, Fig. 19, Table S4), which is considered an important trait in their development as this acts as a source of some nutrients that cannot be obtained from gaseous sources, chiefly phosphorous but also micronutrients[30]. This is likely to be traced to changes in the content of microbial exopolysaccharides that can act as a glue in the process. Exopolysaccharide production has been correlated with cyanobacteria[63] and in line with the decrease in cyanobacteria within plaques, exopolysaccharide content decreased to an average of 47% of what was found in healthy biocrusts, $p = 0.002$ (Fig. 16, Table S4). Arguably the most important function of EPS is the absorption and maintenance of water that prolongs periods of physiologic activity[63], especially in environments with infrequent rainfall. The biocrust water holding capacity at high water content of plaques was significantly reduced when compared to healthy biocrusts ($p = 0.03$). Under identical conditions, plaques dried to 80% water content on average 1.5 times faster than heathy biocrusts surrounding them (Fig. 18, Fig. S4).

Impacts on community composition

I conducted composition analyses of microbial communities within and just outside of plaques at sites 1, 4, 5 and 6 on the basis of 16S rRNA ASV counts and phylogenetic

placement, as converted to absolute abundance on the basis of total 16S rRNA gene abundance determined by qPCR. Detailed results for these analyses with resolution at the phylum level are presented in Fig. S1 and Table S6. The ratio of absolute abundances of each phylum was obtained from pair-wise samples, and the collection of ratios used to test the hypothesis of significant effects of infection by Wilcoxon tests on the median. Among all phyla, the only consistent changes in absolute terms involved a decrease in cyanobacteria in the infected areas, as expected. No changes in other phyla reached a significance of $p < 0.05$ (Fig. S2, Table S6). I also conducted similar analyses with genus/species level resolution within cyanobacteria (detailed analyses by site and sample are presented in Fig. S3 and Table S7). Here, among the 29 taxa identified, only *Microcoleus vaginatus*, *Allocoleopsis* spp., *Potamolinea* spp. and *Xeronema* spp. suffered demonstrably significant decreases ($p < 0.05$). I also assessed if there were any specific drivers of community composition shifts at the ASV level and found that results were site dependent but inconsistent. The taxonomic identification of population declines due to infection in my survey is thus consistent with the prey range demonstrated in culture. In sum, the effects of infection seem to be restricted to cyanobacteria and within them to some of the filamentous, non-heterocystous forms that are considered foundational to biocrusts. It is still likely, if not rigorously demonstrated by molecular tallies, that other cyanobacterial taxa suffered from indirect effects of predation (i.e. from a loss of nutrient supply induced by the infection), as is the case of heterocystous forms like *Scytonema* or *Tolypothrix*; while the molecular approach did not reach significance, the evaluation of the areal contents of the pigment scytonemin (a marker for these genera in biocrusts) did reach significant (if moderate) declines (Fig. 16, Table S4).

Discussion

After discovery of a predatory bacterium of cyanobacteria within biocrusts, *Candidatus* Cyanoraptor togatus[35], I set out to determine its ecological relevance using molecular surveys at the worldwide and local (southwestern US) scales and field surveys of plaques, visible areas of disease. There is potential for bacterial disease to have far reaching consequences because biocrusts have a worldwide distribution[24] and contribute so heavily to the health of arid and semiarid environments through soil stabilization[25,26] and nutrient additions[27–30]. I found that *Candidatus* C. togatus is indeed widely present and detrimental to the functioning of biocrusts via the clearing of healthy biocrusts, forming plaques.

Supporting my conclusion that this predator is a universal factor in biocrust health, I found *Candidatus* C. togatus in 90% of the deserts surveyed via my meta-analysis and within plaques containing predatory bacteria in every site scouted at an identity of 94% or greater, which is not surprising considering the pervasiveness[2,4–8,13] of the predatory *Bdellovibrio* and like organisms. Given the limited molecular data available[35] and lack of visual surveys for plaques outside the southwestern US, I suspect that its distribution is actually far wider. Furthermore, discovery of molecular signatures of Cyanoraptor, and related organisms, outside of plaques may indicate that low levels of infection are present before the symptoms of disease become visible and are undetectable via bioassays. Thus, approximations based solely on visual surveys may underestimate the abundance of predators.

While *candidatus* C. togatus is pervasive, estimates of the extent of predatory damage at the large scale was difficult due to the heterogenous and clumped coverage of plaques, with average disease coverage ranging anywhere from 0.1% to 22% at the m^2 (Table S2). This distribution also gives me insight into the means of dispersal at the large scale, indicating that infection is propagated by something other than random wind dispersal, instead likely by surface water runoff. In support of this, field observations of ephemeral rain runoff paths exhibit high concentrations of plaques, while adjacent areas are devoid of plaques. Dispersal at the small scale is likely influenced by Cyanoraptor's prey preference, non heterocystous motile bundle formers. The formation of bundles[25] by the prey provides a crowded environment in which Cyanoraptor can easily transfer between hosts. Additionally, filamentous cyanobacteria advance laterally along the soil surface[47], providing access to new prey.

In every plaque screened for *Candidatus* C. togatus the most abundant cyanobacterium, *M. vaginatus* and *Schizothrix* spp., experienced the greatest losses (Fig. 16, Fig. S3, Table S7). And though Cyanoraptor does not directly attack heterocystous cyanobacteria such as *Nostoc* spp. and *Scytonema* spp.[35], there appear to be ripple effects of predation demonstrated by the reductions in scytonemin, (Fig. 16, Table S4), a marker for these cyanobacteria[48]. By targeting pioneer cyanobacteria[25,26] specifically, Cyanoraptor may spell disaster for the establishment of biocrusts in denuded locales.

The formation of plaques results in the utter destruction of biocrusts and loss of their characteristic functions. This is hugely detrimental considering that biocrust communities can take decades to centuries to recover from damage[64,65], with the slowest rates of

recovery in the most arid locations[66], resulting in long term ramifications for the contributions of biocrusts to ecological services[67]. Biocrusts are also globally important primary producers[27] and key contributors of fixed N to arid lands[29], but plaques cause a shift in the balance of nutrients. TOC decreases (Fig. 16, Table S5) and biocrusts shift from a carbon sink into net exporters (Fig. 17, Table S4). TON declines as TIN increases.

Plaques are spaces that are depleted in exopolysaccharides, which are crucial to soil moisture retention and dust entrapment. Biocrust water retention has long been speculated to be heavily reliant on polysaccharides[68] but it was not clear who the chief producers were. I was able to demonstrate a clear drop in water retention (Fig. 18, Fig. S4), by an average of 67%, correlated with a decrease in exopolysaccharides (Fig. 16, Table S4). Water is the most limiting factor in arid environments[69], with physiologic activity tied to infrequent, pulsed rain events[69], thus any reduction in water holding capability will negatively impact microbial growth and repair, including the production[70] and maintenance of protective pigments, membrane integrity[71] as well as the photosynthetic machinery[72,73]. Decreases in water retention not only restrict microbial activity but also lessen macro and micronutrient inputs from aeolian dust particle entrapment[30]. Reduced dust entrainment documented in plaques (Fig. 16, Fig. 19, Table S4) will further impoverish biocrusts.

I have demonstrated that *Candidatus* Cyanoraptor togatus constitutes a diverse clade of predatory bacteria that can be found worldwide and identified by the formation of plaques, or circular areas of clearing in otherwise healthy biocrusts. Distribution and cover of plaques within the Southwestern US are heterogeneous at the local and

landscape scale and result in decreases in cyanobacteria, specifically non heterocystous, motile, bundle forming species, which is characteristic of culture based experiments with Cyanoraptor[35]. Infection appears to have a varied impact on heterotrophs and does not shift the overall composition of their microbial community. Plaques also result in the near total loss of beneficial biocrust contributions to the ecosystem, including decreases of exopolysaccharides which then leads to decreases in water retention and dust entrainment. Primary productivity is significantly retarded and shifts in carbon and nitrogen content also occur.

Studies of the environmental impacts of predatory bacteria are few and far between[74] but the obvious signs of disease caused by predatory bacteria of cyanobacteria presents an opportunity to fill this gap within a system that has been thoroughly studied regarding its phototrophic components. Historically, biocrust research has failed to recognize heterotrophic contributions to biocrusts but recent discoveries of beneficial[75] and now detrimental[35] bacterial components demonstrates the importance of a holistic "microbiome" approach to biocrust science. Thus, study of bacterial predation in biocrusts has a dual benefit by providing both a model system for environmental studies of predatory bacteria and also fills gaps in biocrust science.

While I have demonstrated the worldwide presence of *Candidatus* Cyanoraptor togatus and its capacity to alter the characteristic functions of biocrust communities, further work is needed outside the southwestern US. If distribution patterns are similar, the health of biocrusts may vary widely and assessment of biocrusts' ecosystem contributions may need to be modified. As this is the first recognized predatory bacterium within biocrusts,

it is quite possible that there are others preying on other cyanobacteria or heterotrophs. In fact, predatory bacterial abundance as a whole is thought to be underestimated by 2.5 orders of magnitude[76]. Unfortunately, visible signs of disease may not be as obvious as the biocrust plaques and a concerted effort would need to be made to isolate both prey and predator.

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Conflict of Interest

The authors declare no conflict of interest.

Tables

Sites	Coordinates	Type of crust*	Closest City, State	Desert Biome
1	lat 32.59194°, long -106.85286°	Smooth	Las Cruces, NM	Chihuahuan
2	lat 32.50321°, long -106.74097°	Smooth	Las Cruces, NM	Chihuahuan
3	lat 32.56348°, long -106.75795°	Smooth	Las Cruces, NM	Chihuahuan
4	lat 32.51540°, long -106.74269°	Rugose	Las Cruces, NM	Chihuahuan
5	lat 34.33703°, long -106.72910°	Rugose	Albuquerque, NM	Chihuahuan
6	lat 32.9913°, long -111.76130°	Smooth	Casa Grande, AZ	Sonoran
7	lat 33.3923°, long -111.35404°	Smooth	Gold Canyon, AZ	Sonoran
8	lat 33.57293°, long -111.79713°	Rugose	Scottsdale, AZ	Sonoran
9	lat 33.30089°, long -111.68285°	Smooth	Mesa, AZ	Sonoran

***** Type of crust as defined in Belnap and Lange 2001[77].

Table 3. Sampling sites for surveys and sample collection of biocrusts. Biocrusts were largely cyanobacterial, although some contained some lichens as well.

