Earth's Stratosphere and Microbial Life

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Abstract
The Earth's atmosphere is an extremely large and sparse environment which is quite challenging for the survival of microorganisms. We have long wondered about the limits to life in the atmosphere, starting with Leeuwenhoek's observation of "animalcules" collected from the air. In the past century, significant progress has been made to capture and identify biological material from varying elevations, from a few meters above ground level, to the clouds near mountaintops, and the jet streams, the ozone layer, and even higher up in the stratosphere. Collection and detection techniques have been developed and advanced in order to assess the potential diversity of life from very high altitudes. Studies of microbial life in the stratosphere with its multiple stressors (cold, dry, irradiated, with low pressure and limited nutrients), have recently garnered considerable attention. Here, we review studies of Earth's atmosphere, with emphasis on the stratosphere, addressing implications for astrobiology, the dispersal of microbes around our planet, planetary protection, and climate change.

Introduction

Why is it important to study and understand the limits to life at the highest elevations in the atmosphere? Elevation is a limit to life that has not yet been fully explored. It is relevant for space exploration, the search for life

outside our planet, as well as more down-to-Earth concerns such as climate and disease.

In this review, we focus on conditions in the upper atmosphere, especially the stratosphere (Figure 1), and primarily on what is known about how microorganisms can survive there, under extreme conditions. The stratosphere is the highest elevation of the atmosphere where life has been found and hence its importance in studies of microbiology in our atmosphere. Astrobiology research has utilized Earth's stratosphere and its conditions as an analogue to conditions found on Mars, addressing whether extremophilic and other hardy microorganisms that survive in the stratosphere may tolerate conditions on the surface of Earth's sister planet. Here, we explore both the stressors encountered and the associated microbial responses. However, since studies of the stratosphere are relatively few, we also refer to a set of atmospheric analyses conducted in the troposphere or closer to Earth's surface for comparison and contrast. We present a summary of the studies which have contributed to our current level of understanding as well as the implications for astrobiology, climate, and health.

A relatively limited number of microbes have been collected from or exposed to the stratosphere, and most of them were either dormant or otherwise metabolically inactive, such as spores and lyophilized cells (Moeller and Horneck, 2004). In a sole instance, metabolically active microbes in growth media were launched into the stratosphere and returned to Earth (DasSarma and DasSarma, 2018). These cells retained viability, suggesting that ice entrapment may enhance microbial survival. Sampling and experimentation missions in the stratosphere are technically challenging and further work needs to be done to catalog and characterize cells that successfully survive. Technical issues of these studies, such as potential contamination during sample collection, can be a significant concern. Nevertheless, it is imperative to understand microbial survival strategies from the perspective of life in multiple extreme conditions like the stratosphere.

From Aerobiology to Exobiology

Aerobiology is the scientific field that studies the passive transport of biological particles through the atmosphere and its effect on living systems and the environment. In general, it has focused on particles of biological origin (bioaerosols), ranging from 0.2 to 2.5 μm in diameter, which make up 25% of atmospheric particles (Griffin et al., 2018). With higher elevation and the complete disappearance of the atmosphere, aerobiology transitions to space biology or astrobiology (used by several authors as a synonym for exobiology). As early as 1960, Lederberg first used the term "exobiology" to describe the exploration of life in higher elevations, from the stratosphere into the realm of space, during his presentation at the 1st

International Space Science Symposium sponsored by the international Committee on Space Research (COSPAR) (Lederberg, 1960).

Research in aerobiology began with Earth-based questions, focusing on the spread of diseases. In 1546, Girolamo Fracastoro suggested that disease was transmitted not only by direct contact with a sick person, or contagion through contaminated objects, but also by transmission through the air at a distance or *ad distans* (Dubos, 1986). After the discovery of the microscope, Antoine van Leeuwenhoek determined, in 1702, that "animalcules" could be carried by the wind, together with dust floating in the air. John P. Ehrenberg examined air and dust specimens, collected by Charles Darwin on his trip on the H.M.S. Beagle in the 1830s, and found that it was composed of a multitude of "infusoria" (Darwin, 1846), a term commonly used at that time to describe microscopic life. In another study, Ehrenberg concluded the existence of an atmospheric "kingdom" of life, detected 6 km high in the Himalayas (Cunningham, 1873).

In one of the first experimental studies of microbial composition versus elevation, Louis Pasteur used swanneck flasks to demonstrate that the number of microbes found in the air diminished with increasing altitude and also varied by location, time and atmospheric conditions. He found that there were fewer microbes at higher altitudes, such as at the peak of Montan Verte at 2 km height (Pasteur, 1860). Soon after that, H.G. Dyar examined air in New York, in the 1890s, and reported the presence of microorganisms, predominantly *Micrococcus*, *Bacillus* and *Sarcina*, albeit at much more moderate elevations as these were collected closer to the surface (Dyar, 1894).

A number of studies starting in 1921 addressed the spread of fungal diseases of wheat and other agricultural grains in the United States (US). In 1934, a survey conducted dozens of flights over Boston and detected bacteria, molds, yeast and pollen at a height of 5-6 km (Proctor, 1934). Subsequently, the US Department of Agriculture commissioned further studies on the epidemiology of rusts and other plant diseases, leading F.C. Meier and Charles Lindbergh to collect bioaerosols from about 3 km elevation above sea level (ASL). For sample collection, they used an oiled microscope slide extended from the plane by a metal arm. This study collected samples from Maine to Copenhagen (via the Arctic) and tentatively identified microbes belonging to *Macrosporium*, *Cladosporium*, *Leptosphaeria*, *Mycosphaerella*, *Trichothecium*, *Helicosporium*, *Uromyces*, *Camasosporium*, and *Venturia*. They also found diminishing numbers as collections progressed over the sea ice cap of Greenland (Meier and Lindbergh, 1935).

Around the same time, a manned US high-altitude balloon, Explorer II, became the first air sampling mission to reach the stratosphere (up to 21 km ASL), and several viable microbes were isolated within the genera

Bacillus, *Macrosporium*, *Aspergillus*, *Penicillium* and *Rhizopus*, using autoclaved collection tubes (Rogers and Meier, 1936). In 1965, G.A. Soffen flew balloons even higher, up to 40 km ASL, and used an ethylene oxide-sterilized impactor for isolation, but only found *Penicillium* species (Soffen, 1965).

In the 1970s, A. A. Imshenetsky and colleagues collected samples of air from even higher elevations, from the stratosphere to the mesosphere (48-85 km ASL), using γ-radiation sterilized meteorological rockets and investigated the characteristics of the bacterial and fungal strains isolated. They also studied the effects of the various atmospheric stressors, which continues to be one of the most remarkable investigations of microbial isolates from the highest elevations ever reported (Imshenetsky et al., 1976; Imshenetsky et al., 1977; Imshenetsky et al., 1978; Imshenetsky et al., 1979).

These early studies set the stage for more recent endeavors, including collection from the stratosphere by both planes and balloons (DeLeon-Rodriguez et al., 2013; Griffin, 2004; Smith et al., 2010). Balloons continue to be valuable for stratospheric studies as they can remain in place for sampling, exposure and experimentation, and may be flown to higher altitudes carrying larger payloads than most planes. In addition, balloons are advantageous because they can be maneuvered to many different locations and do not require expensive landing strips when returning samples to Earth (Smith et al., 2010; Smith and Sowa, 2017).

The Atmosphere

Many recent atmospheric studies have resulted in significant implications for health, including providing a better understanding of epidemiology, climate patterns and change, and planetary protection. However, the challenges continue to be considerable, with the atmosphere constituting the largest fraction of the biosphere. The mass of the Earth's atmosphere is 5.1×10^{18} kg (or ~1/1,200,000th of the planet), with 50 % of atmospheric mass located above 5.6 km, 10 % above 16 km, and 0.1 % above 100 km (Lutgens and Tarbuck, 1995). The diversity and distribution of atmospheric life are considerably sparse when compared to terrestrial and aquatic environments. Answers to fundamental questions about the nature of life in the atmosphere and its survival are likely to lead to very significant results for both human health and the health of the planet.

Evolution of Earth's atmosphere

Earth's atmosphere has functioned for eons as a protective buffer for life on our planet. After the initial Hadean era, more than 3.4 billion years ago, before the evolution of life, the atmosphere primarily consisted of hydrogen and helium gases. As the atmosphere cooled, it became rich in nitrogen and carbon dioxide gases. During this very early period of the Earth's history, the sun was also dimmer by about 30% compared to the present

day. Around 2.3 billion years ago, the *Great Oxidation Event* resulted in the atmosphere becoming oxygen-rich and evolving into its modern composition over time. Solar radiation catalyzed the conversion of molecular oxygen into ozone in the upper atmosphere, which resulted in heating and temperature inversion, providing the planet with an UV-protective layer in the stratosphere, critical for subsequent development and successful spread of life (Henderson and Salem, 2016; DasSarma and DasSarma, 2018; DasSarma and Schwieterman, 2018).

In the Anthropocene, many environmental factors contribute to further changes in the atmosphere. Jet travel, rocketry, military activities, and industrial and household pollution are all contributing to these changes and the natural barriers that prevent the transport and mixing between the layers of the atmosphere, e.g. troposphere and stratosphere, are being disrupted. Nevertheless, there are still some important questions that remain to be answered: a) Are anthropogenic activities responsible for the eutrophication of clouds and the atmosphere?, b) To what extent are anthropogenic activities contributing to climate change?, c) To what degree is the higher atmosphere, especially the stratosphere, subject to anthropogenic factors?, and d) Is it a habitat/place where life is expanding and serving as conduit for the dispersal of bioaerosols?

Layers of the Atmosphere

The atmosphere is divided into five main layers (from closest to furthest from the Earth's surface) (Figure 1), with this review focusing only on the lowest two (i.e. the ones where life seems to thrive).

Troposphere

The lowest level of the atmosphere, the troposphere, clearly contrasts with the stratosphere in terms of the necessary components for life maintenance: nutrients, in the form of dust particles and chemical compounds, air with all its components, water and water vapor. Like Earth's atmosphere in general, it is composed of 78% nitrogen, 21% oxygen and 1% of trace gases (argon, carbon dioxide, and most of the atmospheric water vapor). Temperature in this layer decreases with increasing height by ~7 ^{o}C/km, ranging from 15 to -60 ^{o}C (Vargin et al., 2015). The troposphere is where most weather events occur and is a characteristically turbulent and well-mixed layer. Jet streams, associated with frontal weather (where two different air masses meet), occur at or below the tropopause.

