Sustainable life support on Mars – the potential roles of cyanobacteria

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Abstract: Even though technological advances could allow humans to reach Mars in the coming decades, launch costs prohibit the establishment of permanent manned outposts for which most consumables would be sent from Earth. This issue can be addressed by *in situ* resource utilization: producing part or all of these consumables on Mars, from local resources. Biological components are needed, among other reasons because various resources could be efficiently produced only by the use of biological systems. But most plants and microorganisms are unable to exploit Martian resources, and sending substrates from Earth to support their metabolism would strongly limit the cost-effectiveness and sustainability of their cultivation. However, resources needed to grow specific cyanobacteria are available on Mars due to their photosynthetic abilities, nitrogen-fixing activities and lithotrophic lifestyles. They could be used directly for various applications, including the production of food, fuel and oxygen, but also indirectly: products from their culture could support the growth of other organisms, opening the way to a wide range of life-support biological processes based on Martian resources. Here we give insights into how and why cyanobacteria could play a role in the development of self-sustainable manned outposts on Mars.

Introduction

Sending humans to Mars within a few decades is now a realistic goal (e.g., Baker & Zubrin 1990; Horneck *et al.* 2006; Drake 2009; Zubrin & Wagner 2011). However, even though leaving a footprint and planting a flag could be achieved with not much more than the current state-of-the-art of engineering, a definite pay-back is still in doubt. On the other hand, if a Mars mission can allow extensive human scientific activity and yield meaningful scientific data, the effort is justified. In such a case, scientists will need to spend a considerable period on site, and multiple short-term missions are not a viable option. Given the time, costs and challenges associated with the journey, long-term human bases will likely be needed.

But while the vision of long-term human presence on Mars is compelling, providing consumables to sustain crews is a challenge. Even though launch costs vary depending on mission scenarios and might be reduced in the coming decades, they have been estimated to be in the order of $300 000 kg^{-1} (Massa *et al.* 2007). Sending from Earth all the needed resources does not seem financially sustainable. Should Mars colonization be consequently deemed too expensive to be realistic? Maybe not. There are alternatives.

One is recycling – using regenerative systems. Such systems should have biology-based components: various life-support functions can be provided by physicochemical processes, but some valuable products such as high-protein food cannot currently be produced by the latter (Drysdale *et al.* 2003; Montague *et al.* 2012). In addition, many components of physicochemical life-support systems are heavy, highly energy-consuming and/or require high temperatures. Even in the case where these are the backbones of life-support systems, biological modules could both complement them and provide safe redundancies. Consequently, various bioregenerative life-support systems (BLSS) are or have been under development for recycling food, water and gases both in space (e.g., Godia *et al.* 2002; Gitelson *et al.* 2003; Drysdale *et al.* 2004; Lobascio *et al.* 2007; Nelson *et al.* 2010; Giacomelli *et al.* 2012) and within lunar and Martian outposts (e.g., Gitelson 1992; Blüm *et al.* 1994; Tikhomirov *et al.* 2007; Nelson *et al.* 2010). This may sound promising: instead of sending resources in amounts almost proportional to the mission length, only a few weeks' worth of consumables would be sent and recycled. The issue is: 100% recycling efficiency cannot be reached and losses are unavoidable. For quantitative information regarding the theoretical recycling efficiencies of the Micro-Ecological Life Support System Alternative (MELiSSA), for example, see Poughon *et al.* (2009).

A regenerative system's running time without re-supply is consequently limited. It also cannot be expanded, as the

mass of cycling components is, at a given time, at most equal to their initial mass. In addition to this, most current BLSS projects represent a large initial volume and mass, as well as a high power consumption. For instance, the mass of consumables needed to sustain a crew of 6 using the MELiSSA system has been assessed to be about 30 metric tons (mt), hygiene water not included, for a 1000-day mission (Langhoff *et al.* 2011). Re-supply is thus needed and the most advanced BLSS projects heavily depend on materials imported from Earth – although a theoretical physicochemical/biological resource production system relying on Martian resources has recently been patented (Cao *et al.* 2014). These projects are consequently not suitable for autonomous, long-term human bases on Mars: the mass problem is reduced, but not solved.

There is however a promising solution: producing resources from local materials. Like all human settlers of previous generations, the Martian pioneers must 'live off the land'. A critical question this fact raises is whether it is possible to feed biological systems with local resources. On the one hand, water (H_2O), solar energy, carbon (C), nitrogen (N) and many other life-supporting nutrients are found on Mars (Meyer & McKay 1989; Olsson-Francis & Cockell 2010; Cockell 2014). But on the other hand, they cannot be directly exploited in the form in which they are found there by key organisms of current BLSS projects. This limitation may lead one to think that BLSS based on local resources are irrelevant for Mars exploration. What if, however, Martian resources could be exploited and processed into suitable forms by an additional biological module? What if there was a biological link between on-site resources and BLSS? This link might be created by cyanobacteria. All the inputs needed to sustain a diazotrophic (N-fixing), bioleaching cyanobacterium's metabolism could in theory be obtained from Mars's mineral resources, water, atmospheric gases and incident solar energy. Firstly, some (e. g., *Anabaena* spp. and various desert species) have the ability to extract and biologically fix nutrients from analogues of Martian rocks (Brown & Sarkisova 2008; Olsson-Francis & Cockell 2010). Owing to these abilities and to their useful products, cyanobacteria have already been suggested as a basis for creating planetary BLSS relying on local resources (Brown 2008a; Brown *et al.* 2008; Stanford-Brown 2011 iGEM team 2011). They are also able to fix C from carbon dioxide (CO_2) and some species can fix N from dinitrogen (N_2), both of which are found in the Martian atmosphere, leading to organic matter and dioxygen (O_2) production without dependency on carbohydrate feedstock. Some species are highly tolerant to extreme environments (e.g., Rothschild & Mancinelli 2001); consequently their culture is less demanding in terms of hardware than that of more environmentally sensitive microbial species. Cyanobacteria could be used directly to produce resources such as food and O_2, but products from their cultures could also be used to feed other living organisms, so opening the way to a wide range of life-support biological processes.

Cyanobacteria could thus provide the key link between BLSS and Martian resources, making the former sustainable and expandable in human bases on Mars. An artist's rendering of such a cyanobacterium-based BLSS ('CyBLiSS') is given in Fig. 1. The present paper is not intended as a specific mission design, but rather as an overview of how and why cyanobacteria could be grown on Mars. We first outline their potential applications there. Then we discuss the associated challenges and possible ways of facing them. Finally, we outline the research efforts needed to design functional, cyanobacterium-based BLSS for human outposts on Mars. A summary of this paper's contents is given in Fig. 2.

Potential roles of cyanobacteria in Mars-specific BLSS

Feeding other microorganisms

Heterotrophic microorganisms have been used throughout human history and would be highly useful on Mars. Potential applications include the production of drugs, food, biomaterials and various industrially useful chemicals, metal leaching and food processing for taste improvement (Cumbers & Rothschild 2010; Langhoff *et al.* 2011; Montague *et al.* 2012; Menezes *et al.* 2014; Verseux *et al.* 2016). As most of these applications require relatively small culture volumes and no solar light, cultures could be performed under Earth-like conditions with reasonable costs. However, heterotrophic microorganisms rely on organic materials whose availability on Mars remain very poorly known and are not expected to be abundant there (Ming *et al.* 2014). Could the local resources be processed into suitable substrates by cyanobacteria? Related phenomena naturally occur on Earth, where cyanobacteria are known to support different heterotrophic communities. They can for instance be the earliest colonizers of desert habitats and allow the development of local ecosystems (including heterotrophic bacteria) through the production of organic compounds, N fixation and rock leaching (e.g., Eldridge & Greene 1994; Danin *et al.* 1998; Herrera *et al.* 2009). In aquatic ecosystems as well, cross-feeding and metabolite exchange occur between cyanobacteria and heterotrophic microorganisms (see, for instance, Stevenson & Waterbury 2006 and Beliaev *et al.* 2014).