Desert	Location	Detected	Number of ASVs	Closest ASV	Range % Match ASVs	Sum % Reads
Pinnacles[78]	Australia	+	1000	99	99-87	2
Simpson[79]	Australia	+	384	98	98-87	5
Kalahari[80]	Botswana	+	255	98	98-87	1
Gurbantunggut[81]	China	+	43	94	94-87	10
Chihuahuan	United States Albuquerque, NM[46]	+	407	99	99-87	11
Great Basin[45]		+	90	95	95-87	1
Mojave[57]		+	423	96	96-87	1
Wahiba[82]	Oman	-	NA	85	NA	NA

Table 4. Worldwide occurrence of *Candidatus* Cyanoraptor togatus, or closely related predatory bacteria, based on field surveys and a meta-analysis of published 16S rRNA gene data. A positive result for the presence of Cyanoraptor is at the family level or above, ≥86.5% similarity.

Figures

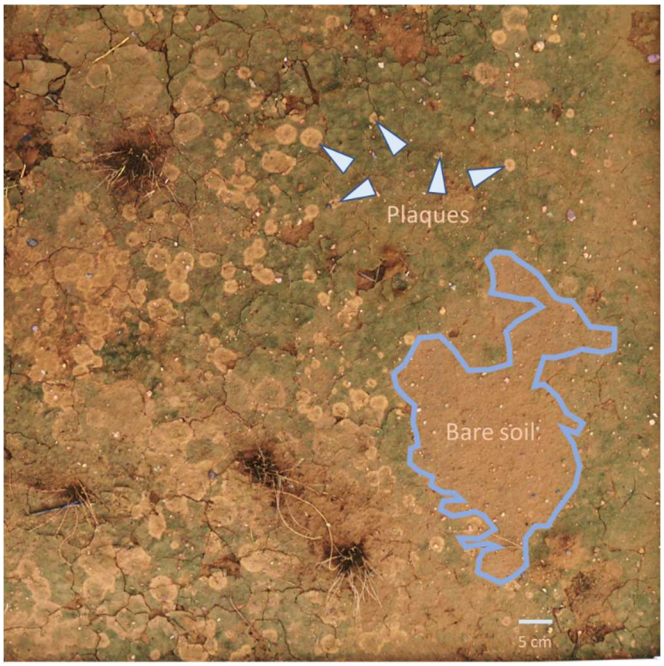


Figure 15. Representative quadrat (site 9) used to derive measures of plaque density, distribution and percentage cover. Arrows indicate individual plaques. Demarcated area is an example of bare soil. Scale bar 5 cm.

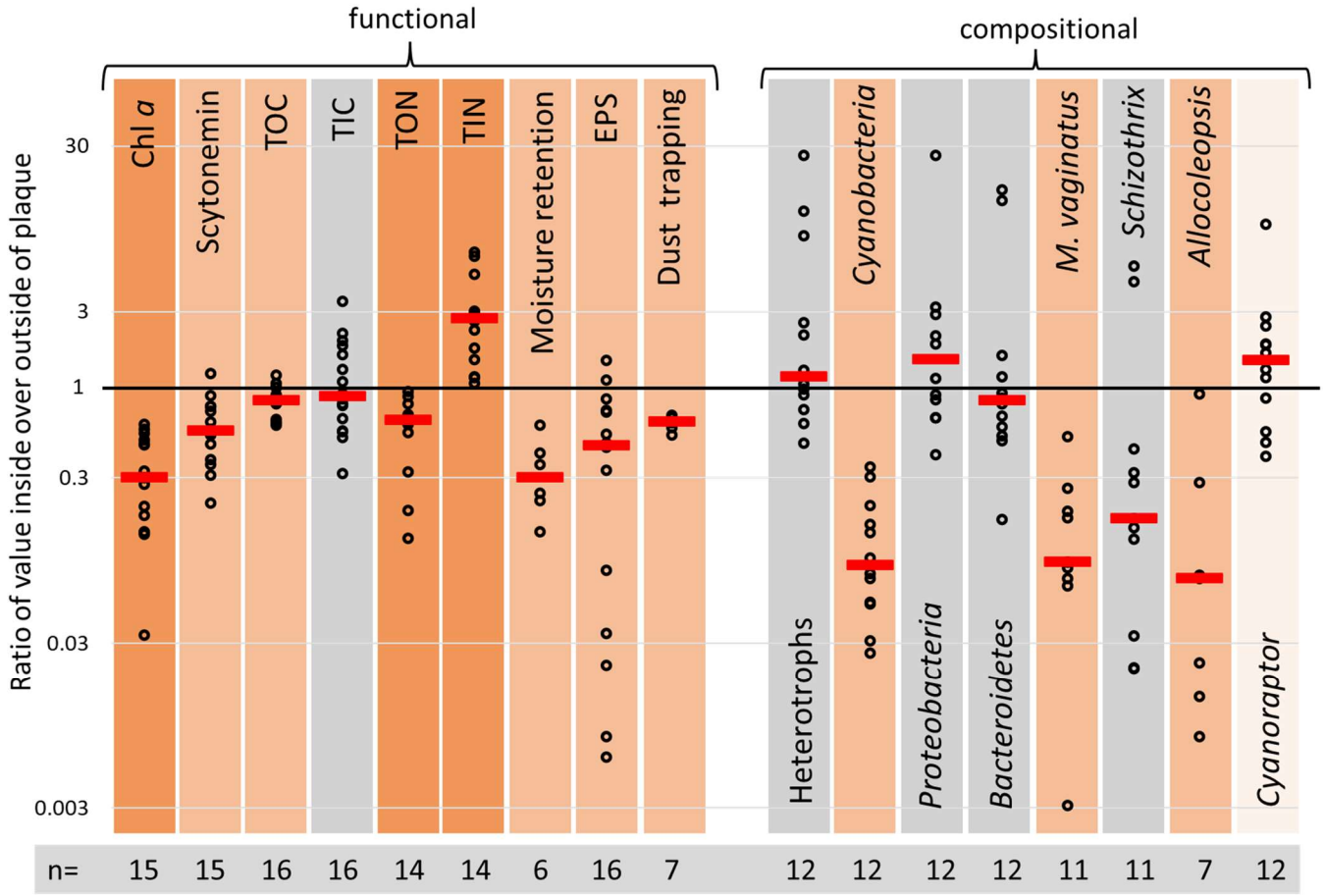


Figure 16. Compilation of effects of Cyanoraptor epidemics on various biocrust parameters as the ratio of the parameter measured outside over that inside plaques. Points indicate ratios, and the red line indicates their median. The level of significance is denoted by the background color where gray is non-significant (ratio $p > 0.1$), light orange is $0.05 > p < 0.1$, medium orange is $0.05 > p < 0.005$ and dark orange is $p < 0.005$ according to Wilcoxon tests. Full set of data is in Supplementary Tables 4, 5, 6 and 7.

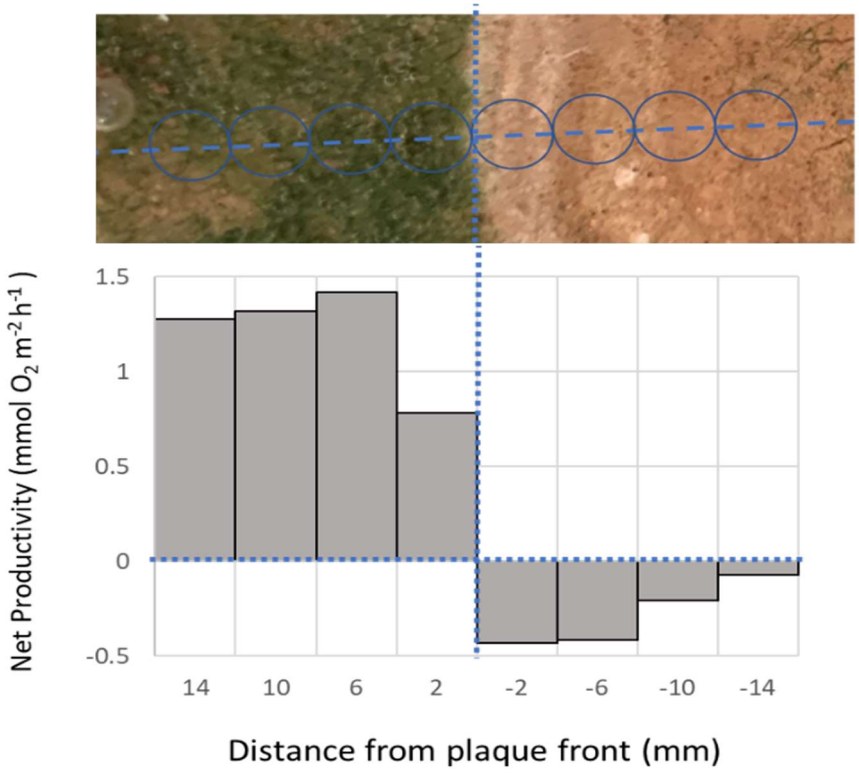


Figure 17. Spatial variation of net productivity (oxygen export) across a plaque boundary showing how a biocrust turns into a net respiratory system within a plaque. Photograph above shows areas measured. Data from similar measurements from a variety of plaques from different sites can be found in Supplementary Table 4.