Most studies carried out thus far on life in the atmosphere have been within the troposphere. These studies provide the foundation for stratospheric analyses, addressing many of the challenges found at the higher altitudes. Tropospheric research has shown not only the presence of microbes, but also their metabolic activity (e.g. Klein et al., 2016). Culturing and metagenomic studies have identified a range of microbes that may be residents or have been transported into the troposphere where they may

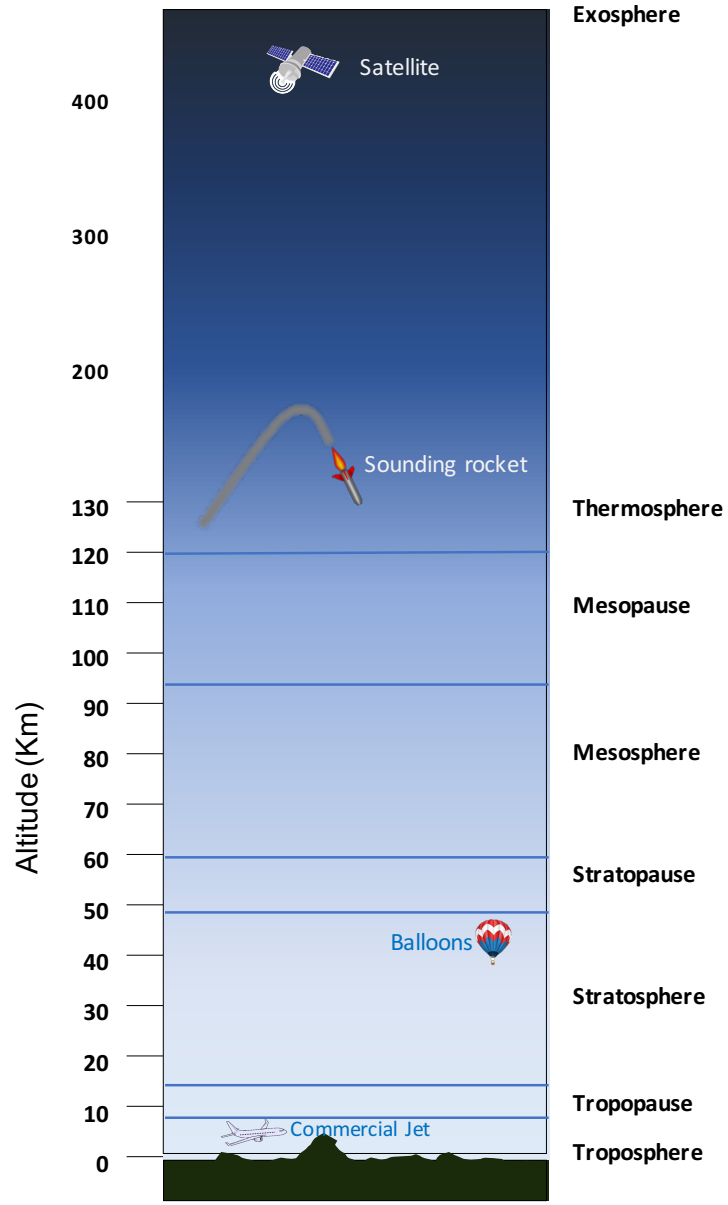

Figure 1. Overview of the different layers of Earth's atmosphere and their characteristics.

Exosphere
The top layer is the exosphere, located above the thermosphere and also considered to be part of space. It extends to at least 10,000 km, where it merges with the Solar wind (a constant stream of plasma and charged particles released from the corona, the Sun's outer layer). The exosphere is where most Earth-orbiting satellites are located.

Thermosphere
The thermosphere is located at heights above the mesopause with temperatures up to 1700 oC. It has a very low density of particles, is cloudless, and lacks water vapor altogether. This is where the International Space Station is located (at 350-420 km). The thermosphere ends with the thermopause, also called the exobase, ~600-1000 km, depending on solar activity.

Mesosphere
The mesosphere occupies heights from the top of the stratopause to 95-120 km, where the temperature decreases with height, at which point the mesopause, known as the coldest place on Earth with average temperature of -85oC is located. This is the layer where meteors burn up, sounding rockets and rocket-powered aircraft travel, and where the highest clouds, noctilucent, or night-glowing, made of ice crystals, and seen only during astronomical twilight exist.

Stratosphere
The stratosphere occupies heights from the top of the troposphere up to 50-60 km. Here, the temperature increases with height. This is the highest region where jet planes fly (~10-13 km). The stratosphere ends at the stratopause (Figure 1).

Troposphere
The troposphere occupies 15-18 km heights in the tropics and 10-12 km at the Polar Regions, with the temperature decreasing with increasing height. At the top of the troposphere, the tropopause separates the troposphere from the stratosphere.

be dormant or dead. The troposphere is considered a microbial ecological niche and ecosystem that requires study with efforts similar to that used in terrestrial and aquatic environments.

Clouds
About 15% of the first 6 km of the atmosphere consists of clouds, and several studies have focused on the importance of nucleation in both cloud and fog formation (Delort et al., 2010). Both homogeneous and heterogeneous particles are found near clouds and close to the tropopause. Ultrafine particles (3-15 nm in diameter) can serve as cloud condensation nuclei, which can grow to 100 nm within a few days (Kulmala et al., 2004).

Culturing, phylogenetics, and metagenomics have been used to extensively analyze cloud matter (Xu et al., 2017). Culture-based studies have identified bacteria (including α-, β- and γ-*Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*), and fungi (Basidiomycetes and Ascomycetes) 1.5 km above the volcanic dome of Puy de Dôme. They

found that less than 1% of bacteria and about 10% of fungi (e.g. *Dioszegia* and *Udeniomyces*) were reported to be culturable (Vaïtilingom et al., 2012). Another study reported that ~17% of known fungal species can be grown in culture (Amato et al., 2017).

Microorganisms are also able to act as cloud condensation nuclei and their presence is conditioned by several factors, including local presence of carbon sources and *in situ* pH. Data from the troposphere includes carboxylic acids and alcohols at concentrations of up to 1 mg/L, and a variety of hydrocarbons at concentrations ≤4 ng/L and pH ranging from 3-7, with acidity resulting from dissolved gases and compounds from aerosols in cloud water. Sulfate and nitrate nutrients in cloud water and rainwater can be relatively high and even reach levels typically found in oligotrophic lakes. The microorganisms previously detected in clouds include: *Micrococcus agilis*, *Mycoplana bullata*, and *Brevundimonas diminuta*, as well as plant pathogens such as *Erwinia carotovora* (Pérez-Díaz et al., 2017). Furthermore, species of the genus *Pseudomonas* detected in clouds have been shown to have nucleation properties, as they produce biosurfactants that facilitate the condensation of water on the surface of their cells. As a result, they are able to induce cloud formation, enhancing precipitation in the form of rain or snow (Amato, 2012).

Studies quantifying ATP concentrations and using differential staining have found that as much as ~1 million tons/year of organic carbon is metabolized by bacteria in the clouds (Vaïtilingom et al., 2013). Among these are the metabolically active oligotrophic, pigmented *Sphingomonas* spp. which have a high resistance to UV, cold and salinity, and can tolerate relatively high concentrations of oxidants. In addition, *Pseudomonas* spp. were found to use a variety of carbon compounds, though they were found to be less resistant to UV and oxidants. These microbes may use the atmosphere and clouds for both residence as well as for transport, until rain or snow brings them back to the Earth's surface (Delort et al., 2010; Xu et al., 2017). In another study, metagenomic analysis of cloud water samples from Mt. Tai (~1.5 km elevation), in China, also identified *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* as predominant bacterial groups. Here, researchers also concluded that ozone and sulfur dioxide (SO_2) contributed to the variability of populations in these environments (Xu et al., 2017).

Dust

While microbes may be expected in the humid environment of clouds, studies into the transport of microbes by dust have led to some unexpected discoveries. Sources of aerosolized particulate matter can originate from e.g. vehicular pollution, construction, and industry as well as wind erosion (Griffin et al., 2018). Erosion sources include the ~10 million acres of farmland annually that are lost due to poor agricultural techniques and weather, dust storms from deserts, overgrazing, and deforestation. Most

wind-borne bacteria may be transported for relatively short distances, <1 km from their source, although some seem to be transported for extremely long distances, reportedly over 5,000 km (Kellogg and Griffin, 2006). Each year, more than $90x10^9$ kg of dust are lifted from the Sahara Desert into the atmosphere, making up the Saharan air layer at about 6 km ASL, and crossing the Atlantic in 5–7 days (Di Liberto, 2018; Griffin et al., 2003). As an additional example, each year, ~8 million metric tons of lake-bed sediment from the dry Lake Owens bed in California are transported into the atmosphere and make up the primary source of atmospheric dust in the continental USA (Griffin et al., 2002).

It may be possible for microorganisms to take a ride in the tropospheric and stratospheric wind currents (Creamean et al., 2013; Barberán et al., 2015). Dust-associated bacterial and fungal spores have been reported to be transported across the Atlantic, from Africa to the Caribbean (Kellogg and Griffin, 2006). It has been calculated that the Sahara and Sahel regions of North Africa account for approximately 50-75% of the annual total atmospheric dust load (Griffin et al., 2018). In one study, halophilic endospores associated with Asian dust, or KOSA (the Japanese term for yellow sand), particles and other bioaerosols were mentioned as being transported across Asia from the Gobi Desert to Japan by dust storms (Echigo et al., 2005). Furthermore, it was found that these KOSA particles can act as enhancers of microbial growth (Maki et al., 2011).

Bacillus and *Microbacterium* were reported as the predominant bacteria isolated in several studies from African dust storms (Kellogg and Griffin, 2006). Metagenomic analyses of Caribbean and African dust storm events found that the microbes identified were the same. For example, the 18S sequences of *Cladosporium* isolates from the Caribbean samples were 99–100% identical to an isolate from African dust, indicating that these microorganisms may have been transported through the atmosphere (Kellogg and Griffin, 2006).