The question of water is addressed in subsection 'Water'. Heterotrophic organisms also need, first of all, organic compounds as a source of C and energy. Lysed cyanobacterial biomass could be used as such (and as a source of other critical macronutrients such as hydrogen [H], oxygen [O], phosphorus [P] and sulphur [S]; for N, see the following paragraph). Consistently, a filtrate of grinded *Anabaena* sp. PCC7120 (100 mg of dried biomass ml^{-1} before filtration) has been used as the only source of organic compounds and fixed N to grow *Escherichia coli* K-12 MG1655 in phosphate-buffered saline, reaching more than 10^9 cells ml^{-1} within 24 h (Verseux, unpublished data). Note that these results are preliminary and that no optimization step (e.g., choice of a strain that can metabolize sucrose, alteration of culture conditions to modify cyanobacterial biomass's composition and/or more efficient extraction method) has yet been performed. Lysed cyanobacterial biomass has also been shown to be a suitable substrate for ethanol production in yeasts (Aikawa *et al.* 2013; Möllers *et al.* 2014). In lysogeny broth (LB), the most
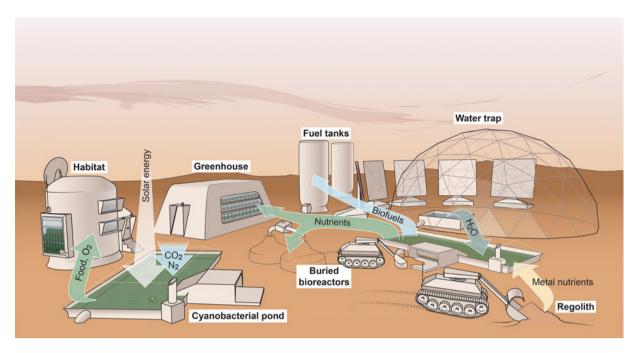
Fig. 1. Artist's rendering of a cyanobacterium-based biological life-support system on Mars. Figure design: Cyprien Verseux and Sean McMahon (Yale University). Layout: Sean McMahon.

common growth medium for heterotrophic bacteria in laboratories, the concentration of fermentable sugars and sugar equivalents (sugar phosphates, oligosaccharides, nucleotides, etc.) utilizable by *E. coli* is very low (<100 μM), constraining bacteria to use amino acids as C sources (McFall & Newman 1996; Sezonov *et al.* 2007). However, non-destructive ways of harvesting nutrients could lead to more efficient processes. Substrates could for instance be secreted. This solution has been investigated in Lynn Rothschild's laboratory (at NASA Ames Research Center, Moffett Field, CA) since the 2011 Stanford-Brown iGEM team engineered *Anabaena* sp. PCC7120 to secrete sucrose (Stanford-Brown 2011 iGEM team 2011), which was then used as a C source to grow *Bacillus subtilis* (Ryan Kent *et al.*, unpublished data). Prior to this, *Synechococcus elongatus* PCC7942 was engineered to produce and secrete either glucose and fructose, or lactate, then used as a substrate for *E. coli* (Niederholtmeyer *et al.* 2010). In this kind of system, a major limitation arises from low sugar yields, which are due to relatively low synthesis rates and to the consumption of sugars by the cyanobacteria themselves. Production rates could be increased by further genetic engineering to either increase synthesis or decrease processing of these products by the producer strains. External conditions could also be modified; for instance, osmotic stress induces sucrose accumulation in many cyanobacteria, especially when synthesis of other osmoprotectants is impaired (Miao *et al.* 2003).

Then, fixed N is needed. Heterotrophic bacteria can obtain N from various organic and inorganic sources such as single amino acids (e.g., Crawford *et al.* 1974) and amino acid chains (e.g., Hollibaugh & Azam 1983; Coffin 1989), nucleic acids (Paul *et al.* 1989) and ammonium (NH_4^+). Diazotrophic cyanobacteria can produce all these compounds after N fixation and, here again, a simple way of making this N available to heterotrophic bacteria is to lyse cyanobacteria. But NH_4^+ can be naturally released by some diazotrophic cyanobacteria, without cell lysis; for instance, extracellular NH_4^+ can reach several mM in cultures of *Anabaena* spp. (mutants or wild-type, depending on species) relying on atmospheric N_2 as a sole N source (Spiller *et al.* 1986; Subramanian & Shanmugasundaram 1986). NH_4^+ is the preferred N source for most microorganisms and becomes limiting at extremely low concentrations (e.g., below a few μM for *E. coli*; see Kim *et al.* 2012), several orders of magnitudes below the above-mentioned concentrations. Released NH_4^+ could be used for feeding not only heterotrophic microorganism, but also some phototrophic species of interest which cannot fix N. Within the MELiSSA loop (e.g., Godia *et al.* 2002), NH_4^+ (there resulting from human and plant waste processing by thermophilic anaerobic bacteria) is converted into nitrates by nitrifying bacteria before being transferred to *Arthrospira* sp. cultures. Even though nitrate is considered the preferred N source for this species (as for most non-N fixing filamentous cyanobacteria), using NH_4^+ instead of nitrates has been shown not to reduce growth rates (Filali *et al.* 1997). A drawback is that NH_4^+ becomes growth-limiting from relatively low concentrations (a few mM) and should consequently, if limiting below these concentrations, be regularly (or continuously) added to the culture medium. Semi-separated cultures, where cyanobacteria's and heterotrophic microorganisms' culture vessels communicate through membranes that allow medium but not cell exchange, can also be considered. In such a case, extensive system characterization is needed to predict outputs, and processes could be optimized by improving the culture setup and by performing evolutionary selection of the co-culture to improve metabolic
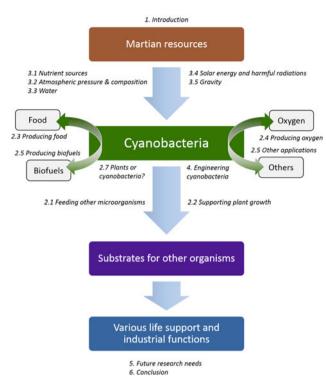
Fig. 2. Visual table of contents.

interactions. In any case, the rates of NH_4^+ release by strains of interest under Mars-like constraints, as well as the use of this NH_4^+ as an N source for non-N fixers, should be further investigated.

Many heterotrophic microorganisms also need O_2. *E. coli*, for example, roughly needs 1 g of O_2 per gram of dry weight (gdw) (Shiloach & Fass 2005). O_2 consumption and cell mass vary according to strain and cultures conditions, but this figure corresponds to approximately 1 g of $O_2 \, l^{-1}$ to reach a culture of 2×10^9 cells ml^{-1}. Cyanobacteria produce O_2 through photosynthesis; details are given in subsection 'Producing oxygen'.

Finally, metals must be provided. Some (macronutrients) are needed in relatively large amounts; these are mostly potassium (K), magnesium (Mg) and iron (Fe) and, for some species, sodium (Na) and calcium (Ca). Others (micronutrients) are needed in trace amounts by some microorganisms and include, for instance, chromium (Cr), manganese (Mn), nickel (Ni) and zinc (Zn) (Madigan *et al.* 2000). As an example, *E. coli* needs about 10^8 atoms $cell^{-1}$ of K and Mg; 10^5 atoms $cell^{-1}$ of Ca, Zn and Fe; and 10^4 atoms $cell^{-1}$ of Mn, molybdenum (Mo) and selenium (Se) (Finney & O'Halloran 2003). All the needed elements seem to be present in Martian regolith (Cockell 2014), but some of them may be poorly available to most organisms with no leaching abilities. However, rock-dwelling cyanobacteria can extract metal nutrients from a wide range of rocks (see Olsson-Francis *et al.* 2012). Besides mobilizing them in their own cells, they help release them in the aqueous phase, increasing their concentration there (Olsson-Francis & Cockell 2010; Olsson-Francis *et al.* 2012) and availability for non-leaching organisms. *Anabaena cylindrica* has for instance been shown to release elements including K, Mg, Na, Ca,

Fe, Mn, Ni and Zn from a Mars basalt analogue (Olsson-Francis & Cockell 2010). The metals needed in highest amounts by *E. coli*, namely K and Mg, reached concentrations of 125 and 55 μM, respectively, in large excess compared with *E. coli*'s needs (roughly, 0.3 μM to reach 2×10^9 cells ml^{-1}). Even though lysing cyanobacterial biomass is the most straightforward option for transferring nutrients from cyanobacteria to heterotrophic microorganisms, it may thus not be necessary. What solution minimizes the cost-to-productivity ratio is still to be determined.