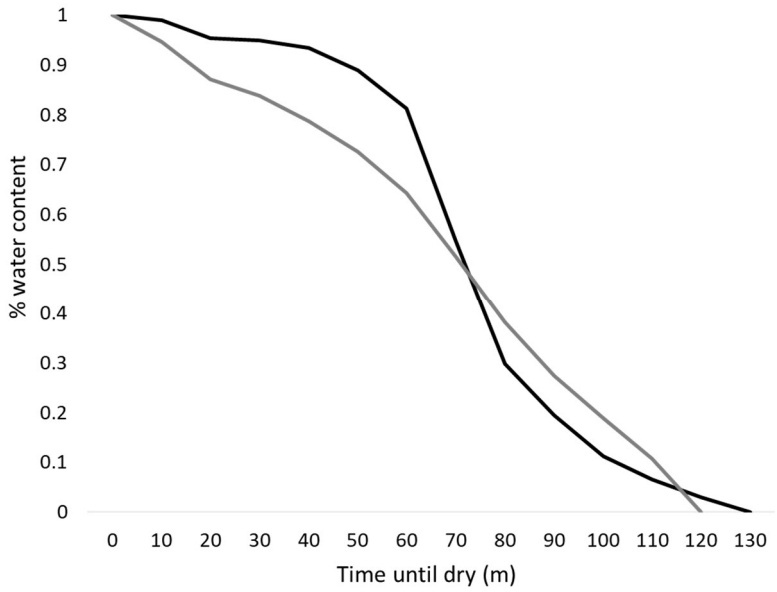


Figure 18. Representative dynamic profile of water loss from site 1 in healthy biocrust (black line) and plaque (grey line), showing a delay in initial water loss in the healthy biocrust. Profiles for other 5 plaques tested are in Supplementary Fig. 4.

Figure 19. Representative image of aspects of the differential dust trapping and binding assay. Top: Dry sample of biocrust containing a central plaque before assay. Bottom: Differential patterns of dust binding revealed during the drying process. Data from measurements can be found in Table S4.

Supplementary Information

Supporting Tables

Site	EMMA In/Out	Number of positives: Inside	Number of positives: Outside	Replicates
1	+/-	6	0	n=6
2	+/-	6	0	n=6
3	+/-	6	0	n=6
4	+/-	6	0	n=6
5	+/-	6	0	n=6
6	+/-	6	0	n=6
7	+/-	6	0	n=6
8	+/-	6	0	n=6
9	+/-	6	0	n=6

Supplementary Table 1. Correlation between plaque symptomology and the presence of pathogenic bacteria.

Sites	Average Plaques/m^2	Standard Deviation	Average % Disease Coverage	Standard Deviation
4	1.3	2.3	.1%	.2%
6	4.8	5.5	1.2%	1.4%
9	16.4	34.3	22.0%	33.9%

Supplementary Table 2. Representative number of plaques and plaque cover at sites 4, 6 and 9. Expanded data in Table S8 and S9.

Desert	Site	Replicate	Number of ASVs	Closest ASV (% similarity to Cyanoraptor)	Range of ASV Match (%)	% Reads
Chihuahuan						
	1	1	23	100	100-88	1.2
		2	21	96	96-88	0.3
		3	63	96	96-88	3.4
	4	1	39	96	96-87	1
		2	33	94	94-87	0.5
		3	24	93	93-87	0.7
	5	1	68	94	94-88	0.8
		2	56	94	94-88	2.1
		3	41	94	94-88	3.6
Sonoran						
	6	1	15	94	94-87	0.2
		2	16	100	100-87	0.2
		3	14	94	94-88	1.9

Supplementary Table 3. Molecular detection of *Candidatus* Cyanoraptor togatus and allied organisms, inside of plaques from different locations based on 16S rRNA similarity, with relative abundance of positive ASVs.

		Chl a (mg/m^{-2})			Scytonemin (mg/m^{-2})			Productivity (mmol O_2*m^{-2}*h^{-1})			EPS (mg/cm^{-2})			Difference in Dust Trapping (relative units)			Moisture Retention (m until 80% water content)		
Site	Plaque	Out	In	Ratio	Out	In	Ratio	Out (avg)	In (avg)	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio
1	1	64.1	30.3	0.47	145.3	136.2	0.94	1.5 ± 0.4	-0.4 ± 0.2		0.02	0.04	1.53	1.2	0.8	0.72	60.5	37.6	0.62
	2	82.2	48.3	0.59	174.9	141.9	0.81	1.4 ± 0.4	-0.1 ± 0.0		0.04	0.05	1.16	2.1	1.3	0.60	49.5	20.9	0.42
	3	67.8	34.4	0.51	166.0	107.5	0.65	0.6 ± 0.2	-0.7 ± 0		0.07	0.00	0.03				34.6	8.3	0.24
	4										0.03	0.00	0.08						
	5																		
	6																		
	Average	71.4	37.7	0.52	162.0	128.5	0.80	1.20	-0.3		0.04	0.02	0.70	1.65	1.06	0.66	48.18	22.27	0.43
	SD	9.6	9.4		15.2	18.4		0.60	0.3		0.02	0.02		0.69	0.32		12.98	14.66	
	p =	0.01			0.07			<0.001			0.29						0.08		
4	1	95.3	52.8	0.55	489.0	279.6	0.57	0.4 ± 0.2	-0.1 ± .01		0.16	0.12	0.76	2.3	1.6	0.69	41.2	14.8	0.36
	2	114.5	72.2	0.63	898.8	321.9	0.36	0.6 ± 0.4	-0.2 ± 0.3		0.26	0.13	0.48				51.9	11.3	0.22
	3	77.4	37.2	0.48	387.2	81.2	0.21	0.4 ± 0.2	-0.1 ± 0.1		0.29	0.00	0.01						
	4										0.11	0.00	0.02						
	5																		
	Average	95.7	54.1	0.56	591.7	227.6	0.38	0.5	-0.1		0.21	0.06	0.32				46.55	13.05	0.29
	SD	18.6	17.5		270.8	128.5		0.3	0.1		0.09	0.07					7.61	2.42	
	p =	0.05			0.13			<0.001			0.04								
5	1	137.5	24.4	0.18	203.7	97.6	0.48	0.7 ± 0.2	0 ± 0										
	2	85.0	23.3	0.27	142.3	115.1	0.81	1.3 ± .3	-0.2 ± 0										
	3	89.3	17.9	0.20	162.1	207.7	1.28	0.7 ± 0	0 ± 0										
	4																		
	5																		
	Average	103.9	21.9	0.22	169.4	140.1	0.86	0.9	-0.1										
	SD	29.2	3.4		31.3	59.1		0.4	0.1										
	p =	0.04			0.50			<0.001											
6	1	49.7	14.9	0.30	85.4	46.3	0.54	0.8 ± 0.4	-0.0 ± 0.0		0.12	0.04	0.33	1.2	0.8	0.65	57.0	8.0	0.14
	2	74.2	10.2	0.14	130.2	40.6	0.31	0.9 ± 0.2	-0.4 ± 0.3		0.07	0.06	0.90	1.4	0.921	0.64			
	3	75.0	24.7	0.33	180.4	105.5	0.58	0.2 ± 0.2	-0.1 ± 0.1		0.29	0.21	0.75						
	4										0.21	0.10	0.49						
	5																		
	Average	66.3	16.6	0.26	132.0	64.1	0.48	0.7	-0.1		0.17	0.10	0.62	1.33	0.86	0.65			
	SD	14.4	7.4		47.5	35.9		0.4	0.2		0.10	0.08		0.15	0.08				
	p =	0.01			0.12			<0.001			0.33								
9	1	105.1	5.0	0.05	231.5	89.4	0.39	0.3 ± 0.2	-0.1 ± 0.1		0.65	0.36	0.55	2.0	1.1	0.54			
	2	56.8	8.0	0.14	116.6	87.7	0.75	0.4 ± 0.2	0 ± 0		0.89	0.41	0.46	1.6	1.0	0.65			
	3	69.0	9.7	0.14	146.2	82.0	0.56	0.6 ± 0.3	-0.1 ± 0.2		0.90	0.00	0.00						
	4										0.39	0.00	0.01						
	Average	77.0	7.6	0.11	164.8	86.4	0.57	0.4	-0.1		0.71	0.19	0.25	1.78	1.05	0.60			
	SD	25.1	2.4		59.7	3.9		0.3	0.1		0.24	0.22		0.29	0.04				
	p =	0.04			0.15			<0.001			0.02								
	Grand Average	82.9	27.5	0.33	244.0	129.4	0.62	0.7	-.1		0.28	0.10	0.47	1.69	1.08	0.64	49.10	16.81	0.33
	SD	30.4	19.5		211.7	84.3		0.5	0.2		0.29	0.13		0.46	0.28		9.72	11.24	
	p =	**<0.001**			**0.002**						**0.002**			**0.02**			**0.03**		

Supplementary Table 4. Impacts of disease on chlorophyll *a*, scytonemin, productivity, EPS, dust trapping and moisture retention. n≥3 inside and n≥3 outside, see Fig. 16.