Results of such studies vary depending on numerous factors, including sampling methods, analysis and likely variability in the atmosphere at any given location and weather condition. For example, in two studies from Taiwan, dust from various time points was collected, including storms that transport dust from China and Mongolia to Taiwan, and spores were morphologically identified using microscopy-based analysis. In one study, ascospores and spores from *Cladosporium*, *Penicillium*, and *Aspergillus*, were found to be dominant in dust; though, only *Cladosporium* spore levels increased during dust storms. In addition, *Ganoderma*, *Arthrinium*, *Papularia*, *Cercospora*, *Periconia*, *Alternaria, and Botrytis* spores were also identified (Ho et al., 2005). In another study, the dominant spores of *Penicillium*, *Aspergillus*, *Nigrospora*, *Arthrinium*, *Curvularia*, *Stemphylium*, *Cercospora*, and *Pithomyces*, were found 15 m above ground level in calm conditions; and, only *Penicillium*, *Aspergillus*, *Nigrospora*, and some

Figure 2. View from the window of a commercial airliner at 12 km altitude. Below are tropospheric clouds, above, the clear zone of the stratosphere.

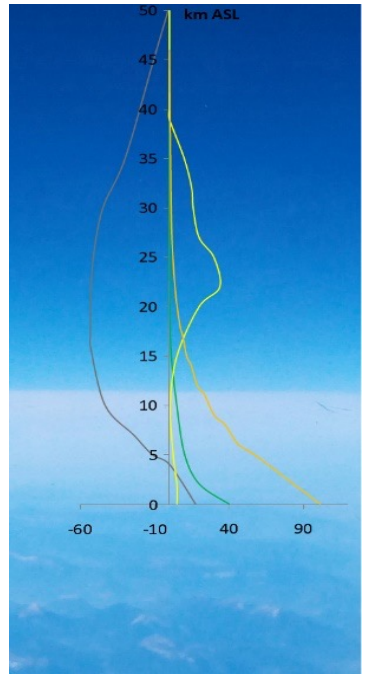

Figure 3. Selected characteristics of the troposphere and stratosphere. Average temperature (^{o}C), gray; average water vapor (g/m^3), green; barometric pressure (kPa), orange; and ozone (mPa), yellow on x-axis, plotted against km above sea level (ASL) on y-axis.

unidentified spores were found to have increased concentrations during dust storm events (Wu et al., 2004).

The numbers of culturable airborne microorganisms were found to increase 2 to 3-fold during African dust-events. Direct microbial counts of air samples using epifluorescent microscopy, determined that bacteria- and virus-like particle counts were ~10-fold greater during these events than during clear conditions. Also, autofluorescence was exhibited by bacteria-like particles during an African dust-event, further supporting the presence of microorganisms (Griffin et al., 2001). Also, epifluorescent microscopy of nucleic acid stained filters of material from dust events showed that the bacterial and viral counts were the same, in contrast to results from soil and marine environments, where the viral counts were an order of magnitude higher. This suggests that viral particles are more susceptible to the high UV radiation and dry air associated with long-distance transport in dust events (Kellogg and Griffin, 2006).

At the top of the troposphere, the tropopause acts as a barrier to particulate matter, with peak heights over the equator and minimum heights over Polar Regions. Temperature inversion occurs in the tropopause, from decreasing with increasing height in the troposphere, to increasing with increasing height in the stratosphere (negative lapse rate) (Mohanakumar, 2008; Gettelman et al., 2011). The temperature in the tropopause is isothermal, and effectively stops the transfer of most aerosols from the troposphere into the stratosphere. It is also the region where the atmosphere becomes exceedingly dry (Gettelman et al., 2011).

Stratosphere

There is some variability in the height of different layers in the atmosphere, depending on factors such as e.g. latitude, season, atmospheric conditions and solar activity. The stratosphere (Figure 2), located above the troposphere and tropopause, can start as low as ~7 km near the poles and as high as 20 km at the equator and extends up to approximately 50-60 km ASL. Conditions here are among the most extreme on Earth and comparable to conditions found on the surface of Mars, making it valuable as a Mars analog (Figure 3). This Martian analogue status is supported by data from several studies. Indeed, the stratosphere has low nutrient availability, reduced atmospheric pressure (between 0.1 to 10 kPa), low temperatures (around -50 °C), presence of toxic chemical species, and intense solar radiation and is extremely dry (relative humidity ~23%) (Smith et al., 2011; Smith et al., 2013).

There is some drastic variation in conditions within the stratosphere. Near the top of this layer, the pressure is extremely hypobaric (~0.1% of that at sea level). Furthermore, its high concentrations of ozone and ozone layer restrict weather-producing turbulence and mixing. Temperature increases from -70^{o}C at the tropopause, to 0^{o}C at the top of the stratosphere due to

Table 1. Overview of microbes isolated from and/or tested in the stratosphere*.				
Isolate name	**Domain**	**Height (km ASL)**	**GC-composition***	**Spore former?**
Actinobacteria	Bacteria	18-29[I]	Variable	Some
Actinomyces sp.	Bacteria	19[T]	High	May form endospores
Aspergillus fumigatus, Aspergillus niger	Eukarya	11-21[I], 48-77[I], 19-22[T]	~ 50%	Yes
Bacillus endophyticus, Bacillus luciferensis, Bacillus pumilus SAFR-032, *Bacillus simplex, Bacillus* (*Lysinibacillus*) *sphaericus*, *Bacillus subtilis*	Bacteria	20-77[I], 20-30[T]	Low	Endospore forming
Brachysporium sp.	Eukarya	22[T]	ND	Spore-forming
Brevibacterium luteolum	Bacteria	20[I]	High	No
Circinella muscae	Eukarya	48-77[I]	ND	Spore-forming
Cladosporium sp.	Eukarya	22[T]	~ 50%	Spore-forming
Deinococcus aetherius, *Deinococcus aerius TR0125*	Bacteria	10-12[I,T]	High	No
Diplodia sp.	Eukarya	22[T]	High	ND
Engyodontium albus	Eukarya	41[I]	ND	Spore-forming
Escherichia coli	Bacteria	40[T]	~ 50%	No
Eurotiomycetes sp.	Eukarya	20[I]	ND	ND
Exophiala sp. 15LV1	Eukarya	25-30[T]	ND	ND
Fusarium sp.	Eukarya	19[T]	~ 50%	ND
Halobacterium species NRC-1	Archaea	36[T]	High	No
Halorubrum lacusprofundi	Archaea	36[T]	High	No
Helminthosporium sativum	Eukarya	22[T]	ND	ND
Hysterium sp.	Eukarya	22[T]	~ 50%	ND
Macrosporium sp.	Eukarya	11-21[I], 19[T]	ND	ND
Micrococcus albus	Bacteria	48-77[I]	High	No
Monilia sitophila	Eukarya	19[T]	ND	ND
Mycobacterium luteum	Bacteria	48-77[I]	ND	May form spores
Naganishia (*Cryptococcus*) *friedmannii* 16LV2	Eukarya	25-30[T]	ND	ND
Paenibacillus sp.	Bacteria	12-35[I]	~ 50%	Spore-forming
Papulaspora anomala	Eukarya	48-77[I]	ND	ND
Penicillium cyclopium, *Penicillium chrysogenum* (formerly *notatum*), *Penicillium* sp.	Eukarya	11-77[I],19[T]	~50%	
Pestalozzia sp.	Eukarya	19[T]	ND	ND
Proteobacteria	Bacteria	18-29[I]	ND	ND
Proteus mirabilis	Bacteria	40[T]	Low	No
Pseudomonas aeruginosa	Bacteria	40[T]	High	No
Puccinia graminis	Eukarya	19[T]	Low	ND
Rhizopus sp.	Eukarya	11-21[I], 19[T], 22[T]	Low	ND
Salmonella enterica Serovar Typhimurium	Bacteria	40[T]	~50%	No
Staphylococcus pasteuri, *Staphylococcus aureus MRSA*, *Staphylococcus aureus*	Bacteria	41[I], 40[T]	Low	No

ozone absorbing UV rays and consequently releasing heat (Smith et al., 2011).

The stratosphere is almost devoid of clouds, with the exception of the tall cumulonimbus clouds, also called thunderheads, observed during storms, which can penetrate from the troposphere through the tropopause into the stratosphere. In the coldest polar regions, nacreous clouds may also be observed (Henderson and Salem, 2016). Cloud formation can sometimes occur at the tip of the boundary layer under extremely polluted conditions or during the winter in polar regions at temperatures below -78°C and form polar stratospheric clouds at 15-25 km (Ursem, 2016).

Chemical components of the stratosphere include: water vapor, methanol, nitrogen oxides and bromide, as well as a background aerosol layer consisting mainly of binary sulfuric acid-water aerosol droplets (Vargin et al., 2015; Smith and Sowa, 2017). Molecular hydrogen (H_2) is an atmospheric trace gas and acts as a source of water vapor in the stratosphere (Meredith et al., 2017). Most of the known aerosol nanoparticles in the stratosphere are found at 17-50 km ASL, and result from large volcanic eruptions and major meteorite impacts. They are known to have global effects for months or years and have been directly related to the Earth's climate alterations (Ursem, 2016).

For nearly 100 years, humans have been able to enter the stratosphere. In 2014, the Air Transport Action Group estimated the number of scheduled flights to be close to 100,000 per day. Adding to this number, other human sources of stratospheric breaches include orbital launches (which in 2017 consisted of 29 launches from the United States, 20 from Russia and 18 from China; Leary, 2018), and launches of weapons and other military material. All of these can contribute to the dispersal of hitchhiking microorganisms and organic elements into the upper troposphere, lower stratosphere, or higher, and is termed "artificial panspermia" (Lederberg, 1960).

In addition to potential microbial "hitchhikers" on these vehicles, there may also be microbial matter that made its way there via natural means. Natural sources include volcanic activity, storms, wind, fires, and clouds. Whether any of these microbes are metabolically active or even reproduce in the stratosphere is yet to be determined. To date, bacterial and fungal spores dominate the isolates found (Table 1). In addition, experiments that involve launches of microbes into the stratosphere have shown that a trip into the stratosphere is indeed non-fatal to metabolically inactive spores, lyophilized cultures of bacteria and fungi, and active cultures of halophilic archaea (Table 1) (DasSarma and DasSarma, 2018).