Supporting plant growth

The majority of BLSS projects include plants for air and water regeneration and food production. Interestingly, all of the nutrients needed to grow plants (C, H, N, O, P, S, K, Mg, Fe, Na, Ca and micronutrients) seem to be present on Mars. Banin (1989) proposed using Martian regolith to grow plants and this approach is still under investigation (e.g., Silverstone *et al.* 2003, 2005; Nelson *et al.* 2008; Maggi & Pallud 2010; Wamelink *et al.* 2014). However, even though Martian regolith is mostly basaltic and weathered basalt can yield extremely productive soils on Earth (Dahlgren *et al.* 1993), regolith will require physicochemical and/or biological treatment before it can be used as a growth substrate for plants. Besides excess salts, oxides and toxins, the main limiting factors are its low nutrient bioavailability and poor water-holding potential caused by low organic C contents (Maggi & Pallud 2010; Cockell 2011). Enrichment of N, in particular, is critical: most plants cannot fix atmospheric N (a few, mainly legumes, benefit from symbiotic N fixation by specific bacteria harboured in their root tissues).

Some elements could be obtained by recycling human waste and inedible biomass from previous crops. However, relying solely on this would strongly limit sustainability and scalability of the system: without adding new components to the loop, the pool of supplies can only decrease over time. Again, inputs for plant cultivation should rather be provided from local resources. An interesting approach has been proposed in the context of lunar exploration. Following this approach, plant species which are tolerant to harsh growth conditions would be grown in local regolith, after inoculating seeds with a carefully chosen consortium of bioleaching bacteria to deliver nutrients to plant roots and to protect them against excessive accumulation of toxic elements. Once these 'first-generation plants' would have been grown, their biomass would be converted by microorganisms into a fertile protosoil used to support the growth of more demanding plants (Kozyrovska *et al.* 2006; Zaets *et al.* 2011). An alternative strategy could rely on cyanobacteria to process elements from rocks and fix N. Even though no plant cultures are currently produced using cyanobacteria as exclusive nutrient sources, the latter are used in agriculture to improve soil fertility, balance mineral nutrition and release biologically active substances that promote plant growth and increase plant resistance (Singh 2014). In the context of space exploration, cyanobacterial cultures have already been proposed as a way of releasing chemical elements from rocks to hydroponic solutions (Brown & Sarkisova 2008).

N fixed by cyanobacteria could also be used in hydroponic and/or soil-based systems. It could come from two main sources: from processed biomass containing for instance amino acid chains, and from released NH_4^+.

Within the MELiSSA loop, NH_4^+ is converted into nitrates before being transferred to plants, but plants can also efficiently uptake NH_4^+ (see, e.g., Howitt & Udvardi 2000). In theory, plant growth rates can even be higher with NH_4^+ than with nitrates due, first, to the energy needed to reduce nitrates to NH_4^+ before its incorporation into organic compounds (Bloom et al. 1992) and, second, to a reduced need for photons, water and catalytic metal use per unit of C fixed when relying on NH_4^+ (see Raven et al. 1992). However, experimental data often do not support this hypothesis (e.g., Raven et al. 1992). NH_4^+ induces toxicity at lower concentrations than nitrate, with a threshold which varies widely among species (Britto & Kronzucker 2002). As a consequence, preference for NH_4^+ over nitrate is species-dependent (Barker & Mills 1980; Zhou et al. 2011). Even though NH_4^+ toxicity is not fully understood, it can presumably be explained in large part by the effect of the absorbed N's form on the uptake of other ions: NH_4^+ leads to higher anion uptake and lower cation uptake. It also affects pH: nitrate and NH_4^+ absorption induce, respectively, a release of hydroxyl ions (HO^-) and a release of protons (H^+), and for some plants NH_4^+'s negative effects can be reversed by buffering the medium (Britto & Kronzucker 2002). Harmful effects could consequently be mitigated by controlling pH and adjusting the supply of other nutrients (Muhlestein et al. 1999), and by keeping NH_4^+ concentrations below toxic levels. Further studies could ascertain to what extent the growth-limiting effects of high NH_4^+ concentrations can be mitigated; more generally, work is needed to determine whether the benefits brought by nitrifying bacteria would justify the complexity and costs associated with their culture. Besides NH_4^+, organic N from cyanobacterium biomass can be considered as an N source: plants can uptake amino acid chains (e.g., Lipson & Näsholm 2001; Näsholm et al. 2009). Uptake of single amino acids and short peptides, and possibly di- and tri-peptides, occurs via membrane transporters in root cells (Rentsch et al. 2007). Some plants can also uptake proteins after degradation by fungal symbionts (e.g., Bajwa et al. 1985) and at least some can do it without prior degradation by other organisms, either directly (presumably by endocytosis) or via the secretion of proteolytic enzymes (Godlewski & Adamczyk 2007; Paungfoo-Lonhienne et al. 2008).

Symbioses between plants and diazotropic cyanobacteria naturally occur. The best-known instances involve aquatic ferns from the genus *Azolla* and their symbionts from the genus *Anabaena* (Peters & Meeks 1989), and angiosperms from the genus *Gunnera* and their symbionts from the genus *Nostoc* (Bergman et al. 1992), but interactions naturally occur between cyanobacteria and representatives of most plant groups. Artificial symbioses between diazotrophic cyanobacteria and plants which normally do not harbour cyanobacterial symbionts, where the former provide fixed N to the latter, have also been successfully created with various plants including cereals (Gusev et al. 2002). In a hydroponic system, an *Anabaena*

variabilis strain has even been shown to provide fixed N to wheat grown in an otherwise N-free medium, yielding plant growth comparable with that of the control plants grown in a nitrate-containing medium (Spiller & Gunasekaran 1990). Assessments of the cyanobacterium-to-plant biomass conversion efficiency, when all substrates besides CO_2 and water come from cyanobacteria, require further experiments.

Even though experimental research is needed to quantify the nutrient composition of culture supernatants (with and without cell lysis), to determine the most efficient way of transferring nutrients from cyanobacterial cultures to other organisms and to get quantitative estimates of the system's requirements, cyanobacteria could thus be considered as a useful tool for processing local inorganic resources into a form which is available to other organisms (for a visual summary, see Fig. 3). But cyanobacteria could also be used directly for various applications. The most critical are outlined below.

Producing food

If all food came from Earth and assuming the easiest option of providing shelf-stable, prepackaged food similar to International Space Station's provisions, the amount to be sent would be about 1.8 kg day^{-1} crewmember^{-1} (Allen et al. 2003). Adding the needed vehicle and fuel weight to carry it and assuming a 10:1 vehicle-to-payload ratio (Hoffman & Kaplan 1997), 1-year food supply for a crew of 6 would add more than 29 mt to the initial mass of the transit vehicle. Worse, the load needed for a healthy diet would be much higher: even though convenient due to reduced need for storage facilities and contamination risks, a diet composed exclusively of this type of food would be nutritionally incomplete and thus not adequate in the long term. Frozen food could be seen as an interesting complement but would be extremely demanding in terms of storage facilities, could not be kept at commercial temperatures for more than about 1 year without losing palatability and would be unreliable due to potential freezing failure. Thus, even though current space food systems are relevant for short-term space missions and missions close to Earth (in low Earth orbit and possibly on the Moon), they must be developed further to meet the requirements of a manned mission to Mars (Perchonok et al. 2012). Establishing long-term human settlement on Mars seems unrealistic without local food production systems.

Growing plants might appear as an obvious option, even though creating an adequate environment on Mars would be particularly challenging at the first steps of colonization (details are given in the 'Plants or cyanobacteria?' section below). However, cyanobacteria could be an interesting alternative. Some species are edible, can be grown at a large scale and are expected (based on comparisons of metabolic pathways and human nutritional requirements) to be a nearly complete nutritional source, lacking only vitamin C and possibly essential oils (Way et al. 2011). Some species, notably from the genus *Arthrospira*, have consequently been studied as a potential food source in life-support systems (Hendrickx et al. 2006; Lehto et al. 2006). *Arthrospira platensis* is already used on Earth as a food supplement, and its nutritional value and
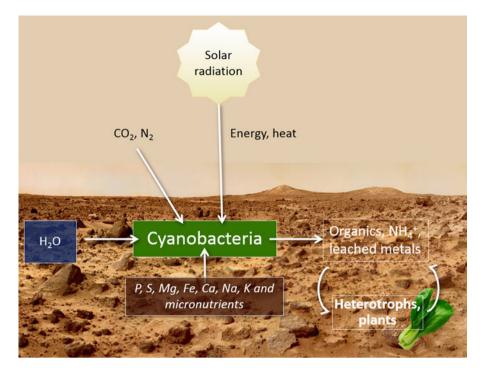
Fig. 3. Using cyanobacteria to process Martian resources into substrates for other organisms. In this scheme, cyanobacteria are fed with various nutrients obtained from the regolith, gaseous carbon and nitrogen from the atmosphere, energy from solar radiation, and water from various possible sources including ice caps, subsurface ice, atmosphere and hydrated minerals. Additional organic material, CO_2 and water could be provided from metabolic and manufacturing waste resulting from human activity. Products from cyanobacterial cultures are then used as a substrate for heterotrophic microorganisms and plants.