		TOC (g/kg soil)			TIC (g/kg soil)			TON (g/kg soil)			TIN (g/kg soil)		
Site	Plaque	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio
1	1	16.9	11.4	0.67	1.2	2.3	1.89	0.8	0.3	0.32	0.1	0.3	3.03
	2	17.1	15.7	0.92	0.7	2.3	3.46	0.8	0.5	0.63	0.1	0.2	1.22
	3	28.3	18.5	0.65	10.3	17.2	1.67	1.6	1.2	0.72	0.1	0.2	2.88
	4	24.1	30.1	1.25	10.8	6.1	0.57	1.3	1.3	0.99	0.1	0.1	1.81
	5	10.6	9.2	0.87	0.7	1.5	2.23	0.9	0.1	0.13	0.5	1.1	2.33
	6	11.9	10.0	0.84	2.9	0.9	0.32	1.0	0.7	0.70	0.3	0.4	1.11
	Average	18.2	15.8	0.87	4.4	5.0	1.69	1.1	0.7	0.58	0.2	0.4	2.06
	SD	6.9	7.9		4.8	6.2		0.3	0.5		0.2	0.4	
	p =	0.60			0.85			0.14			0.31		
4	1												
	2												
	3												
	4												
	5												
	Average												
	SD												
	p =												
5	1	25.8	23.1	0.90	4.20	2.20	0.52	1.4	1.0	0.68	0.0	0.1	2.65
	2	30.4	24.5	0.81	9.75	13.20	1.35	1.9	1.2	0.64	0.1	0.4	6.87
	3	29.4	26.9	0.91	10.85	9.75	0.90	1.7	1.1	0.64	0.1	0.3	2.97
	4	26.2	27.4	1.05	13.05	14.90	1.14	1.7	1.5	0.92	0.1	0.4	5.05
	5	15.5	10.0	0.65	7.35	14.85	2.02	0.8	0.6	0.83	0.0	0.2	6.52
	Average	25.5	22.4	0.86	9.0	11.0	1.19	1.5	1.1	0.74	0.1	0.3	4.81
	SD	5.9	7.1		3.4	5.3		0.4	0.3		0.0	0.1	
	p =	0.48			0.5			0.14			0.02		
6	1	12.4	11.8	0.95	2.20	1.50	0.68	0.5	0.4	0.68	0.1	0.1	1.20
	2	9.8	6.3	0.64	1.05	0.60	0.57	1.2	0.7	0.56	0.5	0.7	1.55
	3	9.2	5.7	0.62	0.60	0.50	0.83	0.7	0.1	0.19	0.3	1.0	2.79
	4	23.7	25.0	1.05	13.55	11.05	0.82						
	5	7.2	8.0	1.11	3.30	3.15	0.95						
	Average	12.5	11.4	0.88	4.1	3.4	0.77	0.8	0.4	0.48	0.3	0.6	1.85
	SD	6.6	8.0		5.4	4.4		0.3	0.3		0.2	0.4	
	p =	0.82			0.8			0.16			0.35		
9	1												
	2												
	3												
	4												
	Average												
	SD												
	p =												
	Grand Average	18.7	16.5	0.87	5.8	6.4	1.25	1.2	0.8	0.62	0.2	0.4	3.00
	SD	8.1	8.5		4.9	6.0		0.4	0.5		0.2	0.3	
	p =	**0.02**			**0.59**			**<0.001**			**<0.001**		

Supplementary Table 5. Impacts of disease on soil nutrients, n≥6.

		Heterotrophic Bacteria (millions 16S rRNA gene copy number/cm^{-2})*			*Cyanobacteria* (millions 16S rRNA gene copy number/cm^{-2})**			*Proteobacteria* (millions 16S rRNA gene copy number/cm^{-2})			*Bacteroidetes* (millions 16S rRNA gene copy number/cm^{-2})			Cyanoraptor. (millions 16S rRNA gene copy number/cm^{-2})		
Site	Plaque	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio
1	1	33.4	115.7	3.46	85.6	56.8	0.66	8.2	50.4	6.17	4.5	22.0	4.86	2.7	5.6	2.07
	2	51.0	36.2	0.71	125.7	36.2	0.29	14.9	10.4	0.70	13.5	6.8	0.50	5.9	1.6	0.27
	3	18.3	153.7	8.41	47.0	31.2	0.66	6.8	66.9	9.88	5.2	32.9	6.33	2.4	14.7	6.07
	4															
	5															
	6															
	Average	34.2	101.9	4.20	86.1	41.4	0.54	9.9	42.6	5.58	7.7	20.5	3.90	3.7	7.3	2.80
	SD	16.4	60.0		39.4	13.5		4.3	29.1		5.0	13.1		1.9	6.7	
	p =	0.18			0.18			0.19			0.23			0.45		
4	1	147.5	111.8	0.76	101.6	23.4	0.23	49.2	50.1	1.02	19.3	13.5	0.70	4.8	3.2	0.68
	2	132.1	72.0	0.54	43.9	18.3	0.42	44.4	26.1	0.59	13.6	8.1	0.60	4.6	1.5	0.32
	3	139.9	82.1	0.59	88.4	15.6	0.18	49.5	31.4	0.63	18.7	8.9	0.48	5.3	2.2	0.41
	4															
	5															
	Average	139.8	88.6	0.63	78.0	19.1	0.27	47.7	35.9	0.75	17.2	10.2	0.59	4.9	2.3	0.47
	SD	7.7	20.7		30.2	3.9		2.8	12.6		3.1	2.9		0.4	0.9	
	p =	0.04			0.08			0.24			0.05			0.02		
5	1	129.9	85.8	0.66	129.6	12.9	0.10	45.4	26.3	0.58	23.1	10.1	0.44	14.4	6.8	0.48
	2	252.5	168.7	0.67	206.1	53.7	0.26	129.8	75.2	0.58	41.4	26.3	0.64	19.2	17.4	0.91
	3	317.2	454.7	1.43	266.7	71.0	0.27	133.9	216.4	1.62	63.3	76.7	1.21	29.9	30.4	1.02
	4															
	5															
	Average	233.2	236.4	0.92	200.8	45.9	0.21	103.0	106.0	0.93	42.6	37.7	0.76	11.2	12.1	0.69
	SD	95.1	193.5		68.7	29.8		50.0	98.7		20.1	34.8		10.0	7.5	
	p =	0.98			0.04			0.97			0.84			0.74		
6	1	92.0	32.4	0.35	127.8	13.3	0.10	48.4	14.2	0.29	18.5	8.3	0.45	3.0	1.8	0.58
	2	23.4	21.6	0.92	58.8	6.0	0.10	12.1	10.3	0.85	4.8	3.1	0.64	0.4	1.6	3.56
	3	191.3	607.1	3.17	196.5	59.2	0.30	62.2	146.4	2.35	69.2	37.1	0.54	18.0	14.3	0.79
	4															
	5															
	Average	102.2	220.3	1.48	127.7	26.2	0.17	40.9	57.0	1.17	30.8	16.2	0.54	7.2	5.9	1.64
	SD	84.4	335.0		68.9	28.8		25.9	77.5		33.9	18.3		9.5	7.3	
	p =	0.61			0.11			0.76			0.56			0.86		
9	1															
	2															
	3															
	4															
	Average															
	SD															
	p =															
	Grand Average	127.4	161.8	1.81	123.1	33.1	0.30	50.4	60.3	2.11	24.6	21.1	1.45	9.2	8.4	1.43
	SD	92.9	181.2		68.9	21.9		42.5	62.2		21.9	20.7		9.1	9.0	
	p =	**0.85**			**0.002**			**0.85**			**0.25**			**0.08**		

*I assume all non-cyanobacteria are heterotrophs. **Oxyphotobacteria

Supplementary Table 6. Impacts of disease on bacterial composition, as determined by 16S rRNA gene amplicon sequence variant with qPCR on major phyla, n=3 plaques per site.

		Microcoleus vaginatus (millions 16S rRNA gene copy number/cm^{-2})			*Schizothrix* spp. (millions 16S rRNA gene copy number/cm^{-2})			*Allocoleopsis* spp. (millions 16S rRNA gene copy number/cm^{-2})			*Potamolinea* spp. (millions 16S rRNA gene copy number/cm^{-2})			*Xeronema* spp. (millions 16S rRNA gene copy number/cm^{-2})		
Site	Plaque	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio	Out	In	Ratio
1	1	40.8	16.8	0.41	4.3	3.0	0.70	10.7	0.0	0.00	2.3	0.9	0.40	3.0	0.7	0.22
	2	56.7	7.5	0.13	1.4	8.9	6.50	15.9	0.0	0.00	4.0	3.3	0.82	4.3	0.3	0.07
	3	23.8	6.7	0.28	0.2	5.3	24.16	8.3	0.3	0.03	2.4	1.1	0.47	2.7	0.2	0.07
	4															
	5															
	6															
	Average	40.5	10.3	0.28	2.0	5.8	10.45	11.7	0.1	0.01	2.9	1.8	0.56	3.3	0.4	0.12
	SD	16.5	5.6		2.1	3.0		3.9	0.2		1.0	1.3		0.9	0.3	
	p =	0.07			0.15			0.04			0.30			0.02		
4	1	27.8	11.2	0.40	9.6	0.5	0.05	0.8	0.5	0.66	3.1	0.2	0.08	4.7	0.7	0.15
	2	9.1	5.9	0.65	2.7	1.5	0.55	0.3	0.3	1.18	0.9	0.6	0.67	3.3	0.8	0.24
	3	16.2	3.3	0.21	3.6	1.3	0.36	1.2	0.0	0.03	1.3	0.5	0.39	9.5	0.1	0.01
	4															
	5															
	Average	17.7	6.8	0.42	5.3	1.1	0.32	0.7	0.3	0.62	1.7	0.4	0.38	5.8	0.5	0.13
	SD	9.5	4.0		3.8	0.5		0.5	0.2		1.2	0.2		3.2	0.4	
	p =	0.17			0.19			0.23			0.20			0.10		
5	1	71.2	0.8	0.01	0.0	0.0		13.6	1.2	0.09	0.0	0.0		1.2	0.2	0.15
	2	69.0	33.2	0.48	10.5	5.5	0.52	11.3	1.5	0.14	0.6	1.4	2.28	3.9	0.0	0.01
	3	65.8	16.5	0.25	0.5	0.2	0.43	74.1	19.4	0.26	11.5	0.6	0.05	4.6	0.2	0.04
	4															
	5															
	Average	68.7	16.8	0.25	3.7	1.9	0.47	33.0	7.4	0.16	4.0	0.6	1.17	3.2	0.1	0.07
	SD	2.7	16.2		5.9	3.1		35.7	10.4		6.5	0.7		1.8	0.1	
	p =	0.03			0.68			0.34			0.46			0.10		
6	1	0.0	0.0		15.9	5.4	0.34	0	0		3.0	1.4	0.46	1.3	0.0	0.01
	2	0.2	0.0	0.00	19.0	2.1	0.11	0	0		5.5	0.6	0.10	1.3	0.1	0.06
	3	3.0	0.0	0.00	82.4	5.0	0.06	0	0		17.5	1.2	0.07	2.0	0.0	0.00
	4															
	5															
	Average	1.0	0.0	0.00	39.1	4.2	0.17	0.0	0.0		8.7	1.0	0.21	1.5	0.0	0.02
	SD	1.7	0.0		37.6	1.8		0.0	0.0		11.5	0.6		1.6	0.0	
	p =	0.39			0.25			NA			0.23			0.02		
9	1															
	2															
	3															
	4															
	Average															
	SD															
	p =															
	Grand Average	32.0	8.5	0.26	12.5	3.2	3.07	11.3	1.9	0.27	4.3	1.0	0.53	3.5	0.3	0.08
	SD	27.8	9.8		22.9	2.8		20.7	5.5		5.1	0.8		2.3	0.3	
	p =	**0.003**			**0.32**			**0.01**			**0.05**			**<0.001**		

Supplementary Table 7. Impacts of disease on major cyanobacterial taxa, as determined by 16S rRNA gene amplicon sequence variant with qPCR, n=3 plaques per site.