Extreme conditions in the Stratosphere

There are a multitude of extreme conditions that make it difficult for most microbes to survive stratospheric conditions, including: irradiation, desiccation, freeze-thaw cycles, low pressure, and lack of water and nutrients. Some of these stressors can also be observed on the Earth's surface, but there is a complex combination of factors in the stratosphere that more closely mimics conditions on Mars than alternative analogue surface sites. This makes the study of the stratosphere of great interest to astrobiologists, who use this natural environment as a laboratory to study both a) the effects of Martian-like conditions (as proxy), e.g.: by testing the viability of organisms, and b) native microbial communities and inhabitants found in these environments. Research into microbial viability in simulations has shown that sun-illuminated bioaerosols are quickly inactivated in the laboratory through application of these stressors (Griffin et al., 2018).

Among the deadliest stressors in the stratosphere are the cosmic rays that constantly bombard our planet. Most of these are either deflected by the Earth's magnetic field or interact with air molecules. Cosmic rays include galactic cosmic rays (from outside the solar system), anomalous cosmic rays (from interstellar space at the edge of the heliopause), solar energetic particles from solar flares and other energetic solar events, and other types of cosmic rays, which include X-rays, γ-rays, and the short wave ultra-violet (UV) portion of the electromagnetic spectrum (Christian, 2012). Cosmic rays are primary sources of ionizing radiation (IR) and carry enough energy to liberate electrons from atoms or molecules (UNSCEAR, 2008).

Damage from IR includes radiolysis of water that generates reactive oxygen species [ROS; e.g.: hydroxyl radicals ($HO^{\bullet}$), superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2)], which cause damage to nucleic acids, generating oxidized DNA bases and sugar moieties, abasic (apurinic or/and apyrimidinic) sites, and single-stranded breaks (SSBs) (Hutchinson, 1985; Imlay, 2006). With increasing doses of IR, the linear density of DNA base damage and SSBs increases on both strands and gives rise to double stranded breaks (DSBs). ROS damages proteins by introducing carbonyl residues, amino acid radical chain reactions, cross-linking, and ultimately results in protein inactivation and denaturation (Stadtman and Levine, 2003). O_2^{-} does not easily cross membranes, and is not metabolized by the cell or react with DNA or most proteins; but it damages and inactivates enzymes with exposed 2Fe–2S or 4Fe–4S clusters causing release of Fe^{2+}, which in turn reacts with H_2O_2 and catalyzes Fenton reactions (oxidation of organic substrates) (Imlay, 2006). IR can also cause 40 times more SSBs than DSBs when macromolecules absorb X-ray and γ-ray photons, (von Sonntag, 1987; Daly et al., 1994).

Of all the stressors present in the stratosphere, short-wave UV radiation causes the greatest damage to cells. It causes an increase in ROS

production, resulting in damage of biomolecules such as proteins, lipids, DNA and RNA. Stratospheric ozone reduces penetration of wavelengths <320 nm and completely excludes those <290 nm. Thus, UV-A (315-400 nm) which constitutes 95% of total energy of UV spectrum that reaches the Earth's surface does not damage organic material. The remaining 5% is UV-B (280-315 nm), which has the greatest biological impact on Earth. Some studies have predicted that aerosols are crucial factors involved in blocking UV-B radiation (Bais et al., 2018). UV-C (100-280 nm) is completely blocked by Earth's ozone layer in the stratosphere. However, above the ozone layer in the stratosphere, any bioaerosols present would experience high fluxes of UV-C radiation.

The destructive power of UV radiation was first determined to be an order of magnitude higher in air than in liquids (Wells and Fair, 1935). Whisler found that, effectiveness of UV to kill was determined not only by the source of radiation, but also by the type of organisms irradiated, with some air-borne microorganisms like *Micrococcus luteus, Staphylococcus aureus, and Bacillus subtilis* found to be considerably more tolerant of UV than *Escherichia coli*. *M. luteus* was found to be 100 times more resistant than *E. coli*, potentially due to clustering of their cells and shielding effects. *S. aureus* and sporulating cultures of *B. subtilis* were 3 and 8 times more resistant than *E. coli*, respectively. This lethal effect of UV radiation was also remarkably dependent on the relative humidity of the air, with cells being an order of magnitude more sensitive in dry air when compared to humid air (Whisler, 1940).

As conditions in the stratosphere also include low temperatures, low pressures and desiccating conditions, microorganisms in this environment would need to have cold- and freeze-adaptations, be able to tolerate hypobaric conditions and be xerotolerant (Rothchild and Mancinelli, 2001; Fletcher et al., 2014). Due to the sparseness of material in the stratosphere, it would be difficult for any living cells to find the building blocks of life needed to metabolize and reproduce, though attachment to particulate matter and the potential for a buildup of such materials, from natural or anthropogenic sources, may provide some resources.

Entry and return of material

Some of the natural uplift mechanisms, capable of driving vertical transport of aerosols into the atmosphere, include convective overshooting and strong up-drafting forces generated by processes associated with thunderstorms, typhoons, monsoons, hurricanes, and cyclones. Large-scale storms, especially hurricanes and cyclones, may reach into the lower stratosphere as they cross oceans. Blue jets, which are optical flashes above thunderclouds, propagate upwards from thunderclouds to ~70 km. Stratosphere-troposphere exchange occurs by deep convection in the tropics, tropopause folding, convective overshooting, as well as by meteors (Wilson et al., 1978; Pasko et al., 2002; Smith and Sowa, 2017; Berera,

2017, Griffin et al., 2018). Volcanic eruptions can send ash and other material 2-45 km and possibly higher (55 km) into the atmosphere, with a dust load of 4-25x10^6 tons in a single eruption (Griffin et al., 2018). Particle loads, from large eruptions, are known to force climate change through the resulting changes in planetary solar irradiance and contribute to bioaerosols being swept into the stratosphere (Rohatschek, 1984).

Aerosol particles' lifetime (such as those from volcanic eruptions) in the stratosphere, has been calculated to be 1 to 2 years and results in reduction of solar radiation to the Earth's surface and, in turn, reduction of surface temperature. This is unlike the troposphere, where particles are rapidly removed via precipitation (Vargin et al., 2015). Other calculations indicate that bacteria remain aloft for 2-10 days, and can travel over thousands of kilometers, while viruses are believed to be associated with particles in the nano- to micrometer range, with a predicted 2-188 days residency time (Cuthbertson and Pearce, 2017; Amato et al., 2017). Eventually, in months to years, depending on the aerosol size, stratospheric aerosols return to the surface by Brewer Dobson circulation which is believed to move air masses towards the poles (Smith and Sowa, 2017).

Micron scale particles can be elevated to altitudes of 80 km, due to irradiation of particles by sunlight through gravitophotophoretic effects and electrostatic levitation. In photophoresis, small particles, suspended in liquid or gas, start to migrate when illuminated by a sufficiently intense beam of light because of non-uniform temperature distribution. This levitation occurs only with negative photophoresis, and it has been predicted that pointing towards the sun would create a lifting component exceeding the gravitational force. Furthermore, it was also concluded that some particles in the stratosphere may rise against the force of gravity (Orr Jr and Keng, 1964; Rohatschek, 1996). Experimental evidence indicated that gravitophotophoresis in sunlight causes the ascent or the suspension in the middle atmosphere of carbonaceous, mineral, and metallic particles, mainly in the 1-10 μm size range, which otherwise would fall with considerable velocity (Rohatschek, 1984). Particles in the 1-100 μm size range were shown to levitate due to photophoretic forces in a laboratory simulation of solar and middle atmospheric air densities (Rohatschek, 1984). The studies of gravitophotophoresis suggested it may be the reason why microorganisms are found in the stratosphere and mesosphere (Imshenetsky et al., 1977). They also indicated that radiation absorbed by pigments produces photophoretic forces sufficient to overcome gravity (Rohatschek, 1984).

Bioaerosols

Materials of organic origin in the atmosphere are called bioaerosols, and can include microbes, spores and pollen. Some of this material is free-floating, while other can be found attached, trapped in, or on larger

particles or aggregates (Smith et al., 2012; Smith et al., 2013). Primary biological aerosols represent 5-10% of the total number of atmospheric particles >0.2 μm in diameter (Amato et al., 2017). It has been predicted that 40-1800 Gg of organic matter is aerosolized annually (Cuthbertson and Pearce, 2017). Bioaerosols may consist of raindrop-bubbles from soil which has been calculated to aerosolize ~0.01% of soil surface bacteria (Joung et al., 2017). In this phenomenon, raindrops impacting the soil, form tiny bubbles and rupture at the raindrop/air interface, and cause emission of tiny water jets that are subsequently broken into aerosols containing soil-associated bacteria that are released into the air column (Joung et al., 2017; Jang et al., 2018). Microbes and viruses can also be transferred from the ocean surface to the marine aerosol (Rastelli et al., 2017). Some bioaerosol microbes in the troposphere are believed to be metabolically active and capable of reproduction, with a calculated doubling time of 3.6-19.5 days (Cuthbertson and Pearce, 2017). The metabolic processes identified include nitrogen processing, sulfur oxidation and reduction, and photosynthesis (Cuthbertson and Pearce, 2017).

A survey of virus-like particles in the Sierra Nevada Mountains, Spain, above the atmospheric boundary layer (1.7 ± 0.5 km ASL), determined that the flux of viruses ranges from 0.26 to >7 × $10^9/m^2$ per day. These deposition rates were considerably greater than the rates for bacteria, which ranged from 0.3 to >8 × $10^7/m^2$ per day. The highest relative deposition rates for viruses were associated with atmospheric transport from marine rather than terrestrial sources. Virus deposition rates were positively correlated with organic aerosols <0.7 μm, whereas, bacteria were primarily associated with organic aerosols >0.7 μm, implying that viruses could have longer residence times in the atmosphere and, consequently, may be dispersed further (Reche et al., 2018). A similar survey of stratospheric material would be useful to expand our understanding of this phenomenon.

Studies of aerosolized material in the atmosphere have been undertaken in recent years using metagenomic techniques. In a study of aerosol material before and after heavy rains in Korea, researchers used pyrosequencing to identify *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, and lesser quantities of *Planctomycetes*, *Chloroflexi*, *Gemmatimonadetes*, and *Cyanobacteria* in post-rain samples (Jang et al., 2018). Surprisingly, no archaeal sequences were reported; and, levels of the marine bacterial sequences (*α-Proteobacteria*, *Actinobacteria*, and *Firmicutes*) decreased after rainfalls. Sequences corresponding to the non-spore forming *Actinobacteria* and the phytopathogens *Clavibacter michiganensis*, *Staphylococcus saprophyticus,* and the human pathogen, *Propioni-bacterium acnes*, were often observed in post-rain air samples, whereas the *Firmicutes* decreased in abundance. The *Firmicutes* were more abundant in coarse particles than in fine particles and the study considered that precipitation may selectively remove particle-associated bacterial

groups, causing changes in airborne bacterial community composition after rainfall, despite increases in overall bioaerosol concentration.