lack of toxicity are well-characterized. Its dried biomass has been categorized 'Generally Recognized As Safe' (GRAS) for human consumption by the Food and Drug Administration (FDA) of the United States (FDA GRAS Notice No. GRN 000127). It has high protein contents (roughly, 50–70% of the dry weight) and a high productivity: cultures in outdoor ponds under non-optimized conditions typically yield from 1 to 3×10^7 gdw ha^{-1} yr^{-1} (Jiménez *et al.* 2003) and produce 20 times more proteins per hectare than soya's (Hendrickx *et al.* 2006; Henrikson 2009). Yields can be much enhanced in more controlled conditions. Cultures in photobioreactors can produce about 1 gdw l^{-1} day^{-1} and, with such yields and assuming energetic contents of 3.75 kcal gdw^{-1} (Tokusoglu & Unal 2003), 2000 kcal day^{-1} can be produced with about 0.53 m^3. That being said, this figure is a very rough approximation of the actual needs: even though it covers mean caloric needs for a 30–50-year-old adult, it does not represent the amounts needed to cover nutritional needs. On the other hand, production rates can be further increased; for instance, a productivity of 16.8 gdw m^{-2} h^{-1} (there corresponding to 1.2 gdw l^{-1} h^{-1}) has been attained for a short period of time using high *A. platensis* cell densities, a very short light path (14 mm), a very high photon flux (8000 μmol m^{-2} s^{-1}; about four times the solar flux on Earth when the Sun is directly overhead) and highly turbulent mixing (Qiang *et al.* 1998). *Arthrospira* spp. have health-promoting properties, including antiviral and antimutagenic functions which are especially relevant in Mars's highly irradiated environment (Lehto *et al.*

2007). They are also much more digestible than eukaryotic microalgae from the genus *Chlorella*, their main competitor as a photosynthetic microorganism-based food source, which have poorly digestible cellulose-containing cell walls.

However, *Arthrospira* spp. biomass currently cannot be used as a staple food: in addition to a taste that very few people would qualify as appealing, its carbohydrate-to-protein ratio is low, it contains very high levels of vitamin A, and it lacks vitamin C and possibly essential oils. Due to this nutritional imbalance, it is generally not recommended to consume more than 10 g day^{-1}. Cyanobacteria could be mixed with other microalgae and plants to optimize nutrient and fibre contents, as well as to diversify taste and texture, but limitations could also be addressed using synthetic biology (Way *et al.* 2011). Modifying the sugar, protein and lipid proportions, as well as introducing molecules that humans require (e.g., vitamin C) could be achieved using metabolic engineering and other genetic manipulations. Preliminary work has been done in this direction; for instance, mutant strains of *A. platensis* have been selected that are richer than the wild-type in compounds including essential amino acids, phycobiliproteins and carotenoids (Brown 2008b). Novel taste, smell and colour molecules could be produced by cells to increase palatability, attractiveness and meal diversity. It should also be taken into account that, depending on its adjusted pressure, Martian atmosphere can strongly affect the composition of cyanobacterial cultures: *A. cylindrica* cells grown under low pressure and high CO_2 concentrations showed decreased protein contents

and increased sugar contents compared with cells grown under ambient conditions (Lehto *et al.*, unpublished data), shifting the protein/carbohydrate ratio closer to humans' needs.

Research has been extensively focused on *Arthrospira* spp., but other cyanobacterial species such as *Nostoc commune*, *Nostoc flagelliforme* and *Anabaena* spp. in symbiosis with *Azolla* spp. are traditionally consumed and more are edible. *Arthrospira* cultures require extensive nutrient supply (including fixed N), a high temperature with an optimum around 35°C and an alkaline pH. Other species which are less demanding in terms of culture conditions might thus be more suitable on Mars and, even within the genus *Arthrospira*, species could be screened for the highest productivity under on-site constraints.

Cyanobacteria could also be used for food complementation without being directly eaten or even inactivated, as they can be engineered to secrete nutritional compounds (Way *et al.* 2011). As mentioned above, the possibility of engineering them to produce and secrete sugars has already been demonstrated (Niederholtmeyer *et al.* 2010; Stanford-Brown 2011 iGEM team 2011).

Finally, high-protein food could be suggested to come from animals. Granted, it is unrealistic to envision growing large species on Mars during its earliest colonization steps, due for instance to the need for an Earth-like environment, to low protein yields-to-allocated resources (area, water, vegetal-originated proteins, working time, etc.) ratios and to the risk of pathogen transmission to humans. However, aquaculture of densely growing fish species (e.g., *Tilapia* spp.), crustaceans and shellfish may be considered (McKay *et al.* 1993) and cyanobacteria could be used for feeding them. Cyanobacteria are already used on Earth as a main food source for larvae of many species of fish, zooplankton (itself used for feeding fish larvae), crustaceans and shellfish (Pulz & Gross 2004).

Producing oxygen

O_2 will be one of the most critical resources in human bases on Mars, the most obvious reasons being human respiration and fuel oxidation. But it represents only 0.13% of Mars's atmosphere, against 21% of Earth's. Given the total pressures of both atmospheres, its partial pressure on Mars is more than 20 000 times lower than on Earth. O_2 thus needs to be either brought to Mars or produced there and, as for other resources, the second option is likely the most viable in the long term. On-site O_2 production could be performed using physicochemical methods: by processing regolith, water and/or atmospheric CO_2 (e.g., by combining a CO_2 scrubber, a Sabatier reactor and an electrolyser). However, cyanobacterium-based O_2 production from H_2O and CO_2 could provide a safe redundancy (Sychev *et al.* 2003) and complement physicochemical methods. It could also be less energy demanding, and faster to set up and expand to face unexpected needs.

How much O_2 is needed? Each crewmember consumes about 1 kg of O_2 per day for respiration, assuming 2 h a day of intensive physical exercise (Horneck *et al.* 2003). If produced photosynthetically, this requires the fixation of more than 1.3 kg of CO_2 a day, part of which could be provided, if useful, by

recycling CO_2 produced by the crew's metabolic activity (about 1 kg day^{-1} crewmember^{-1}) and manufacturing activity in addition to using atmospheric CO_2.

Cyanobacteria are very efficient O_2 producers: whereas trees release about 2.5–11 t of O_2 ha^{-1} yr^{-1}, industrial cultivation in open ponds of *Arthrospira* species in Southeastern California release about 16.8 t of O_2 ha^{-1} yr^{-1}. Cultures have been stated to be about 2.5 times more productive in the tropics (Henrikson 2009); under these conditions, about 80 m^2 of culture per crewmember would be needed to cover human respiration needs. However, O_2 production rates can be dramatically increased by photobioreactor-like culture systems which optimize for instance temperature, nutrient flow rates, cell density and illumination. Photosynthetic microorganisms from a eukaryotic microalgal genus, *Chlorella*, have been well-studied in the context of O_2 production for life-support systems. For instance, an experimental system ('BIOS-I') was designed and tested in the 1960s where a man living in a sealed volume had his atmosphere and water regenerated by a *Chlorella vulgaris* culture. This work was preceded by experiments aimed at investigating the potential use of *Chlorella* spp. for air regeneration, performed by Y. Y. Shepelev and G. I. Meleshko at the Institute of Aerospace Medicine, Moscow, in 1960–1961 (Salisbury *et al.* 1997). In BIOS-I, CO_2 excreted by the man and biogenous elements from his urine (N, P, S and K) were fed into the algal culture, which in turn produced O_2 and purified water. Investigators showed that producing 500 g of dry algal biomass per person and per day was enough for water and air generation, and could be achieved in 20 litres only (with a 0.5 cm thick cultivation compartment between 8 m^2 plane parallel walls to maximize light absorption), using one-side illumination with photosynthetically active radiation at 250–300 W m^{-2} (Kirensky *et al.* 1968; Gitelson 1992). The system was further developed, plants were added and various manned closure experiments were performed within 'BIOS-2' and 'BIOS-3', where *Chlorella* sp. was used to recycle part of the air (even though the *Chlorella* compartment was later replaced with additional plant compartments, mainly for food-related issues; see Salisbury *et al.* 1997).