SITE	TRANSECT	QUADRAT	PLAQUE DENSITY (m^-2)	BIOCRUST COVER (%)	PLAQUE DENSITY IN BIOCRUST (m^-2)	PLAQUE DENSITY IN BIOCRUST (m^-2), TRANSECT MEAN	PLAQUE DENSITY IN BIOCRUST (m^-2), SITE MEAN
4	1	1	5	85.5%	5.8		
		2	9	98.3%	9.2		
		3	0	97.5%	0.0		
		4	0	100.0%	0.0		
		5	0	95.3%	0.0		
		6	0	100.0%	0.0		
		7	0	92.2%	0.0		
		8	1	100.0%	1.0		
		9	0	100.0%	0.0		
		10	0	98.3%	0.0	1.60	
	2	1	2	100.00%	2.0		
		2	2	100.00%	2.0		
		3	0	100.00%	0.0		
		4	0	99.00%	0.0		
		5	0	100.00%	0.0		
		6	0	100.00%	0.0		
		7	2	92.00%	2.2		
		8	7	97.27%	7.2		
		9	0	97.68%	0.0		
		10	0	100.00%	0.0	1.34	
	3	1	0	100.00%	0.0		
		2	1	100.00%	1.0		
		3	3	100.00%	3.0		
		4	0	94.52%	0.0		
		5	5	99.36%	5.0		
		6	1	100.00%	1.0		
		7	0	100.00%	0.0		
		8	0	100.00%	0.0		
		9	0	100.00%	0.0		
		10	0	100.0%	0.0	1.00	1.31
6	1	1	15	56.91%	26.4		
		2	2	75.11%	2.7		
		3	1	71.52%	1.4		
		4	2	75.90%	2.6		
		5	0	100.00%	0.0		
		6	20	96.46%	20.7		
		7	6	98.33%	6.1		
		8	12	77.99%	15.4		
		9	1	79.90%	1.3		
		10	0	100.00%	0.0	7.65	
	2	1	1	54.71%	1.8		
		2	9	78.75%	11.4		
		3	2	100.00%	2.0		
		4	3	100.00%	3.0		
		5	0	100.00%	0.0		
		6	9	99.36%	9.1		
		7	14	87.81%	15.9		
		8	0	100.00%	0.0		
		9	10	100.00%	10.0		
		10	3	100.00%	3.0	5.63	

	3	1	1	95.13%	1.1		
		2	1	96.09%	1.0		
		3	0	100.00%	0.0		
		4	3	100.00%	3.0		
		5	4	94.40%	4.2		
		6	2	100.00%	2.0		
		7	0	100.00%	0.0		
		8	5	99.28%	5.0		
		9	5	98.94%	5.1		
		10	14	95.96%	14.6	3.60	5.63
9	1	1	0	42.8%	0.0		
		2	0	47.6%	0.0		
		3	74	100.0%	74.0		
		4	226	85.7%	263.8		
		5	8	100.0%	8.0		
		6	1	100.0%	1.0		
		7	0	100.0%	0.0		
		8	1	100.0%	1.0		
		9	3	100.0%	3.0		
		10	176	100.0%	176.0		
		11	61	100.0%	61.0		
		12	180	100.0%	180.0		
		13	8	93.7%	8.5		
		14	1	100.0%	1.0		
		15	1	100.0%	1.0		
		16	2	100.0%	2.0		
		17	2	100.0%	2.0		
		18	8	100.0%	8.0		
		19	17	100.0%	17.0		
		20	0	100.0%	0.0		
		21	1	100.0%	1.0		
		22	0	78.3%	0.0		
		23	0	58.8%	0.0		
		24	0	0.0%			
		25	0	22.5%	0.0		
		26	0	100.0%	0.0		
		27	0	79.5%	0.0		
		28	0	100.0%	0.0		
		29	10	88.3%	11.3		
		30	0	100.0%	0.0		
		31	2	100.0%	2.0		
		32	7	100.0%	7.0		
		33	0	100.0%	0.0		
		34	0	4.5%	0.0		
		35	0	22.9%	0.0		
		36	0	4.7%	0.0		
		37	0	78.6%	0.0		
		38	0	100.0%	0.0		
		39	0	100.0%	0.0		
		40	1	100.0%	1.0		
		41	0	100.0%	0.0		
		42	0	100.0%	0.0		
		43	1	100.0%	1.0		
		44	0	100.0%	0.0		
		45	0	81.9%	0.0	18.88	

	2	1	1	100.00%	1.0		
		2	0	62.74%	0.0		
		3	29	100.00%	29.0		
		4	86	100.00%	86.0		
		5	85	100.00%	85.0		
		6	9	95.21%	9.5		
		7	9	58.55%	15.4		
		8	16	92.38%	17.3		
		9	4	73.34%	5.5		
		10	6	100.00%	6.0		
		11	0	49.41%	0.0		
		12	1	42.46%	2.4		
		13	1	76.75%	1.3		
		14	4	60.50%	6.6		
		15	4	67.59%	5.9		
		16	11	100.00%	11.0		
		17	17	100.00%	17.0		
		18	5	100.00%	5.0		
		19	0	100.00%	0.0		
		20	0	34.39%	0.0		
		21	0	75.91%	0.0		
		22	0	66.96%	0.0		
		23	1	80.39%	1.2		
		24	23	81.89%	28.1		
		25	9	80.07%	11.2		
		26	45	100.00%	45.0		
		27	26	100.00%	26.0		
		28	53	93.45%	56.7		
		29	6	100.00%	6.0		
		30	0	67.58%	0.0		
		31	1	96.93%	1.0		
		32	0	100.00%	0.0		
		33	0	61.55%	0.0		
		34	0	51.86%	0.0		
		35		no biocrut			
		36		no biocrut			
		37		no biocrut			
		38	0	100.00%	0.0		
		39	0	100.00%	0.0		
		40	0	100.00%	0.0		
		41	0	91.64%	0.0		
		42	8	78.53%	10.2		
		43	67	87.64%	76.4		
		44	90	100.00%	90.0		
		45	65	100.00%	65.0		
	3	1	17	71.51%	23.8	17.70	
		2	61	100.00%	61.0		
		3	36	27.52%	130.8		
		4	69	54.57%	126.4		
		5	0	3.85%	0.0		
		6	0	41.70%	0.0		
		7		no biocrut			
		8	0	43.14%	0.0		
		9	0	23.72%	0.0		
		10	0	27.18%	0.0		

		11		no biocrust			
		12	0	15.50%	0.0		
		13		no biocrust			
		14	0	100.00%	0.0		
		15	4	15.19%	26.3		
		16	22	100.00%	22.0		
		17	23	87.50%	26.3		
		18	1	86.41%	1.2		
		19	0	70.30%	0.0		
		20	28	98.22%	28.5		
		21	10	75.22%	13.3		
		22	0	11.52%	0.0		
		23	0	12.37%	0.0		
		24	12	56.06%	21.4		
		25	0	100.00%	0.0		
		26	3	23.19%	12.9		
		27	17	80.72%	21.1		
		28	3	65.71%	4.6		
		29	15	100.00%	15.0		
		30	24	100.00%	24.0		
		31	4	98.88%	4.0		
		32	1	28.26%	3.5		
		33	4	78.59%	5.1		
		34	5	59.32%	8.4		
		35	5	61.53%	8.1		
		36	45	100.00%	45.0		
		37	37	100.00%	37.0		
		38	48	100.00%	48.0		
		39	49	100.00%	49.0		
		40	36	100.00%	36.0		
		41	4	100.00%	4.0		
		42	19	71.72%	26.5		
		43	68	100.00%	68.0		
		44	47	100.00%	47.0		
		45	27	100.00%	27.0	23.22	19.93
				Grand Means:	14.55	8.96	8.96
				Standard deviation:	33.30	8.63	9.75

SITE 4: lat 32.51540°, long -106.74269°, Las Cruces, NM, Chihuahuan Desert
SITE 6: lat 32.9913°, long -111.76130, Casa Grande, AZ, Sonoran Desert
SITE 9: lat 33.30089°, long -111.68285°, Mesa, AZ, Sonoran Desert

Supplementary Table 8. Spatial survey of density of Cyanoraptor plaques on biocrust of the southwestern US.