Collection and analysis of material for microbiological studies

Over 10^{21} cells/year are estimated to be lofted into the atmosphere, with only a small fraction (<0.1%) believed to survive (Aguilera et al., 2018). How many of these actually exist in the stratosphere is still to be determined. Thus far, only a limited number of studies have been performed to address this question, and they include direct isolation from the stratosphere and simulations in the laboratory (DasSarma and DasSarma, 2018).

Analysis of collected material is conducted in two main ways: a) culturing and characterization, and b) DNA extraction and identification (Cuthbertson and Pearce, 2017; Griffin et al., 2018). Culturing results are dependent on both the collection method and media used, while DNA extraction will vary based on the methods used, which may selectively be easier for some microbes and more difficult for others. Polymerase chain reaction (PCR) and sequencing methods may also introduce biases based for example on the primer sequences used. Given the limited number of studies thus far, no preferred methods for stratospheric collection studies have arisen.

One of the earliest methods used, the plate-fall/drop-plate technique, involves leaving agar plates open to allow material to drop into it. This method is hard to implement when material is scarce, or gravity is less effective. It is probably not feasible for the stratosphere, since centrifugation would be a necessary step difficult to perform at this height, but has been used widely for work done in the past for lower levels of the atmosphere, as early as 1894, when the air of New York was sampled (Dyar, 1894). It limits the findings to only those microorganisms which are viable and can grow in the media used.

Impaction has also been used since early investigations (Meier and Lindbergh, 1935; Solomon et al., 1983). In this method, material is collected by collision of aerosols onto Petri dishes or other surfaces, which may be coated with sticky material (e.g. glycerol). For example, the Life's Atmospheric Microbial Boundary (LAMB) balloon payload collected ~100 cells per glycerol-coated rod, at ~38 km, in a passive sampling mission (Bryan et al., 2014; Griffin et al., 2018). The NASA Cosmic Dust Group were able to collect viable bacteria and fungi, on an impactor plate, in a housing located on the underside of a Lockheed Martin plane that was flown at 20 km ASL for 2.5 hours from New Mexico to California, USA (Griffin et al., 2004). The method allows for both culturing, as long as the media is suitable, and DNA analysis.

Filtration has been a more common isolation method performed on air samples and lower atmospheric materials. For example, in a study of

tropospheric dust from dust storms, volumes of 139.5 and 269.7 liters were vacuumed during specific times (15 and 29 minutes, respectively) through pre-sterilized nylon filters with 0.2 μm pore size, and a portion of each filter was placed on agar plates for culturing. Using 0.02 μm pore size filters, air was also vacuumed for 10 to 15 minutes at a rate of 14.65 to 29.98 L/minute, for direct counting of average numbers of microbe-like particles using SYBR Gold staining (Griffin et al., 2001). Some problems with this method include possible damage of cellular material during the vacuum stage, and loss of cell viability from impaction with filter materials or media that does not support growth. This method is, at present, hard to perform at the stratospheric altitudes, though collected stratospheric material can be processed over filters. The method allows for both culturing as well as DNA analysis. Radosevich and colleagues (2002) collected air in Utah for which they developed an apparatus based on membrane filtration. From the particles collected, and through bacterial genomic DNA analysis, they were able to find a wide variety of species, many belonging to the phylum Proteobacteria. In other studies, sterile quartz and glass fiber filters were used to collect aerosols from the troposphere over the period of one year and identified archaeal DNA from *Thaumarchaeota* or *Euryarchaeota* with seasonal variation (Fröhlich-Nowoisky et al., 2014). Fungal spores had already been determined as being a large part of air particulate matter, and it had already been described a rate higher than initially expected, with a species richness of 368. These studies concluded that airborne diversity was closely linked to soil diversity, and that soil and soil dust might be the primary source of airborne microorganisms (Fröhlich-Nowoisky et al., 2009; Fröhlich-Nowoisky, 2014).

Cryosampling has also been used in the modern era with cooling of samples during the collection process. This process is expected to increase cell viability and facilitate both culturing and DNA extraction. In one study, sampling of air above Hyderabad, India, employed a cryosampler made of a 16-probe stainless steel manifold with remote-controlled motorized valves (Wainwright et al., 2003). The payload had a 2-meter-long intake tube which was tethered by a rope, both previously sterilized, behind the balloon gondola, in order to avoid the collection of any materials from the balloon surface. Throughout the ascent trip (up to 41 km), in order to create a cryopump effect, sequence probes were immersed in liquid neon allowing surrounding air to be collected when the valves were opened. Once all the collections were made, the cryosampler manifold was parachuted back to the ground. Collection of 38.4 and 18.5 liters of air at normal temperature and pressure, was accomplished at 30-39 and 40-41 km elevations, respectively. The aerosols were aseptically extracted by sequentially filtering the air through 0.45 and 0.22 μm micropore cellulose nitrate filters, and then injecting the probes with sterile phosphate buffer solution and agitating in order to release particles adhered to the walls. The subsequent liquid was then sequentially filtered through 0.7 μm glass, 0.45 μm and 0.2 μm cellulose acetate filters before analysis. Several different

shaped bacteria were found to be present and one fungal species was isolated (Wainwright et al., 2003).

Another recent method, impingement, involves air collection by suction and then impinged/forced into liquid solutions such as water, buffer or oils. This method allows for the retrieval of the collection media for both microbial culturing (of selected microbes) as well as DNA extraction and subsequent analysis. However, this method is selective for only the cells that can survive in the collection medium. Many studies of the troposphere have used this method since the collection step allows for rapid sampling of large volumes and potential collection of both viable and nonviable material. Though more expensive than membrane filtration, high-flow rate impingers have the advantage of speed. When comparing different air sampling methods, impingers were considered as not appropriate or efficient for the cultivation of certain microorganisms (Cooper et al., 2019). Nevertheless, a study of atmospheric dust collection showed that impingers allow for microbial recovery rates 20x higher than membrane filtration (Griffin et al., 2011). In order to verify the absence of contaminations, impingers – like other air sampling equipment – can initially be analyzed in a sterile test chamber (Cooper et al., 2019).

The more complex cyclonic methods are designed to separate airborne dust into fractions. Air is drawn into a cylindrical chamber by rotation of the air flow, without the use of filters. As a result, aerosols of given sizes move toward the walls by centrifugal force, where they can be rapidly collected (Huard et al., 2010). The force of the collection process may result in some cellular damage and the composition of the walls may further impact microbial survival. This collection method is therefore more suited for DNA sequence analysis of samples than culturing. Furthermore, different designs will have different efficiency values (e.g.: Saunders et al., 2003; Chen et al., 2012).

Sterilization and aseptic techniques are obviously key to any microbiological work, as well as in the field of high-throughput sequencing (Spring et al., 2018). Many methods have been used to clean and sterilize collection vessels and materials, such as use of alcohol wipes, UV-, and ionizing-radiation. For example, in a cryosampling mission to the stratosphere, probes were flushed with acetone and heat sterilized to temperatures of 180 °C for several hours (Wainwright et al., 2003). More recently, enhanced techniques including the use of sodium hypochlorite for sterilization of refined sampling devises and other culturing and non-culturing methods have also been described (Bryan et al., 2014; 2019).

Some studies of the troposphere have involved using multiple approaches, e.g. both impingement and membrane-filter methods to collect material. For example, Jang et al. (2018) utilized such an approach before and after rainfalls in South Korea, albeit at the low altitude of just 16 m. Combination

approaches may have the advantage of being more effective than using only a single method to gain broader insight. Nevertheless, collection and culturing of samples from high altitudes remain challenging and further studies are needed to establish standard protocols in high altitude microbiology.

Culturing has been used to identify microbes collected from the stratosphere. Some additional work has been done in comparing phenotypic traits of microbes collected from or exposed to the stratosphere (Table 1). For example, in the early 1930s, several microorganisms were sent up to the stratosphere, on the Explorer II balloon, in order to test the effect of drying, extreme cold, ozone, strong light rays, and low air pressure in the stratosphere on them. These microorganisms were isolated at various times, from the stratosphere (*Brachysporium* sp., *Hysterium* sp., *Rhizopus* sp. from 1.2 km; *Diplodia* sp., from 1.3 km; and, *Aspergillus niger*, and *Cladosporium* sp., from ~3 km), as well as from diseased sorghum plants (*Helminthosporium sativum*). When they returned to Earth, after a flight that lasted ca. 8 hours (Kennedy, 2018), most were able to grow to varying degrees, but one, *Hysterium* sp., did not (Meier, 1936). This early study illustrated differential effects of the stratosphere on different organisms.

As mentioned before, the equipment used for collection of aerosols may select for and against certain organisms as will culture media and cultivation conditions. Once samples have been collected, they can be inoculated into various media, which so far have mainly been restricted to common bacteriological growth conditions, rather than a wide range needed for culturing diverse microbes, especially those with specific or unusual requirements, such as anaerobic conditions or high salinity. For example, cryosampling of the stratosphere above Hyderabad (India), resulted in the isolation of four new species from the genus *Bacillus*: *B. aerius* sp. nov., *B. aerophilus* sp. nov., *B. stratosphericus* sp. nov., and *B. altitudinis* sp. nov. (Shivaji et al., 2006). In many studies, such as this one, the selection of microbes cultivated was dependent on the method of collection (cryotubes were flushed with buffer that was then spread on culture media) in this case and the medium used (e.g. Luria-Bertani agar or Nutrient agar), and effectively allowed for the recovery and cultivation only of specific microbes.