Productivity will depend on conditions which will be provided on site. It should also be noted that the needed resources do not need to be dedicated to O_2 production, as O_2 will anyway be a side product of other processes relying on cyanobacteria and could be coupled with, for instance, food production.

Producing biofuels

If sending it from Earth, fuel would represent most of the load's mass to be sent from Earth and (for the journey back, assuming a return mission) from Mars. If it could be produced on-site, costs and technical challenges would be much reduced.

The next question concerns fuel-type. Dihydrogen (H_2) can be used for reducing local CO_2 to hydrocarbons, and it has been proposed to bring some to Mars to produce methane (CH_4) and H_2O (the latter can then be hydrolysed into O_2 and H_2). H_2 could theoretically be directly used as a fuel: a mixture of liquid H_2 and O_2 have suitable propulsion performances. It is

however much less attractive than CH_4, among other reasons because of its very low density which induces a need for specialized combustion apparatus. In the Mars Direct plan (Baker & Zubrin 1990; Zubrin *et al.* 1991; Zubrin & Wagner 2011), 6 t of H_2 would be used to generate enough CH_4 and O_2 to bring the return vehicle back to Earth and to power ground vehicles on the Martian surface. Mass could be further reduced if H_2 was also obtained on Mars. Many cyanobacteria can produce it; among them, N-fixing heterocystous species such as *Nostoc* and *Anabaena* spp. are the most promising candidates. Their H_2 production activity comes from their nitrogenases. These enzymes mainly catalyse the reduction of N_2 to NH_4^+ but also, in the absence of N, reduce H^+ to H_2. On Earth, though, H_2 photoproduction is currently too low for it to have practical applications. As an example, *A. cylindrica* grown in a standard medium for cyanobacteria (BG11), at ambient air composition and pressure, yields only about 0.2 μmol H_2 mg Chl a^{-1} h^{-1} (Murukesan *et al.* 2015). One of its main limitations is the inhibition by atmospheric O_2 and N_2: nitrogenases are irreversibly inactivated by O_2, while N_2 strongly inhibits H^+ reduction. The high CO_2, low O_2 and N_2 composition of the Martian atmosphere could thus be an advantage here. Consistently, yields above 20 ml l^{-1} h^{-1} (about 0.8 mmol l^{-1} h^{-1}) were obtained with concentrated cultures of about 50 mg Chl a l^{-1} of *A. variabilis* (so, roughly, 16 μmol H_2 mg Chl a^{-1} h^{-1}) under a 99% argon (Ar), 1% CO_2 atmosphere (Liu *et al.* 2006). Recently, levels of about 25 μmol mg Chl a^{-1} h^{-1} were also reached with *A. cylindrica* cultures under high CO_2/low N conditions (Murukesan *et al.* 2015). With a concentration of 50 mg Chl a ml^{-1}, these rates could lead to 1.5 mmol l^{-1} h^{-1} (about 35 ml l^{-1} h^{-1} at room temperature, with 30 μmol mg Chl a^{-1} h^{-1}) of H_2. Producing 6 t of H_2 would then require about 228 m^3 yr. It corresponds, for example, to 22 days in a culture system the size of 3 m-deep Olympic swimming pool, or to 2 years and 4 months in 100 m^3, assuming yields can be maintained at this scale. But these figures are very rough approximations: rates that would effectively be obtained on Mars depend on the mission scenario and technology choices for culture systems. Finally, current productivity is still far from its maximum, and increases are regularly obtained by culture conditions' optimization and metabolic engineering.

Leaving H_2 aside, cyanobacteria can generate various biofuel precursors and components including alkanes, ethylene, hydrogen peroxide (H_2O_2; which can be used as a monopropellant) and lipids, without relying on organic precursors (see, for instance, Quintana *et al.* 2011). They could also provide organic substrates for production of biofuel precursors by other organisms, particularly relevant on Mars where importing substrates from Earth would be impractical. For instance, as mentioned above, lysed cyanobacterial biomass has been used as a fermentation substrate for ethanol production in yeasts (Aikawa *et al.* 2013; Möllers *et al.* 2014). However, genetic engineering could remove the need for other organisms even for production of biofuel precursors which are not naturally produced by cyanobacteria in adequately large amounts – if at all. For instance, some cyanobacteria have been engineered to produce ethanol. Whereas yeasts rely on a sugar-based pathway and thus on the availability of agricultural substrates, engineered cyanobacteria produced it directly from CO_2 and solar energy, following a much simpler process where pyruvate is first converted to acetaldehyde by a pyruvate decarboxylase and then to alcohol by an alcohol dehydrogenase (Deng & Coleman 1999; Dalton & Roberto 2008; Dexter & Fu 2009). Generally speaking, a lot of work has been done to engineer new biofuel (or biofuel precursor) production pathways – or to improve existing ones – in cyanobacteria (see, e.g., Ducat *et al.* 2011). There is still much room for improvement, especially under Mars-like conditions.

Thus, even though extensive development and optimization is needed, cyanobacterium-based biotechnologies could represent original contributions to the production of rocket fuel for the return flight, for powering surface vehicles and, more generally, for powering equipment that can be operated by combustion engines.

Other applications

Various other applications involving cyanobacteria have been proposed for human outposts on Mars. One, for instance, is biomining. Microorganisms are used on Earth to extract metals of industrial interest (e.g., copper and gold) from rocks, and their use on Mars to mine basalt and potential ores has been suggested (Cockell 2010, 2011; Navarrete *et al.* 2012). Cyanobacteria are known to leach a wide range of rock types, including terrestrial volcanic rocks with compositions similar to Mars's regolith (Brown & Sarkisova 2008; Olsson-Francis & Cockell 2010; Olsson-Francis *et al.* 2012). The use of cyanobacteria, possibly engineered to increase their ability to dissolve rocks and harvest specific elements (Cockell 2011), can thus be considered for simple bioleaching processes that would not rely on imported C sources (needed for heterotrophic microorganisms) or toxic elements such as cyanide (used in non-biological leaching processes). Extracted elements could be used within a wide range of chemical and manufacturing processes such as, for instance, CO_2 cracking, electroplating, production of alloys and manufacturing of solar cells (Dalton & Roberto 2008; Cockell 2011).

Cyanobacteria have also been suggested for controlling Mars's surface dust, through the production of biological crusts, in enclosed structures such as greenhouses and habitats (Liu *et al.* 2008). Indeed, some can grow and form biofilms within the interstices of desert minerals and produce extracellular polysaccharides that bind the particles together, preventing wind-induced dust release. Such crusts could also be used as an air filter to remove dust from the atmosphere (Cockell 2010). Then, the use of dehydrated *Chroococcidiopsis* cells as a gene repository for on-site molecular biology has been suggested (Billi *et al.* 2013), as they can preserve plasmids during long-term desiccation (Billi 2012) and presumably repair them when damaged. Cyanobacteria could also be used to process human waste products and recycle their organic C, water, nitrates and mineral nutrients. Cultures could be used directly (Filali *et al.* 1997; Godia *et al.* 2002; Lehto *et al.* 2006; Yang *et al.* 2008) but also indirectly; for instance, H_2O_2 generated from cyanobacterium-produced O_2 and H_2O could be used to oxidize

human wastes following a physicochemical process developed by researchers of the Institute of Biophysics of the Siberian Branch of the Russian Academy of Sciences (Kudenko *et al.* 2000); nutrients could then be recycled in cyanobacterial cultures (Tikhomirov *et al.* 2007). Cyanobacteria have also been suggested for the production, beyond Earth, of various chemicals including nutritional molecules, drugs, bioplastics and cellulosic building materials (Way *et al.* 2011; Menezes *et al.* 2014).

Finally, the ability of cyanobacteria to produce organic material from Martian resources, coupled to our increasing abilities in metabolic engineering, make it possible to consider many other applications ranging from performing basic life-support functions to generating comfort products.

Plants or cyanobacteria?

Assuming that both plants and cyanobacteria can be grown on Mars, some functions such as food and O_2 production could be performed by either or both of them. For these functions, plants are the most commonly proposed photosynthetic organisms. This choice, however, is mostly due to our historical reliance on – and experience with – them: they have been a highly available food source throughout most of human history. Matters need to be reconsidered where environmental conditions, resources and other constraints are different.