SITE	TRANSECT	QUADRAT	BIOCRUST AREA INFECTED (%)	BIOCRUST AREA INFECTED (%), TRANSECT MEAN	BIOCRUST AREA INFECTED (%), SITE MEAN
4	1	1	0.3%		
		2	0.6%		
		3	0.0%		
		4	0.0%		
		5	0.0%		
		6	0.0%		
		7	0.0%		
		8	0.1%		
		9	0.0%		
		10	0.0%	0.10%	
	2	1	0.12%		
		2	0.06%		
		3	0.00%		
		4	0.00%		
		5	0.00%		
		6	0.00%		
		7	0.05%		
		8	0.53%		
		9	0.00%		
		10	0.00%	0.08%	
	3	1	0.00%		
		2	0.15%		
		3	0.36%		
		4	0.00%		
		5	0.63%		
		6	0.02%		
		7	0.00%		
		8	0.00%		
		9	0.00%		
		10	0.00%	0.12%	0.10%
6	1	1	2.42%		
		2	0.61%		
		3	0.15%		
		4	0.73%		
		5	0.00%		
		6	3.67%		
		7	1.74%		
		8	4.87%		
		9	0.79%		
		10	0.00%	1.50%	
	2	1	0.21%		
		2	1.43%		
		3	0.57%		
		4	0.59%		
		5	0.00%		
		6	4.42%		
		7	3.22%		
		8	0.00%		
		9	2.41%		
		10	0.10%	1.29%	

	3	1	0.19%		
		2	0.33%		
		3	0.00%		
		4	0.43%		
		5	0.75%		
		6	0.07%		
		7	0.00%		
		8	1.02%		
		9	0.62%		
		10	3.46%	0.69%	1.16%
9	1	1	0.0%		
		2	0.0%		
		3	27.7%		
		4	88.8%		
		5	4.6%		
		6	0.1%		
		7	0.0%		
		8	0.9%		
		9	2.9%		
		10	88.4%		
		11	30.3%		
		12	74.3%		
		13	3.8%		
		14	4.9%		
		15	0.3%		
		16	1.3%		
		17	0.8%		
		18	2.5%		
		19	12.8%		
		20	0.0%		
		21	0.3%		
		22	0.0%		
		23	0.0%		
		24			
		25	0.0%		
		26	0.0%		
		27	0.0%		
		28	0.0%		
		29	14.3%		
		30	0.0%		
		31	1.3%		
		32	10.8%		
		33	0.0%		
		34	0.0%		
		35	0.0%		
		36	0.0%		
		37	0.0%		
		38	0.0%		
		39	0.0%		
		40	0.1%		
		41	0.0%		
		42	0.0%		
		43	1.3%		
		44	0.0%		
		45	0.0%	8.47%	

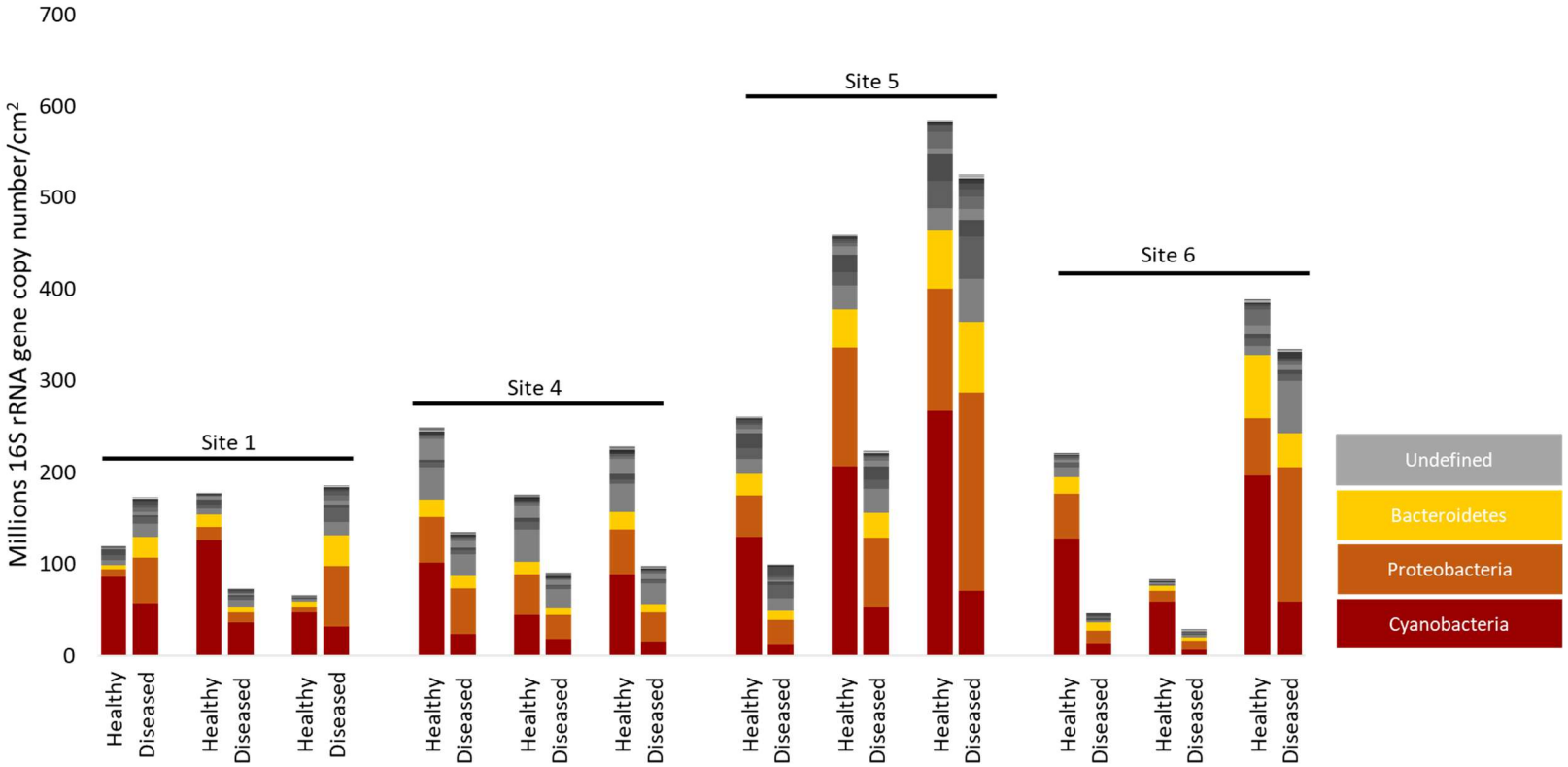
	2	1	0.11%		
		2	0.00%		
		3	20.37%		
		4	82.58%		
		5	91.16%		
		6	3.78%		
		7	50.42%		
		8	22.39%		
		9	1.54%		
		10	2.48%		
		11	0.00%		
		12	0.12%		
		13	0.08%		
		14	0.97%		
		15	1.52%		
		16	8.73%		
		17	41.66%		
		18	3.12%		
		19	0.00%		
		20	0.00%		
		21	0.00%		
		22	0.00%		
		23	0.35%		
		24	58.91%		
		25	4.03%		
		26	69.95%		
		27	28.25%		
		28	80.30%		
		29	2.58%		
		30	0.00%		
		31	0.69%		
		32	0.00%		
		33	0.00%		
		34	0.00%		
		35			
		36			
		37			
		38	0.00%		
		39	0.00%		
		40	0.00%		
		41	0.00%		
		42	28.05%		
		43	65.70%		
		44	86.93%		
		45	85.35%		
	3	1	34.15%	20.86%	
		2	62.83%		
		3	97.76%		
		4	89.57%		
		5	0.00%		
		6	0.00%		
		7			
		8	0.00%		
		9	0.00%		
		10	0.00%		

		11			
		12	0.00%		
		13			
		14	0.00%		
		15	85.51%		
		16	95.62%		
		17	81.13%		
		18	0.46%		
		19	0.00%		
		20	87.26%		
		21	26.94%		
		22	0.00%		
		23	0.00%		
		24	69.42%		
		25	0.00%		
		26	3.36%		
		27	38.85%		
		28	10.74%		
		29	92.73%		
		30	55.06%		
		31	2.25%		
		32	0.48%		
		33	18.18%		
		34	8.16%		
		35	33.47%		
		36	92.49%		
		37	90.56%		
		38	94.00%		
		39	92.83%		
		40	81.38%		
		41	7.89%		
		42	46.87%		
		43	86.57%		
		44	87.86%		
		45	80.83%	41.79%	23.20%
			16.00%	8.32%	8.15%
			30.15%	14.29%	13.04%

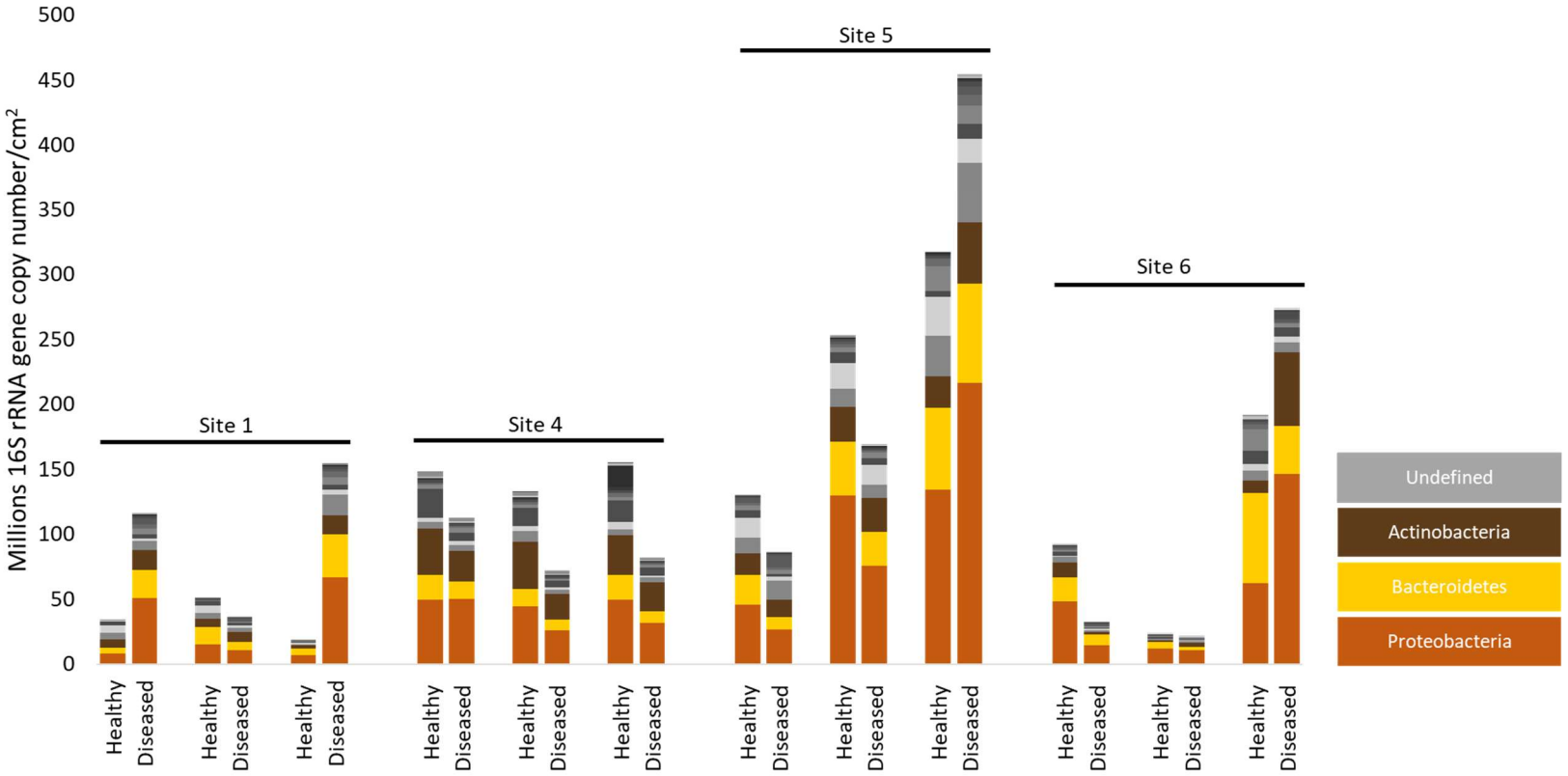
SITE 4: lat 32.51540°, long -106.74269°, Las Cruces, NM, Chihuahuan Desert
SITE 6: lat 32.9913°, long -111.76130, Casa Grande, AZ, Sonoran Desert
SITE 9: lat 33.30089°, long -111.68285°, Mesa, AZ, Sonoran Desert

Supplementary Table 9. Spatial survey of area infected of Cyanoraptor plaques on biocrust of the southwestern US.