A common method for analysis of recovered samples is the use of light, phase, electron and epifluorescence microscopy. In addition to SYBR gold, another analysis used to determine cell viability of material collected, involves using 0.45 µm micro-pore filters, treated with either a fluorescent cationic carbocyanine or an anionic oxonol dye, both membrane potential-sensitive (or voltage sensitive) probes (Harris et al., 2002). While determining the viability of cells using dyes may sometimes lead to ambiguous results, generally cationic dyes will penetrate the cell

membranes of viable, but not of dead cells, while anionic dyes will penetrate the membranes of only non-viable cells (Johnson et al., 2013). Viable cells can usually be visualized using epifluorescence microscopy. Harris and colleagues (2002), discovered clumps of cocci-shaped sub-micron-sized particles on filters recovered from an earlier stratospheric probe. They were identified as prokaryotic microorganisms, using both scanning electron and epifluorescence microscopy. The epifluorescence microscopy used a membrane potential-sensitive dye (carbocyanine), and fluorescence was suggestive of the presence of viable cells (Wainwright et al., 2003; DasSarma and DasSarma, 2018).

Sequencing analyses of the 16S rRNA gene have been largely used in microbial identification. These allow to simultaneously estimate gene abundances and diversity between samples (Smets et al., 2016). In isolates from the stratosphere, *Bacillus luciferensis*, collected at 10 km ASL, was 99% similar to a volcanic soil *B. luciferensis* isolate, suggesting that the source of the stratospheric isolate might have been from volcanic eruption (Griffin, 2004; Griffin et al., 2018). In another large-scale study of aerosols from 20 km ASL, in which samples were collected on an impactor Petri dish, four fungal isolates were observed, all of which belonged to the genus *Penicillium* (Soffen, 1965). In another study, a cryosampler elevated up to 41 km ASL was used to collect air samples, and viable, but initially non-cultivable, microbes were found. In subsequent work, two Gram-positive bacteria, *Bacillus simplex* and *Staphylococcus pasteuri*, and one fungus, *Engyodontium album*, were cultured and identified by: 16S rRNA sequencing – for bacteria, and morphological traits – for fungi (Wainwright et al., 2003). Griffin (2008), used a sterile impacter device with a layer of glycerol to collect air, from 20 km ASL, during a flight of 3.6 hours. Several bacteria were isolated after a long incubation period of 7 weeks. And, interestingly, most of the isolated *Micrococcaceae* and several of the Microbacteriaceae strains were similar to strains previously identified in volcanic soils.

Recently, atmospheric projects have included metagenomic analysis on bioaerosols, but they have been restricted to the troposphere. Metagenomic analysis may not strictly prove the existence of living organisms, since isolated DNA may be associated with dormant or nonviable cell types. However, it is consistent with their presence in the environment and results in a catalog of operational taxonomic units (OTUs) found in a particular sampling of the atmosphere. Moreover, metagenomic analysis can be used on material from nearly all methods of collection and provides considerable depth of data for analysis. For example, filtration may allow for DNA extraction directly from the filter without the need for isolation or culturing. However, those that require growth of microbes, such as the plate-drop method, only provide the opportunity for genomic analysis of cultivatable isolates, but not whole community analysis.

Effect of stratospheric stressors on microbes

Several studies have been performed to determine the effects of stratospheric stressors on isolates from the stratosphere as well as terrestrial ones. *Bacillus* spores could be shielded from damaging UV radiation either by neighboring spores, or by microniches in the platforms they were placed on (mainly metal coupons) or dust particles (Khodadad et al., 2017). Resistance to stratospheric conditions was also studied by exposing two *B. subtilis* strains, one isolated from 20 km ASL over the Pacific Ocean, and the other from a desert basal outcrop in Arizona (USA). For these two strains, no differences in survival were observed after exposure (Smith et al., 2011). Monolayers of *B. pumillus* spores (from a spacecraft clean room) were tested for the effects of UV exposure in the stratosphere. The exposure occurred sequentially from 2 to 8 hours, at ~31 km ASL, with half the spores exposed to sunlight and the other half shielded from it. Spores with increasing sunlight exposure were more affected, presenting an increase of inactivation directly related to the exposure time. After 8 hours of exposure with direct sunlight, viability decreased more than 99.9%, suggesting that UV was the determining factor. Multi-layers of spores resulted in greater survival, due to shielding, and this finding was later confirmed in the laboratory (Khodadad et al., 2017).

Other research has involved exposing desiccated or lyophilized strains, including several potential pathogens to the stratosphere on balloons. Though many survived, changes in protein expression and metabolic pathways were observed (Chudobova et al., 2015). In a study on the impact of sending microbes in liquid media into the stratosphere, two halophilic archaea, the mesophilic *Halobacterium* sp. NRC-1 and the biofilm-forming psychrotolerant Antarctic *Halorubrum lacusprofundi,* both survived launches into the stratosphere on weather balloons. Laboratory experiments showed that the mesophile, was more UV resistant than the psychrophile, while the psychrotolerant strain was more resistant to low temperatures (Anderson et al., 2016; DasSarma et al., 2017). Interestingly, in this study, the psychrotolerant strain, *H. lacusprofundi*, was found to have a 10-fold better survival when compared to the UV tolerant strain NRC-1 overall. Moreover, other investigations have addressed resistance of halophilic archaea to UV-radiation (including the use of both light and dark repair systems) (Crowley et al., 2006; Boubriak et al., 2008), and the high degree of resistance found has been associated to the presence of multiple copies of the genome within each cell, and the high intracellular concentration of halide ions that act as chemical chaperones. The intracellular salts are able to scavenge ROS, protecting the cells against radiation damage (Oren, 2014). Additional studies of genes involved in IR protection, including extensive molecular biological examinations via knockout and overexpression, have shown the importance of ssDNA binding for survival (DeVeaux et al., 2007; Karan et al., 2014).

A recent study of the effect of multiple stratospheric stressors used two balloon flights, up to ~25-30 km, to expose yeast desiccated onto polytetrafluoroethylene strips and *B. subtilis* spores as a control. One yeast strain, *Naganishia* (*Cryptococcus*) *friedmannii* 16LV2, found in volcanic soils, was shown to grow through freeze-thaw conditions down to -6.5°C, and belongs to a clade previously isolated from the troposphere. Another, *Exophiala* sp. 15LV1, was previously shown to survive UV-B and C radiation. These two strains survived stratospheric exposure better than *B. subtilis* spores, although ~90% of the viable cells were inactivated. A third yeast strain, *Holtermanniella watticus* 16LV1, isolated from the high-altitude volcanic area of the Atacama Desert, lost most of its viability due to desiccation. When desiccated, *H. watticus* was further weakened by low pressure and temperature and did not survive the stratospheric UV exposure. Additionally, an environmental simulation chamber was used to evaluate effects of desiccation combined with other stressors. Desiccation plus exposure to stratospheric low pressure and temperature had a greater impact on the yeasts than the spores (Pulschen et al., 2018).

Additional microbial related parameters analyzed in the laboratory

Radiation

UV, desiccation and cold conditions exposure in the stratosphere can result in DNA damage including single- and double-stranded breaks. In order to survive damaging UV exposure, terrestrial microbes have been known to use a variety of DNA repair mechanisms (Cuthbertson and Pearce, 2017). Some of the best-known examples of extreme resistance to UV and IR are species in the genus *Deinococcus* (originally *Micrococcus*). These include *Deinococcus radiodurans* and *Deinococcus geothermalis* – nonsporulating microbes known for their efficiency in repairing damaged DNA. These can typically survive acute exposures to ionizing radiation ≥12,000 Gy (with *D. radiodurans* having survived up to 20,000 Gy; Krisko and Radman, 2013), compared to 8,000 Gy of γ-radiation survived by some fungi, while *E. coli* is killed by only 200-800 Gy (Harris et al., 2009). *Deinococcus* species are also resistant to desiccation by maintaining homeostasis using DNA repair mechanisms, ROS detoxification and accumulation of compatible solutes (Ranawat and Rawat, 2017). Recent stratospheric *Deinococcus* isolates include a radioresistant orange-pigmented, desiccation-tolerant, UV- and γ-radiation resistant bacterium *Deinococcus aerius* TR0125 (from 0.8-5.8 km), and *Deinococcus aetherius* ST0316 (from 10-12 km above Japan). They were found to have similar radiation resistance traits as *D. radiodurans* in laboratory studies (Yang et al., 2008; Yang et al., 2009; Yang et al., 2010; Satoh et al., 2018). *D. aerius* was found to encode DNA photolyase involved in UV resistance, as well as, radiation/desiccation response system genes including *pprI*, *pprA*, *recA*, *ddrA*, and *ddrO* also found in *D. radiodurans, D. grandis,* and *D. geothermalis*. (Du and Gebicki, 2004; Daly et al., 2007; Yang et al., 2008; Satoh et al., 2018). Some species form aggregates, e.g. *D. aerius* and *D. aetherius* (Kawaguchi et al.,

2013), and *D. radiodurans* is usually found as tetrads (Eltsov and Dubochet, 2005).

Laboratory experiments on UV tolerance, have been largely based on the premise that solar radiation and high vacuum are the main factors affecting the incidence of microorganisms in the stratosphere and mesosphere. Laboratory research on microorganisms isolated from the upper layers of the atmosphere, showed that conidia of *Aspergillus niger* are highly resistant to UV irradiation. Moreover, the conidia of *Penicillium* spp., *Papulaspora anomala*, and *Circinella muscae*, or vegetative cells of *Micrococcus* spp. and *Mycobacterium* spp. are resistant to high vacuum. Their inactivation varied within the range of 2 to 16%, with an exception of *Micrococcus* sp., which was higher, ~40% (Lysenko, 1980). In another study, stratospheric *Bacillus* isolates (*B. aerius*, *B. aerophilus*, *B. stratosphericus* and *B. altitudinis*) were found to be more UV-B-resistant than terrestrial *B. licheniformis* MTCC 429T and *B. pumilus* MTCC 1640T based on CFU, when 100 µl culture samples were spread onto nutrient agar plates and exposed to a UV-B lamp (15Wx4) with the lids open (Shivaji et al., 2006).

In a different experiment, the impact of UV-C radiation on the non-heterocystous cyanobacterium *Microcystis aeruginosa* was examined (Sahu and Šimek, 2013; Phukan et al., 2018). Effects were observed on photo-absorbing pigments such as chlorophyll a, carotenoids, phycocyanin, allophycocyanin and phycoerythrin, proteins involved in photosynthesis such as D1 protein and RuBisCO, and enzymes involved in nitrogen metabolism (Phukan et al., 2018). Similar deleterious effects were also observed, after exposure to UV-C of the nitrogen-fixing heterocystous cyanobacterium, *Nostoc muscorum* Meg1.