Photosynthetic microorganisms are more efficient, on a volume basis, at capturing solar energy than plants. Their culture in photobioreactors could yield much more biomass (especially proteins) and O_2 for a given volume and light intensity than greenhouse-type cultures of staple edible plants (Dismukes *et al.* 2008; Way *et al.* 2011; Wang *et al.* 2012). This is critical where resources are scarce and cultivation areas are highly controlled. Moreover, a considerable part of plant biomass (e.g., roots or stems, depending on species) is inedible and hard to recycle: plant cell walls are among the least degradable polymers in BLSS (Hendrickx & Mergeay 2007).

Plants are also much more demanding in terms of environmental conditions. For instance, they are harmed by anoxia (roughly, a partial pressure of O_2 of at least 50 hPa is needed for proper development, mainly for non-photosynthetic tissues; see Thomas *et al.* 2005) and high concentrations of CO_2 (negative effects have been observed in some plants above 4 hPa [Wheeler 2004], while the partial pressure of CO_2 [pCO_2] on Mars is above 6 hPa). Photosynthetic cultures on Mars, be they vegetal or bacterial, will have to be protected from biocidal environmental conditions. The needed level of protection – and the associated costs – will depend on the ability of cultured organisms to withstand these conditions, and many cyanobacteria are much more resistant than staple plants to the Martian surface's environmental stressors. They thrive in the most extreme habitable conditions on Earth, such as in dry deserts and ice lakes of Antarctica, and within ices of high Arctic seas (Wierzchos *et al.* 2006; Scalzi *et al.* 2012). Some have an outstanding resistance to environmental factors occurring on Mars's surfaces, including ultraviolet (UV) and ionizing radiation (see, for instance, Billi *et al.* 2013 and Thomas

et al. 2006) and can survive in space when protected from UV radiation (Olsson-Francis *et al.* 2010).

In habitable compartments, gas consumed and produced by crewmembers must be balanced with gas consumed and produced by an atmosphere regenerating system. Since CO_2 will be available in the atmosphere and since both CO_2 and O_2 will be generated as by-products of other processes, this system could be more flexible on Mars than in places where simple closed systems would be used: losses could be compensated and excess vented out. However, extensive control of the atmospheric balance would both increase safety and reduce resource consumption. By adjusting culture conditions, controlling cultures' assimilation quotient (CO_2 consumed/O_2 produced) to match humans' respiratory quotient (CO_2 produced/O_2 consumed) is much easier with cyanobacteria than with plants, making the atmospheric O_2/CO_2 balance much more manageable with the former (Averner *et al.* 1984; Horneck *et al.* 2003).

As crewmembers should be available for research and colony settlement and maintenance, time spent on culture management will have to be kept to a minimum. Culturing plants requires significant manpower; within Biosphere 2, for instance, agricultural and food-related tasks took about 45% of crew time (Silverstone & Nelson 1996). In this framework, automation should be extensive. Cyanobacterial cultures are much more suitable for automation than plants' due to their culture homogeneity, growth in liquid medium and lack of inedible parts that should be sorted out. They are also much more manageable; culture parameters could be more easily adjusted to cover human needs, with a safety margin but without excess. Outputs could thus be much more controllable and predictable.

The time needed to establish cultures also matters. Even though shelf-stable food can be sent to sustain crews before cultures are first set up, it is important to be able to re-establish cultures in case of accidental loss – likely enough, and something with catastrophic consequences if not rapidly fixed. Being able to quickly extend cultures to cover unexpected needs (e.g., to compensate an O_2 loss) can also be critical. Even though some plants can be grown faster, staple crops can take 3–4 months to mature even under favourable conditions (Drysdale *et al.* 2003). On the other hand, microbial cultures can be quickly expanded and re-deployed from very small amounts.

Finally, cyanobacteria are much easier to engineer than plants due to their rapid division times, compatibility to transformation, unicellularity and relatively simple genetic background (Koksharova & Wolk 2002; Way *et al.* 2011; Berla *et al.* 2013). They could therefore be much more easily modified for new functions and adaptation to Martian conditions (see section 'Engineering cyanobacteria' below).

It should however be noted that plants have some advantages over cyanobacteria: they could provide tasty and carbohydrate-rich comfort food, and have beneficial psychological impacts on crewmembers (Allen 1991). Establishing small-scale cultures is not obviously unrealistic, especially if nutrients are provided from local resources as described above.

Besides plants, one may wonder why the present paper focuses on cyanobacteria rather than eukaryotic microalgae, which could also perform some of the functions described above. Reasons include cyanobacteria's overall better abilities to use Martian resources (e.g., by N fixation and regolith leaching) and to withstand Martian conditions, the higher digestibility of their edible species and their higher growth and photosynthetic rates. They are also more suitable for genetic engineering, in part due to current transformation systems which are much simpler and well developed for cyanobacteria than for eukaryotic microalgae (Wang *et al.* 2012; Wijffels *et al.* 2013).

What about non-photosynthetic (chemotrophic) microorganisms? Heterotrophs may be useful on Mars, but not as primary producers (see subsection 'Feeding other microorganisms'). Resources needed to feed the metabolism of some chemoautotrophs, on the other hand, may be found on Mars; for example, reduced iron could be used as an energy source by iron-oxidizing bacteria (Nixon *et al.* 2013). Chemoautotrophs could thus be considered for some applications, such as the extraction of industrially useful minerals (Cockell 2010, 2011). However, none has the versatility of cyanobacteria to be the basis for BLSS: none combines, for instance, high N fixation rates, high growth rates, ability to rely exclusively on Martian resources, O_2 production, H_2 production, amenability to genetic manipulation and edible biomass.

Growing cyanobacteria on Mars

All organisms we currently know have evolved on Earth and none of them would be able to grow efficiently on the Martian surface. Cyanobacteria must be provided with shielding and an environment suitable for metabolism and growth. Elaborated hardware systems providing Earth-like conditions have been proposed but they rely on complex technology and require accurate control of all the process parameters (e.g., gases, temperatures and pressures in each compartment), are very demanding in terms of construction materials and energy consumption, need to be constructed on Earth, are very massive and expensive to carry to Mars, and can consequently be applied to small-scale cultures only. In order to be cost-effective and reliable, culture hardware for large-scale, long-term, sustainable BLSS on Mars should be much simpler.

Fortunately, reproducing Earth-like conditions is not needed: cyanobacteria can grow under conditions which are much closer to Mars's. In addition, most inputs – if not all – needed for growing cyanobacteria can be found on-site. An adequate culture system could thus provide a set of parameters (radiation shielding, atmospheric composition and pressure, gravity, nutrient supply, etc.) resulting from a compromise between (i) efficient support to growth and metabolism, and (ii) system feasibility and substrate availability on Mars's surface.

This culture system should be able to resist an inside/outside pressure difference, fine dust, large temperature gradients and strong radiation fluxes. Whatever its final design, efforts should be made to keep its weight, cost and energy consumption as low as reasonably possible given the other

requirements. Ideally, the design should allow manufacturing from on-site compounds (e.g., regolith-based materials for radiation shielding, glass manufactured using silicon dioxide from Martian soil, and metallic parts derived from metal oxides mined in the regolith), assuming that equipment needed for processing is available on site. The potential of ultimately creating many of these facilities with local resources is currently being explored in Lynn Rothschild's laboratory.

Nutrient sources

Most elements needed for feeding plants and microorganisms can be found in Martian regolith.
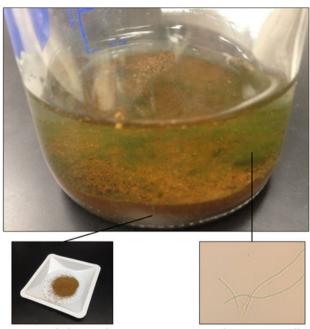
Data on the composition of Martian soils and rocks have been obtained from analyses of the SNC (Shergottites, Nakhlites, Chassignites) group of meteorites (McSween 1994), the Viking (Clark *et al.* 1982), Pathfinder (Rieder 1997) and Phoenix (Hecht *et al.* 2009) landers, the Spirit rover (Morris *et al.* 2004), instruments operated from orbiters – noteworthy the Thermal Emission Spectrometer on Mars Global Surveyor (see Christensen *et al.* 2001), the Gamma Ray Spectrometer on Mars Odyssey (Boynton *et al.* 2007) and the Compact Reconnaissance Imaging Spectrometer for Mars (CRISM) on the Mars Reconnaissance Orbiter (Mustard *et al.* 2008) – and, recently, using X-ray spectrometers aboard the Opportunity and Curiosity rovers. In particular, the latter two have allowed detailed mineral compositions to be deduced at multiple sites in the Endeavour crater (Arvidson *et al.* 2014) and Gale crater (Grotzinger *et al.* 2014; McLennan *et al.* 2014; Ming *et al.* 2014; Vaniman *et al.* 2014). On Earth, basalt is the dominant rock type on the surface. It harbours much of the biosphere and, as an abundant source of redox couples and macronutrients, provides an efficient support to microbial life (for a review in the context of the search for life on Mars, see McMahon *et al.* 2013). Martian regolith is also in large part composed of basaltic minerals; more generally, Mars's surface seems to be mostly basaltic (McSween *et al.* 2009; Taylor & McLennan 2009). All basic elements needed for cyanobacteria and other organisms (C, H, O, N, P, S, K, Mg, Na, Ca, Fe), as well as other elements needed in smaller amounts (Mn, Cr, Ni, Mo, Cu, Zn, etc.), have been detected there.