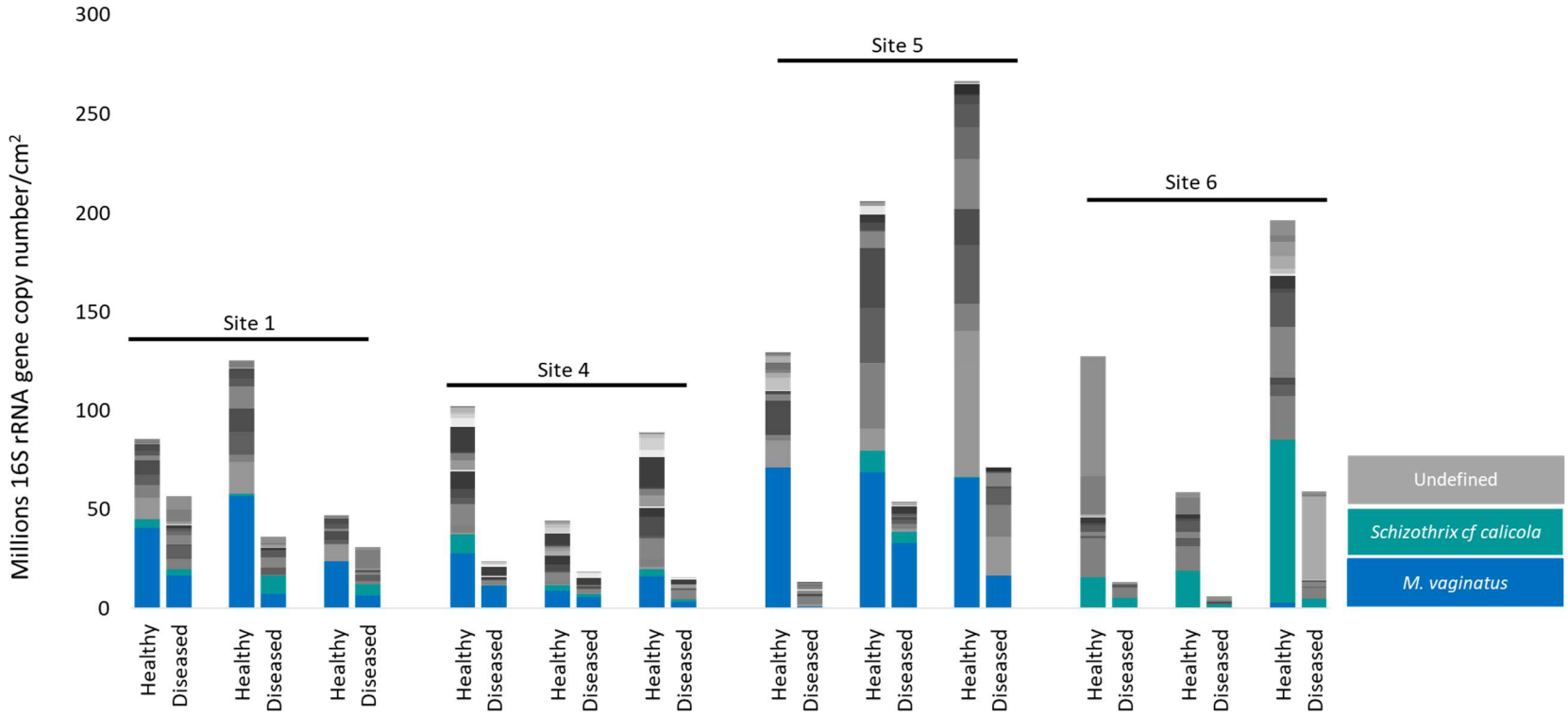
Supporting Figures



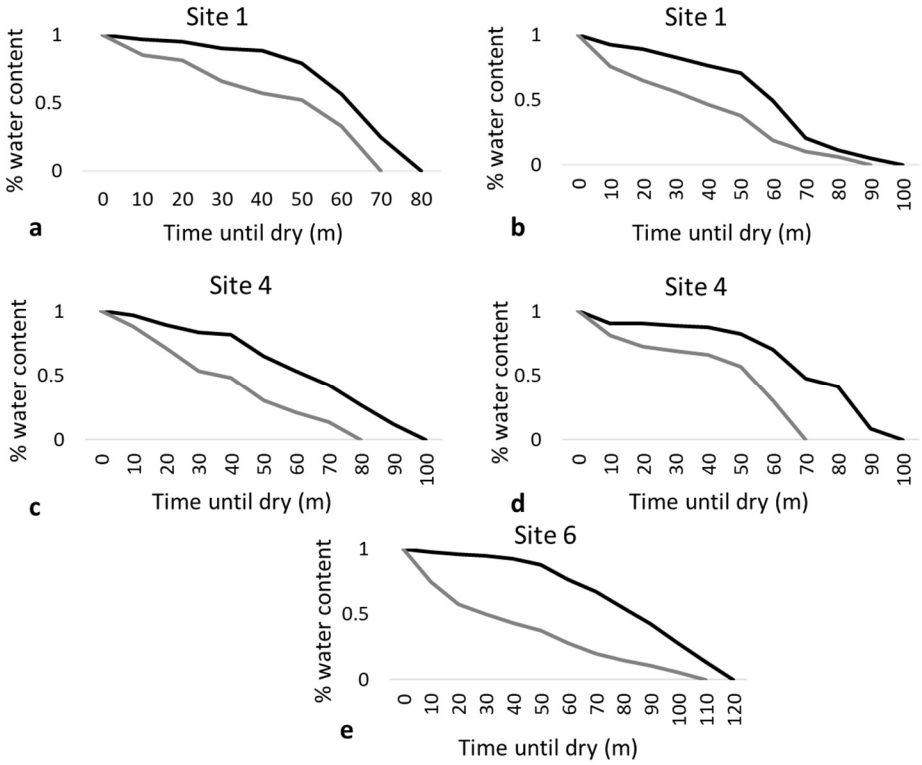
Supplementary Figure 1. Phylum level shifts in community composition in healthy and diseased biocrusts, based on 16S rRNA gene amplicon sequencing.



Supplementary Figure 2. Phylum level shifts of heterotrophs in community composition in healthy and diseased biocrusts, based on 16S rRNA gene amplicon sequencing.



Supplementary Figure 3. Cyanobacterial community composition in healthy and diseased biocrusts, based on 16S rRNA gene amplicon sequencing.



Supplementary Figure 4. Dynamics of water loss across plaque boundaries in plaques from various sites. Out of plaque determinations are black lines and in-plaque are grey lines. See also Fig. 18.

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5 – CONCLUSIONS

Contributions to predatory bacteria and biocrust science

My research on a predatory bacterium of cyanobacteria describes a new microbial functional component of biocrust communities and supplies ample evidence for its deleterious ecological impacts on biocrust ecology.

State of research on predatory bacteria and biocrusts

Research on predatory bacteria has a relatively short history. The first predatory bacterium, *Bdellovibrio bacteriovorus*, was discovered only in 1962, with efforts primarily focusing on describing its morphology and life cycle[1] via transmission electron microscopy. *B. bacteriovorus* research peaked in the late 1970s[2], widening advances to the description of its interactions with prey bacteria and prey physiological responses[3,4], all the while expanding on the now standard methodologies for enrichment and isolation of predatory bacteria[5]. Following this explosion of interest, scientific contributions seemed to have stalled until the new millenium[2] where renewed interest in this bacterial guild has continued to the present day. With advances in molecular techniques and, eventually, -omics based studies[6], a more detailed mechanistic understanding of *Bdellovibrio*'s complex life cycle was made possible, providing further confirmation of its total reliance on prey and attack weaponry, including its an arsenal of hydrolytic enzymes[7]. Transcriptional analysis of *Bdellovibrio bacteriovorus* also elucidated distinguishing characteristics of both attack and growth phases including signals enabling penetration of prey and large increases in protein synthesis upon entry[8,9]. Increasingly, current studies focus on predatory bacteria as a whole, elucidating protein families

common to all[9] and prey signaling that allows prey recognition and predatory attack[10,11]. Yet, investigations of predatory bacteria are, to a great extent, still reliant on culture-based studies of *Bdellovibrio* and *Bdellovibrio*-like organisms[12].

While cyanobacteria, lichens and other components of what are now called biocrusts were described as early as the late 1700s[13], recognition of these components as part of a functional unit and study of their ecological contributions was not comprehensively investigated until the 1950s. Interest in biocrusts expanded greatly in the 1970s and has continued since, identifying new community members[14–18], investigating their important ecological contributions[19–24] and developing restoration strategies[25]. Biocrusts are a functional unit with a wide range of taxonomically and functionally different community members[26], but studies have historically focused on the phototrophic community. While heterotrophic bacteria have been well documented[14,15], their microbial interactions were largely unknown until a recent study identified the importance of heterotrophs in the establishment of pioneer cyanobacteria[27,28]. Work on deleterious relationships is restricted to a single study documenting the presence of bacteriophages infecting *Firmicutes*[29] and there are no reports of the presence of disease agents or their effects on cyanobacteria.