Genomes of UV tolerant non-sporulating microbes isolated from the stratosphere often have been found to contain a high percentage of GC. High GC content has been thought to lead to greater tolerance as a result of avoidance of T-T photoproducts (Kennedy et al., 2001; Griffin, 2004) (Table 1).

Another proposed UV shielding strategy is the formation of aggregates of cells, where the outer cells may protect interior cells against radiation. *M. luteus*, for example, forms cell aggregates and is 100 times more resistant to UV than *E. coli* (Wainwright et al., 2003). *Halorubrum lacusprofundi*, which is able to survive trips into the stratosphere, is known to form biofilms and flocculent material, which might also provide cellular shielding (Reid et al., 2006; DasSarma et al., 2017).

Extensive studies have resulted in an increasing understanding of how radiotolerance (tolerance to UV radiation) is expressed in extremophiles and polyextremophiles (DeVeaux et al., 2007; Karan et al., 2014). Many of

these microorganisms have developed strategies to tolerate radiation like the production of extremolytes and extremozymes which have potential uses for biotechnology and therapeutic industry (Gabani and Singh, 2013). Derivative strains of *Halobacterium* sp. NRC-1, have been shown to have a high tolerance to high energy ionizing radiation, with an LD_{50} greater than 11 kGy. These derivatives were found to overexpress a single-stranded-binding protein operon (*rfa*3, *rfa*8, *ral*), suggesting a novel mechanism of DNA protection and repair (DeVeaux et al., 2007; Karan et al., 2014). Additionally, it was shown that conidia of the insect pathogen *Metarhizium robertsii* accumulate trehalose and mannitol under nutritive stress conditions leading to an increased UV-B tolerance (Ranawat and Rawat, 2017).

Pigmentation

From the earliest, pigmentation of isolates was noted and thought to be important for survival in the stratosphere. It has long been suggested that microbes use pigments like melanin and carotenoids for UV protection (Imshenetsky et al., 1978; Tong and Lighthart 1997; Singh and Gabani; 2011; Koller et al., 2014). Recent microscopic examination of cloud material showed that 55% of detected bacterial cells were pigmented, as well as up to 41% fungal cells (Vaïtilingom et al., 2012).

Microbes isolated from 48-77 km ASL included pigmented conidia: black from *Aspergillus niger*, green from *Penicillium notatum*, and grey from *Circinella muscae* (Imshenetsky et al., 1979). These strains along with vegetative cells of *Micrococcus albus*, and unpigmented mutants were subjected to UV treatment. The unpigmented mutants were more UV sensitive, with resistance restored by addition of *Aspergillus niger* black pigments (which had a maximum radiation absorption range of 210-370 nm) (Imshenetsky et al., 1979). Further surveys showed that albino conidia of a *Metarhizium robertsii* mutant was less UV-B tolerant than the wild-type green conidia (Braga et al., 2001a, 2001b; Rangel et al., 2006; Dias et al., 2018). Interestingly, survival of an unpigmented entomopathogenic fungus, *Metarhizium acridum*, was noted to be similar to that of the pigmented halophilic *Cladosporium herbarum* fungus isolated from the stratosphere and Chernobyl nuclear reactor (Zhdanova et al., 2000; Butinar et al., 2005; Rangel et al., 2005; Rangel et al., 2006; Rangel et al., 2010; Braga et al., 2015; Della Corte et al., 2014). Therefore, even though conidium pigmentation seems to be related to radiotolerance, it is not the only factor contributing to resistance.

It is also believed that melanin plays the role of a radioprotector, since melanized fungi are able to survive exposure to high doses of γ-radiation, lethal to most non-melanized fungi (Dadachova et al., 2008). For example, the melanized fungi *Cryptococcus neoformans* and *C. antarcticus* are resistant to highly energetic and damaging particulate radiation, e.g. deuterons (Pacelli et al., 2017). Melanin may also be used as an energy

transducer, allowing for utilization of ionizing radiation for metabolic processes, and increasing growth rates, compared to non-melanized fungi when exposed to higher than background radiation (Dadachova et al., 2008; Robertson et al., 2012).

Carotenoids are known to be important for protection from oxidative stress and UV irradiation. Not surprisingly, many isolates from the stratosphere, as well as the ones that survive exposure, contain carotenoid pigments. These include halophilic archaea, which contain novel C50 isoprenoids, such as bacterioruberins (DasSarma et al., 2001). In these extremophilic organisms, a number of genes have been shown to be involved in their synthesis, including a cytochrome P450 (Hescox and Carlberg, 1972, Müller et al., 2018). Studies have also been carried out on the fungus *Aschersonia aleyrodis* to show that carotenoids including β-carotene are involved in oxidative stress and UV irradiation protection (van Eijk et al., 1979; Avalos and Limón, 2015; Dias et al., 2018).

Cold temperature

Microbes have developed multiple strategies to protect themselves from the damaging effects of freezing, such as: biofilm-formation (Reid et al., 2006), accumulation of chaotropic metabolites (e.g. fructose and glycerol), increase of polyol levels (e.g. intracellular trehalose), change of membrane lipids and fluidity, secretion of antifreeze proteins, and use of cold-active enzymes (Robinson, 2001; Feller and Gerday, 2003; Chin et al., 2010; Gerday 2013, Martin and McMinn 2018). Many studies have addressed the molecular adaptations that allow growth and survival at cold temperatures on the Earth's surface, but relatively few have addressed this property in the high atmosphere (Karan et al., 2012; Gerday 2013). One important property, for protein function at low temperatures, is greater flexibility and less negative charge at the surface (Laye et al., 2017). Microbial membranes also need to be more fluid, which can be achieved by changing saturation levels of fatty acid modifications and shortening of fatty acid chain length. Synthesis of antifreeze glycoproteins and peptides leads to freezing point depression of water and may improve survival at extremely cold temperatures (Pikuta et al., 2007).

Growth of microbes in the laboratory at subzero temperatures has been observed. The proteomics of *Colwellia psychrerythraea* 34H (Cp34H) at -1 to -10 °C revealed several strategies, including osmolyte regulation and polymer secretion. These appear to be necessary for metabolic activity subzero, while differentially expressed proteins include those involved in DNA repair chemotaxis and sensing (with a drop-in motility-related proteins) (Nunn et al., 2014). The haloarchaeon *Halobacterium lacusprofundi*, isolated from an Antarctic lake, was found to be capable of growth at sub-zero temperatures in high salt brine and also to be more freeze-thaw resistant than mesophilic haloarchaea (Reid et al., 2006; DasSarma et al., 2017). Additional microbes able to withstand the cold,

include *Trichococcus patagoniensis*, which was determined to be able to divide at -5 °C under both aerobic and anaerobic conditions (Pikuta et al., 2007).

Studies suggest that polyhydroxyalkanoates (PHA) storage granules may also be involved in cold stress, osmotic shock, and radiation protection (Pavez et al., 2009; Tribelli and López, 2011; Obruca et al., 2016; Obruca et al., 2017). Strains producing PHA have been found to be more UV radiation resistant than mutants that do not produce PHA (Slaninova et al., 2018). This was also observed in *Azospirillum brasilense* when PHA-rich and PHA poor cells were compared (Tal and Okon, 1985). The granules are believed to scatter UV radiation, shielding bound DNA as well as decreasing intracellular ROS levels. This protection was confirmed using genetically modified *E. coli* (Slaninova et al., 2018).

Osmotic stress

The dry conditions, present in large sections of the atmosphere, reduce water availability and induce osmotic stress in cells exposed to them. Osmotic stress often requires multiple stress responses. For example, fungi have been shown to respond by altering ion transport, homeostasis, sodium extrusion, and melanin synthesis. They also adjust their internal solute potentials by accumulating solutes such as glycerol, erythritol, mannitol, and trehalose, modifying the plasma membrane, decreasing fatty acid saturation in membranes, and increasing cell wall thickness to limit osmotic losses (Hallsworth and Magan, 1994; Serrano et al., 1999; Almagro et al., 2001; Turk et al., 2004; Dijksterhuis and de Vries, 2006; Kogej et al., 2007; Rangel et al., 2008; Kralj Kuncic et al., 2010; Rangel, 2011). Further adaptation strategies to osmotic stress have been studied in halophiles, and both salt-in and salt-out strategies have been identified (for more, see review in DasSarma and DasSarma, 2015).

Metabolic activity

Several observations of microbes from the stratosphere show that they are generally dormant, with suppressed metabolic activity, when isolated. This is consistent with the hypothesis that dormancy conditions prevent excessive DNA damage, which mainly occurs in actively dividing cells. As seen on Table 1, the majority of strains isolated from the stratosphere form spores. For example, in a study of microbes isolated from an altitude of 20 km, several spore-forming pigmented fungi and bacteria were found, including *Penicillium* sp. and several bacilli (Griffin, 2004). Other observations have indicated that cells grow more slowly upon return from the stratosphere. Slowed growth was reported for the orange-pigmented, non-motile, non-spore-forming *Deinococcus aerius* TR0125, isolated from 10 km ASL, which was found to grow at a much slower rate than *D. radiodurans*, *D. grandis*, and *D. geothermalis* (Satoh et al., 2018). This is consistent with other findings of slower growth observed for a stratospheric *Bacillus* sp. isolate (Smith et al., 2010).

Halotolerant fungi, such as melanized *Cladosporium* sp., have been isolated from the stratosphere and have been shown to accumulate mycosporines as a response to stress (Della Corte et al., 2014). *Aspergillus penicilliodes* is able to germinate at very low water activity (0.585 aw, approximately 58.5% of relative humidity), which is now considered the lower limit for life. Studies of this fungus should result in a better understanding of life under severe water limiting conditions, such as found in the stratosphere (Stevenson, 2017; Rangel et al., 2018).