The most convenient sources of C and N will probably be atmospheric CO_2 and N_2 (see 'Atmospheric pressure and composition' section below). Additional C can be found in the CO_2 ice caps, in the surface and subsurface regolith due to exchange with the atmosphere, and possibly in reservoirs formed when the atmosphere was thicker (Kurahashi-Nakamura & Tajika 2006). It has also been suggested that fixed N, derived from Mars's atmospheric N_2, may be buried in the regolith (Mancinelli & Banin 2003; Boxe *et al.* 2012). Consistent with this, N-bearing compounds have been detected there (Ming *et al.* 2014). However, the exact nature and bioavailability of these compounds has not yet been determined.

Thus, all elements needed to support life seem to be present in Mars's rocks (Cockell 2014) and atmosphere. These nutrients can be directly made available to cyanobacteria, as multiple species thrive in a lithotrophic lifestyle, extracting all

their needed mineral nutrients from rocky substrates (including basalt) and obtaining their whole N and C supply via photosynthesis and biological N fixation. Accordingly, some strains (e.g., *A. cylindrica*) have been grown in distilled water containing only powdered Mars basalt analogues (experiments were performed under terrestrial atmosphere; considerations on what atmospheric conditions are suitable are given in the 'Atmospheric pressure and composition' section below). Non-N fixing cyanobacteria could also grow when $NaNO_2$ was added. On Mars, fixed N could come from N fixers (see above sections), while the possibility that nitrate beds are present cannot be ruled out. Supplementing the media with a sulphate source, $(NH_4)_2SO_4$, had a positive impact on some of the tested species. The authors suggested that, on Mars, gypsum ($NaSO_4 \cdot 2H_2O$) could be used as such a supplement (Olsson-Francis & Cockell 2010). Gypsum dunes have indeed been found in the northern polar region of Mars by the OMEGA instrument on ESA's Mars Express orbiter (Langevin *et al.* 2005) and the CRISM and High Resolution Imaging Science Experiment instruments on NASA's Mars Reconnaissance Orbiter (Roach *et al.* 2007), and later confirmed by the rover Opportunity (Squyres *et al.* 2012). Other studies showed that the growth of several siderophilic cyanobacterial species isolated from iron-depositing hot springs in Yellowstone National Park was stimulated by the presence of Martian soil analogues in culture media (Brown & Sarkisova 2008) and that *Nostoc* sp. HK-01 could grow on a Mars regolith stimulant for at least 140 days, without other nutrient source besides atmospheric gas (Arai *et al.* 2008). Other cyanobacteria have been grown using other Martian soil analogues as substrates, in distilled water or spread on gelified water plates. DLR's P-MRS and S-MRS simulants (Böttger *et al.* 2012), and NASA JSC's Mars-1A simulant (Allen *et al.* 1997), for instance, efficiently supported the growth of *Matteia* sp. and *Anabaena* sp. PCC7120 (see Fig. 4), respectively (Verseux *et al.*, unpublished data). The need for providing regolith might be an issue for automation. However, using drilling photobioreactors that extract raw materials from the surface and directly bring them to cultures has been suggested (Cumbers & Rothschild 2010) and technologies have been designed to excavate large amounts of regolith with minimal weight and time (see, e.g., Mueller & Van Susante 2011).

Thus, even though it might be relevant to adequately mix rock types to have all nutrients in appropriate proportions and suitable pH, and even though some salts, oxides and toxins might need to be removed, all nutrients and micronutrients needed to support cyanobacterial metabolism seem to be present on Mars. Additional nutrients could come from human waste. If some micronutrients (e.g., some cofactors) could not be mined or produced on site, bringing them from Earth would add a negligible mass to the initial payload as they are needed in trace amounts only. Methods for physicochemical preprocessing of Martian regolith and atmosphere (to generate, for instance, a broth of pre-leached regolith and nitric acid in which CO_2 is bubbled; see Cao *et al.* 2014) could be considered, if the increased productivity outweighed the increased running cost and complexity.

Mars regolith simulant   *Anabaena* sp. cells

Fig. 4. *Anabaena* sp. PCC7120 growing in distilled water containing JSC Mars-1A regolith simulant, in Lynn Rothschild's laboratory.

Atmospheric pressure and composition

Use of the minimal suitable pressure would greatly lower construction weight and cost of cyanobaterial culture systems on Mars, and would minimize the risk of organic matter leakage (Lehto *et al.* 2006). Relying on a gas composition which is as close as possible to Mars's would make the establishment of such systems even simpler and cost-effective.

What is this minimal suitable pressure? No clear answer has been given to this question, as little work has been focused on microbial growth at low pressure. The lowest pressure at which biological niches are naturally present on Earth is about 330 hPa (at the top of the Mount Everest), way above Mars's surface pressure of about 5–11 hPa (Fajardo-Cavazos *et al.* 2012). Although viable bacteria have been sampled a few times from stratospheric air above 30 km (e.g., Wainwright *et al.* 2003), where atmospheric pressure goes down to Mars's surface pressure, no microbial growth there was indisputably evidenced. Some methanogens can keep metabolic activity (shown by detectable CH_4 production) at 50 hPa of pressure under simulated Martian environmental conditions (Kral *et al.* 2011; Schirmack *et al.* 2014), and a few bacteria such as *Serratia liquefaciens* (Schuerger *et al.* 2013) and isolates from Siberian permafrost samples (Nicholson *et al.* 2013) have been grown under 7 hPa of CO_2-enriched anoxic atmospheres, but a wide range of microorganisms have been shown to be unable to grow on a semisolid agar medium at pressures below 25 hPa of ambient air (Nicholson *et al.* 2010).

It might be possible to decrease the lowest suitable pressure. As no biological niche is naturally present on Earth at pressures close to Mars's, selective pressure is virtually non-existent for current terrestrial microorganisms and the full potential for

growth at low pressures is probably far from being reached. There might thus be much room for improvement by artificially evolving cyanobacteria to grow faster under low (and to grow at lower) pressures, including low pressures of Mars-like gas compositions. Consistently, an isolate of *B. subtilis* showed increased fitness at 50 hPa after a 1000 generation culture at this pressure (Nicholson *et al.* 2010). The minimal suitable pressure can also be dependent on atmospheric composition, as described below, and on medium type (liquid or solid). It should however be noted that a physical limitation derives from the need to maintain a liquid phase at growth-permissive temperatures.

In recent experiments, a decreased atmospheric pressure (50 kPa instead of the ambient 100 kPa) negatively affected the growth of cyanobacteria from several genera (Qin *et al.* 2014). But this was performed under ambient gas composition and, like atmospheric pressure, atmospheric composition matters. CO_2 and N_2 are present in Mars's atmosphere but their partial pressures differ from Earth's (see Table 1). Mars's higher-than-Earth pCO_2 (6.67 versus 0.38 hPa) might actually be beneficial: elevated levels of CO_2 can have a fertilizing effect on cyanobacterial cultures (Murukesan *et al.* 2015). Below one atmosphere of pressure, CO_2 seems to become the limiting factor and cyanobacteria benefit from much higher-than-normal CO_2 concentrations; *Synechocystis* sp. PCC 6803 was shown to grow more than three times faster under 100 hPa with 5% CO_2 (as well as under 1 bar with 5% CO_2) than under 1 bar of ambient air (0.04% CO_2), and to grow under 33 hPa of 100% CO_2 with growth rates close to those obtained under 1 bar of ambient air (Lehto *et al.* 2007; see the culture system in Fig. 5). Later results showed that an increase in CO_2 concentration (at least up to 20%, even though growth rates started to decrease after 10% for the 1-bar samples) under either 1 bar or 100 hPa leads to higher growth rates than ambient air composition at the corresponding pressure (Murukesan *et al.* 2015; unpublished data). Thus, this strain seems to benefit from higher-than-usual pCO_2, with a saturation about 4 hPa, at which level an around 3.5-fold increase in growth rates is observed for cells previously grown in ambient terrestrial atmosphere. When CO_2 is not limiting, its growth rates are not negatively affected by a 10-fold reduction in atmospheric pressure. Similarly, cultures of *A. platensis* and *A. cylindrica* were shown to benefit from higher-than-ambient CO_2 concentrations (Murukesan *et al.* 2015). Finally, at least some cyanobacteria are able to survive (*Synechococcus* PCC7942, *Anabaena* sp.) and even to grow (*Plectonema boryanum*) in liquid culture under 1 bar (1000 hPa) of pure CO_2, at least in the short term, when pCO_2 is gradually increased by 150 hPa day^{-1} (Thomas *et al.* 2005). If needed, bacterial resistance to high CO_2 levels could probably be further increased by preventing pH decrease in the medium, which happens due to carbonic acid (H_2CO_3) formation when CO_2 dissolves in water.