I attempted to fill these gaps in knowledge by identifying, describing, and assessing the ecological impact of a novel predatory bacterium that I discovered during optimization of the production of restoration inoculum. This research expands knowledge of predatory bacteria's functional roles in natural settings and provides a more holistic view of the biocrust microbiome.

Chapter 2

I report on optimization protocols for a "mixed-community" approach to scaling-up production of restoration inoculum using biocrusts collected from the Chihuahuan and Great Basin deserts, evaluating phototrophic growth dynamics and the possibility of recurrent inoculum production. Growth dynamics varied, with wide deviations within locations, though growth was generally faster in crusts from the Great Basin desert. Recurrent inoculum production of biocrusts from the Great Basin desert resulted in cumulative bacterial community shifts over the course of three reinoculations and growth of biocrust phototrophs from the Chihuahuan Desert was so meager that the experiment had to be ended after only one reinoculation. But previously reported inoculum production using the mixed-community approach had resulted in large gains in biomass and steady community composition[30], leading me to suspect that I was facing a yet to be identified loss factor. Depending on biocrust source location and soil texture, 10-80% of growth chambers experienced significant mortality to *M. vaginatus*, supporting my hypothesis that deleterious biological factors were responsible for the high variability in growth and failure of recurrent inoculum production in plots from the Chihuahuan Desert. After a battery of bioassays, I indeed discovered that a predatory bacterium that could prey on *M. vaginatus* PCC9802[31] was present in my production pipeline.

Major conclusions

- Predatory bacteria can be a significant loss factor for biocrust inoculum when using the mixed-community approach to biocrust restoration.

Chapter 3

I obtained a highly purified enrichment of this predatory bacterium using various techniques and used it to describe its morphology and life cycle via transmission electron microscopy, to sequence its entire genome, and to study its basic physiology. I could determine that it is an obligate, endobiotic predator that has both an attack phase and growth phase, much like the predatory bacterium *Bdellovibrio bacteriovorus*[32]. Attack phase cells are nonmotile Gram-negative cocci less than 1 μm with unique double-membrane internal compartmentalization, that attach and gain entry to their prey. Entering the growth phase, they localize to the prey's cytoplasm before beginning filamentous replication and producing copious vesicles. A lack of genes for amino acid biosynthesis reinforced the conclusion that it is an obligate predator and a plethora of genes for hydrolytic enzymes indicate vesicles seen in micrographs are likely used for lysis of its prey. Based on 16S rRNA gene phylogeny, my predatory bacterium was identified to the family *Chitinophagaceae*, in the *Bacteroidetes* phylum. As its identity to the closest cultivated species was less than 90%, I therefore named a new genus, Cyanoraptor and species togatus. Restricting its viability, it only preyed upon non heterocystous bundle forming pioneer cyanobacteria and was highly sensitive to native environmental stressors such as high temperature, repeated desiccation and intense light.

Major conclusions

- Biocrusts contain a novel endobiotic obligate predatory bacterium, *Candidatus* Cyanoraptor togatus, unrelated to other known bacterial predators, that preys upon pioneer biocrust cyanobacteria.
- Native environmental stressors naturally modulate the success of this predator.

Chapter 4

I identified symptoms of disease, plaques, or circular areas of clearing in otherwise healthy biocrusts which allowed me to probe for the presence of Cyanoraptor and ascertain its impact on the bacterial community. Cyanoraptor was found in both plaques and healthy biocrusts but was relatively more abundant in plaques and correlated with decreases in cyanobacteria, specifically pioneer bundle formers. Using an array of tests comparing plaques to healthy biocrusts, I determined that there were indeed significant ecological impacts of predation, most importantly the localized annihilation of primary productivity. But also importantly, exopolysaccharide content was significantly depleted, which was likely behind the demonstrable loss in moisture retention and dust trapping ability. Additionally, there were shifts in the balance of nitrogenous pools, with TON decreasing commensurately with increases in TIN. To assess the global impact of Cyanoraptor, I performed a meta-analysis of 16S rRNA gene datasets from published literature combined with field surveys of plaque prevalence and spatial distribution in the Chihuahuan and Sonoran deserts. Cyanoraptor was present in 8 deserts worldwide but was heterogeneously distributed, aggregated at the local and landscape scales but with the potential to affect up to nearly a quarter of biocrusts present. In the US Southwest it causes significant losses to productivity of biocrusts at the landscape scale.

Major conclusions

- Cyanoraptor causes cm-sized epidemics in the form of plaques.
- Plaques undergo severe and long-lasting functional losses.
- Cyanoraptor is widespread in the southwest US and probably also worldwide.
- Cyanoraptor's ecosystem scale consequences are significant.

Integrative Conclusions

When considered together, the conclusions reached in the previous sections, clearly point out that bacterial predators constitute an ecological force to contend with in biocrusts, able to control major emergent properties and the community composition of soil microbiomes. The fact that this is the case in biocrusts, begs the question to what extent they impact other types of microbiomes, from oceans to the human gut in which they are found.

Regarding disease spread, in natural biocrust communities the heterogenous distribution of predatory bacteria keeps disease in check, preventing widespread community collapse. However, mixed-community approaches to restoration, where whole crusts are homogenized, result in catastrophic failure. Mirroring this, disturbance of natural biocrusts also results in homogenization, increasing the potential for transmittance of predatory bacteria in native communities.

With respect to biotechnological attempts using large-scale cultivation approaches (as in Chapter 2), bacterial predators can also have catastrophic effects. However, in the case of biocrust restoration, the research presented here also provides simple approaches to keep them under check: simply monitor for plaque presence in the early stages and discard the affected areas from downstream inoculation. This straightforward precaution should become standard procedure.

Potential Further Research Avenues

While I was able to make significant advances in my research topic, these efforts also opened new questions regarding the biology and ecology of Cyanoraptor and

Cyanoraptor-like organisms and biocrust diseases in general. With respect to Cyanoraptor, I was only able to enrich for, not isolate, it. Isolation, or a co-culture with predator and prey only, would allow further characterization of its relationship to its prey. Transcriptional profiling of its attack and growth phases would identify specific processes that are key to predation, elucidating the signals that indicate the prey is a sufficient food source, the processes required to gain entry to its prey and triggers to begin replication. Comparisons to the transcriptional profile of the thoroughly studied *Bdellovibrio bacteriovorus* could be made and based on similarities, provide further avenues of research.

All research thus far indicates that Cyanoraptor is nonmotile, with no flagella and no motility genes. I have suggested that its endobiotic lifestyle provides a solution to this roadblock. While ensconced in its motile host it could be carried throughout the soil column[33–35], infecting neighboring cyanobacteria. By way of its prey moving laterally on the soil surface[36] it may be able to spread to new locales that are ripe for attack. Live cell imaging of fluorescently tagged Cyanoraptor could be used to track any assisted motility and transfer of infection from one filament to another. It would thus be interesting to probe if and how, Cyanoraptor takes advantage of cyanobacterial motility more rigorously.

Plaques also provide avenues to further characterize Cyanoraptor and its microbial interactions. Cyanoraptor ASVs have been detected both inside and outside of plaques. This may indicate that cyanobacterial lateral movement creates a front of infection that is yet to be visible and in such low concentrations as to be undetectable via EMMAs.

Research into this phenomenon would be of use to restoration science. Because disease may not be easily visible, practices based on using whole communities as a seed for inoculum production could be at risk for spreading infection rather than sowing healthy biocrust.

Plaques also provide an opportunity to investigate infection dynamics within the native ecosystem. At their center they often contain a circular patch of filamentous cyanobacteria, possibly an area of recovery after Cyanoraptor has moved outwards. In addition, many plaques have concentric rings at their outer edge, possibly a visible demonstration of the pulsed nature of infection based on water availability, with each ring being an infection event. 16S rRNA gene sequencing at the mm scale from the center to the edge of a plaque would provide answers about cyanobacterial recovery and where the highest concentration of Cyanoraptor is found. Combined with sequencing, artificial wetting events could be used to determine if rings at the outer edge of plaques are indicative of pulsed rain events and if these are areas with high concentrations of the predator.

Spatial distribution of plaques at both the meter and landscape scales is clumped, possibly indicating that plaques spread via surface water runoff. Unreported preliminary experiments showed that single plaques grow outwards with an apparent rate of 0.22 cm per cm of rainfall. Depending on rainfall amount this can be a significant increase in plaques. Based on average rainfall in the Great Basin Desert of 15 to 30 cm per year[37] this would result in a 6.75 to 9 cm diameter increase in a plaque, approximately the size of a single large plaque, meaning that the number of plaque coverage in wetter deserts

could double each year. However, this is based on a single observation and only considers total rainfall, not the length of time the biocrust/plaque was wet. Tracking rates of plaque expansion based on water content would provide additional insight into the rate of disease spread at the large scale.

At a broader scale, Cyanoraptor is likely to be a worldwide factor in biocrust health as it was found in nearly every dataset available in my meta-analysis. In order to confirm this, larger visual surveys for plaques combined with 16S rRNA gene sequencing need to be undertaken. This is the first instance of a predatory bacterium within biocrusts but given the prevalence of *Bdellovibrio* and like organisms[38] it is likely there are more to be discovered. As biocrust research expands into understudied locations and sequencing becomes commonplace, the impact of both Cyanoraptor and predatory bacteria may become yet clearer.

Overall, my research provides a comprehensive body of knowledge of a novel predatory bacterium within biocrusts and will serve as a baseline to investigate this bacterium's impact on biocrust constituents and their functions. Given that it is globally distributed and has deleterious effects on biocrust function, this research is a starting point to revise the large-scale ecological contribution of biocrusts. As restoration techniques are refined, it serves as a warning for use of mixed-community approaches, suggesting a that combination of detection methods should be employed.

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