Epidemiology

While most microbiological research has been conducted in the troposphere, it has been established that several pathogens may be viable even after exposure to the stratosphere (Chudobova et al., 2015). Molecular-based studies of airborne microbes in the troposphere have determined that, during African dust events, up to 25% present in the Caribbean air are species of bacteria or fungi that are known to be plant pathogens and about 10% were identified as opportunistic human pathogens (Griffin et al., 2001; Kellogg and Griffin, 2006). Potential pathogens isolated from the troposphere include: *Puccinia melanocephala* and *Hemileia vastatrix*, which cause sugar cane and coffee rust respectively; *Puccinia graminis,* a wheat pathogen; *Mycospherella musicola*, which causes banana leaf spot disease; *Bacillus pumilus*, which causes bacterial blotch in peaches; *Bacillus megaterium*, which causes 'wetwood' disease in trees; *Aspergillus sydowii*, which has been implicated in sea-fan disease; *Karenia brevis*, which is a causative agent for algal blooms; and, the causative agent of meningococcal meningitis, *Neisseria meningitis* (Griffin et al., 2001; Griffin et al., 2002). Since both plant and animal (including human) pathogens have been isolated from the troposphere, the atmosphere may indeed be transporting human pathogenic agents (Griffin et al., 2002). However, how many, if any, are present and transported by the stratosphere is yet undetermined. Further studies are needed to establish the potential epidemiology related to the tropospheric and stratospheric transport of pathogens.

Planetary protection

Studies of the stratosphere are relevant for planetary protection, which is the practice of protecting solar system bodies from contamination by Earth life and protecting Earth from possible life forms that may be returned from other solar system bodies (OSMA, 2019). Understanding microbial survival and adaptation to stratosphere conditions allows for better definition of policies and helps in their development and establishment. For example, the 1958 Committee on Space Research (COSPAR) developed the original guidelines to minimize forward- and backward- contamination and was the basis for the 1967 Outer Space Treaty that provided planetary protection policies. This included quarantining both astronauts and material for the Apollo program (1969-72) (Nicholson et al., 2009). In the 1960s,

acceptable unmanned spacecraft microbial bioloads were 10^4-10^8 CFU/vehicle. The Viking 1 and 2 lander missions to Mars, in 1976, were sterilized using heat, reducing spore forming CFU to 2 x 10^4/lander. These had the search for life as part of their mission, while all other NASA missions have primarily been geology focused (Fairén et al., 2018). Later missions allowed for higher counts, < 3x10^5 CFU bacterial spores, in order to reduce sterilization costs (Nicholson et al., 2009).

Roughly 85–95% of microbial isolates from Spacecraft Assembly Facilities (SAFs) and spacecraft are indigenous to humans, and the remaining have been found in soil and dust. Spore-forming *Bacillus* spp. isolates from SAFs make up ~10% of total cultured bioloads (Nicholson et al., 2009). When considering how to effectively sterilize outbound materials, we have to consider the destination. For example, Mars has been, and still is naturally "sterilized" against terrestrial microorganisms, with broad-spectrum radiation, extreme cold and dryness, and surface soil chemistry containing highly reactive oxidizing agents and low pressure (Nicholson et al., 2009; Freissinet et al., 2015; Khodadad et al., 2017; Fairén et al., 2017; Fairén et al., 2018).

Planetary Protection constraints exploration of Special Regions of Mars, including the potentially aqueous, briny recurrent slope lineae (RSL), in order not to contaminate these potentially habitable environments. On the other hand, long-term plans include human missions, resulting in transport of large numbers of microbes (Fairén et al., 2017; Rummel and Conley, 2017; Fairén et al., 2018). If a Martian mission leads to the discovery of microbial life, how would one know if this is Martian or hitchhikers from Earth? They could be exobiota from the current climate or from when Mars was warmer, or simply contamination from a launch site or a spacecraft clean-room or assembly facility like the highly UV resistant *Bacillus nealsonii*, or *Bacillus odysseyi* (Venkateswaran et al., 2003; La Duc et al., 2009). The stratosphere is likely to be extremely valuable as a Mars simulation region due to their physical-chemical similarities and also serving to test the efficacy of our planetary protection measures. Understanding how Earth's microbes survive stratospheric stressors is likely to help us understand how life from our planet could survive Martian conditions.

In addition to the links to the astrobiological exploration of Mars, such insights will also prove helpful in the future study of the icy moons of the outer solar system and extra solar planets. Discussions on the planetary protection of the exooceans of these moons is an on-going dialogue between different space agencies (Rettberg et al., 2019), and preparations for their future exploration are gaining increased traction and visibility (e.g. Antunes et al., 2020; Jebbar et al., 2020; Taubner et al., 2020).

Climate change

Burning of fossil fuels since the industrial revolution has resulted in increasing concentrations of greenhouse gases to levels not seen for more than a million years. For example, the most abundant of these, CO_2, ranged from 180-280 ppm for most of the past hundreds of thousands of years, but has increased to >400 ppm in 2016. Widespread climate change is occurring as a result, including global warming, sea level rise, extreme storms and flooding, and droughts, wildfires, and desertification. Greenhouse gases and particulate matter affect radiative transfer, changing atmospheric temperature, density and albedo patterns and weather. The role of the stratosphere in climate change has resulted primarily from effects on ozone. The level of ozone in the stratosphere affects the temperature and the height of the tropopause, with significant consequences for the weather and temperature at the Earth's surface (Trickl et al., 2019).

Certain greenhouse gases, especially refrigerants like halocarbon gases (hydrofluorocarbons and chlorofluorocarbons), are known to deplete ozone in the stratosphere via photoreaction (World Meteorological Organization, 2014). Ozone depletion and the subsequent cooling of the stratosphere results in a rise in the temperature of the tropopause with potential impacts on climate (Hoskins, 2003). *Cumulonimbus* clouds may enter the stratosphere as a consequence, bringing moisture to this layer, but at the same time drying the lower troposphere, resulting in less frequent, but longer-lasting thunderstorms and an effective cooling (Ursem, 2016). A difference in humidity at the tip of the boundary layer in the lower stratosphere can result in nucleation on nanostructured colloidal aerosols and formation of droplets and a visible haze effect – especially in the Arctic, where this haze has become almost permanent, with particle lifetime in the stratosphere predicted to be 1-2 years (compared to <2 weeks in the lower troposphere) (Ursem, 2016).

Increased stratospheric cloud formation may in turn increase the rates of ozone depletion, resulting in a damaging cycle. Since the ozone layer within the stratosphere acts as a radiation shield, protecting the Earth's surface from UV-C damage, increased solar UV radiation reaching the Earth's surface harms both plants and animals. Damage to plants suppresses the net photosynthetic rate, and lowers transpiration rate of crops, reducing the overall CO_2 sink (Lou et al., 2017; Pérez et al., 2017). As a result, efforts have been made over the past few decades to reduce ozone depleting greenhouse gases such as refrigerants, through the Montreal Protocol. While these have been quite successful, challenges to increased usage of hydrofluorocarbons and other ozone-reactive gases continue to be of concern (Bais et al., 2018).

The atmospheric trace gas N_2O has also been implicated in climate change, by ozone-depletion in the stratosphere, and is mainly produced by

microbes (~35% from oceanic bacteria and archaea) (Barnes and Upstill-Goddard, 2018). The 100-year global warming potential of N_2O is calculated to be ~300 times stronger than that of CO_2, and its emission has been increasing at a rate of 0.25% per year, with wastewater treatment plants emitting 3.2% of the total anthropogenic N_2O emissions globally (Yan et al., 2017). Methane is another destructive greenhouse gas involved in climate change, although its effects result from ground level, rather than higher atmosphere effects. Natural methane production rates are in turn influenced by climate: as temperature rises, so does methane production in a process known as positive climate feedback (Dean et al., 2018).

Other greenhouse gases are water vapor and molecular hydrogen (H_2) (Meredith et al., 2017). When water vapor enters the lower stratosphere during severe storms, it leads to hygroscopic growth of sulfate aerosols and subsequent ozone loss by up to 17% (Bais et al., 2018). Carbonyl sulfide is the most abundant sulfur compound in the troposphere and can be transported into the stratosphere where it is converted to sulfate by photolysis or reactions with O or OH radicals, resulting in production of sulfate aerosols, which in turn influence the Earth's radiation balance and causes ozone depletion (Ogawa et al., 2017).

There are many natural sources of particulate matter in the stratosphere which decrease heat absorption and result in global cooling, including volcanic eruptions, conical blue jets (from tops of thunderclouds), smoke from large fires, and photophoretic forces. Negatively charged anthropogenic nanoparticles can be transported to the lower stratosphere and accumulate at 18-20 km. These originate from combustion, industry, aircraft, ground transportation, heating with coal and wood and energy production. Proposals for using dust-like substances (like Loess – defined as an eolian sediment, that has been transported and deposited by the wind, and dominated by silt-sized particles of 20–50μm diameter), to cool the environment and limit climate change, have been suggested in a process known as geoengineering (Martínez-Garcia et al., 2011; Lamy et al., 2014).

Clearly, complex dynamics are on-going in the stratosphere. The degree to which microorganisms are affected or are affecting these dynamics is largely unknown. Studies are needed to more fully determine their relevance and impact.

Conclusions

The stratosphere is an extreme environment subject to many simultaneous stressors and is undoubtedly challenging for life. Radiation appears to be the major factor in loss of viability in the stratosphere, but other stressors such as low temperature also limit survival. In addition to being radiation-tolerant and cold-tolerant, cells must also be able to tolerate hypobaric conditions to survive the extremely low pressures (0.1-10 kPa) present in

the stratosphere (Rothchild and Mancinelli, 2001; Fletcher et al., 2014). Stratospheric conditions also lead to rapid desiccation, and only the most xerotolerant microbes are able to cope with such extreme conditions. Due to the sparseness of resources in the stratosphere, it is difficult for any living cells to find the building blocks of life needed to metabolize and reproduce. However, attachment to particulate matter (dust) may provide opportunities to circumvent this limitation.

Whether life can exist for any appreciable length of time in the stratosphere, let alone thrive, is still an open question. What is clear though, is that changes in the stratosphere from human activities are disturbing the troposphere-stratosphere boundary and increasing the exchange of materials between the layers. This reflects the anthropomorphic changes and feedback effects from the troposphere, as well as the surface of the Earth and its oceans, rivers, and lakes. Depletion of the ozone layer represents one of the most destructive potential changes in the stratosphere which may adversely affect animal health, plant life and agriculture.

More detailed investigations involving collection and analysis of stratospheric material will provide better insights into many outstanding questions about the higher atmosphere, including the role of the stratosphere in climate change, long-range dispersal of microorganisms, and implications for planetary protection. In addition, utilizing the stratosphere as a proxy for the surface of Mars will be valuable for astrobiology as we consider the potential for life on the red planet.

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