The most striking shortage of substrates on Mars derives from the low partial pressure of N_2 (pN_2) in the atmosphere and the presumed low availability of N in the regolith. Some work performed with *Azotobacter vinelandii* and *Azomonas agilis* showed that microbial N fixation is possible at a pN_2

Table 1. *Environmental parameters on Mars and Earth surfaces (adapted from Graham [2004] and Kanervo* et al. *[2005])*

Parameter	Mars	Earth
Surface gravity	0.38 g	1.00 g
Mean surface temperature	−60°C	+15°C
Surface temperature range	−145 to +20°C	−90 to +60°C
Mean PAR photon flux	8.6×10^{19} photons m^{-2} s^{-1}	2.0×10^{20} photons m^{-2} s^{-1}
UV radiation spectral range	>190 nm	>300 nm
Atmospheric pressure	5–11 hPa	1013 hPa (mean at sea level)
Atmospheric composition (average)		
N_2	0.189 hPa, 2.7%	780 hPa, 78%
O_2	0.009 hPa, 0.13%	210 hPa, 21%
CO_2	6.67 hPa, 95.3%	0.38 hPa, 0.038%
Ar	0.112 hPa, 1.6%	10.13 hPa, 1%

of 5 hPa, even though below 400 hPa growth rates decreased with decreasing pN_2 (Klingler *et al.* 1989). The lower limit for N fixation might vary among species and is still to be defined, but it has been proposed to be within the range of 1–10 hPa (McKay & Marinova 2001). Crossing this limit using Mars's air composition would require at least 5–50 times the local ambient pressure (reaching a total pressure of approximately 40 hPa to 40 kPa). However, pN_2 would still be limiting and higher values are needed for efficient processes. Rather than only increasing the total pressure to reach adequate pN_2 values, N_2 could be concentrated by separating CO_2 from the other atmospheric gases (mainly N_2 and Ar) and mixing them in different proportions to reach an appropriate pN_2 value in an otherwise optimized total pressure. Gas separation techniques are well developed (see, for instance, Meyer & McKay 1996 and Zubrin & Wagner 2011) and could be based on processes routinely used by industry on Earth. Assuming similar pN_2 needs for cyanobacteria as for *A. vinelandii* and *A. agilis* (see Klingler *et al.* 1989), a pN_2 of 95 hPa would only slightly limit growth (experiments are planned to define the lowest pN_2 allowing efficient growth of diazotrophic cyanobacteria). For wild-type, diazotrophic cyanobacteria, a 100 hPa atmosphere with 95% N_2 and non-limiting (5%) CO_2 could thus lead to higher growth rates than an Earth-like atmosphere. Cultures could also be supplemented with N recycled from human and biomass waste, even though this should not be the only N source as sustainability and expandability would be compromised.

Depending on its adjusted pressure, a Martian-like atmosphere can strongly affect other aspects of the lithotrophic growth of cyanobacteria through indirect effects (e.g., a lowered pH due to H_2CO_3 formation can influence both cell viability and nutrient release from substrates). As for all processes suggested here, extensive and faithful simulations of culture conditions expected to be provided on-site are needed.

Atmosphere of selected gas composition and pressure could be provided within inflatable, tunnel-like containments (Lehto *et al.* 2006). Tight sealing should allow the desired pressure to be maintained, and adjusted gas supply systems could allow CO_2 and some N_2 to be provided from Martian

Fig. 5. Underpressurized culture vials used in Kirsi Lehto's laboratory (at the University of Turku, Finland) to grow cyanobacteria in low-pressure/high pCO_2 atmospheres.

atmosphere – possibly after N enrichment. An onion-like structure, with a pressure gradient throughout layers, has also been proposed. This would offer the additional advantages of a better filtering of the Martian dust and a better thermal insulation (Lehto *et al.* 2006).

Water

One of the most critical resources to provide to cyanobacterial cultures will be water. As water is needed within human outposts, regardless of the use of cyanobacteria, various hardware systems for its extraction and processing on Mars have been suggested elsewhere. There follow a few examples of water sources and mining techniques under consideration. This list is not exhaustive.

Water could be generated on Mars by importing H_2 from Earth and combining it to O from local CO_2 (Zubrin *et al.* 1991) but, even though H represents only 11% by weight of pure H_2O, relying on imported H_2 would strongly limit the autonomy and sustainability of human outposts. However, water can be found in various forms throughout Mars (reviewed in Tokano 2005; Rapp 2007 and Cockell 2014). It is present in large amounts as ice at the north polar ice cap and under the south carbon ice cap, and throughout the planet as near-surface deposits of water ice. The presence of liquid water has to date not been unquestionably established, but several lines of evidence strongly suggest transient liquid brines (Zisk & Mouginis-Mark 1980; McEwen *et al.* 2011; Martín-Torres *et al.* 2015). It might also exist below the cryosphere, in areas where temperatures and pressures are high enough (e.g., Clifford *et al.* 2010), possibly within drilling range. The abundance, location and nature of near-surface water deposits is still to be accurately determined, but mining these to generate usable water may be practical (e.g., Rapp 2013). If it appears that mining liquid water or large ice deposits cannot be done, water could be extracted from the soil's hydrated minerals. Energy requirements for heating soil at 500°C in an oven, before collecting steam, have been assessed to be about 5.2 kWh kg^{-1} of water in a 2%-water soil, plus a small amount (below 0.1 kWh kg^{-1} of water) for excavating and conveying the soil (Stoker *et al.* 1993). Other processes where soil is heated for water extraction are under consideration, including the use

of focused light and of microwaves, the latter being one of the most promising regarding energy requirements, mass and reliability (Wiens *et al.* 2001; Ethridge & Kaulker 2012). A team of the Colorado School of Mines designed a system, referred to as the Microwave Pizza Oven, aimed at extracting water from the soil of Mars using microwaves (generated using about 12 kWh kg^{-1} of water, provided by silicon solar cells) in conjunction with a conveyor belt mechanism to process soil and extract bound water. Expected yields are in the order of 1 kg of water per 2.5 h in a 2%-water soil, for a system's mass below 20 kg (Wiens *et al.* 2001). The use of microwave heating could also reduce dramatically excavation needs, as microwaves could be inserted down bore holes to heat at desired depths (Ethridge & Kaulker 2012). But energy and excavation needs for water extraction from the soil could be further reduced: according to Zubrin & Wagner (2011), a 0.1 mm thick polyethylene dome could be used to farm-selected soil sites by increasing temperature and collecting volatilized water in a cold trap device. Using this approach, a 25 m, 100 kg dome ringed by reflectors could allow about 150 kg of water to be farmed in an 8 h day from a soil with 2% water (Zubrin & Wagner 2011).

There is also water vapour in the atmosphere. Relying on it rather than on soil water would remove the need for processing regolith and/or to move to new locations once a site has been dried up. Concentrations vary through time and location and are very low, but total atmospheric water has been assessed to amount to approximately 1.3 km^3 (1.3×10^9 litres), in very large excess compared with human need for a research base, and extracted water would be renewed naturally by exchange with regolith and polar caps (McKay *et al.* 1993). In spite of the large volumes of atmosphere that need to be processed, water extraction from the Martian atmosphere could be done with an energy consumption below 100 kWh kg^{-1} using a process of atmosphere cooling/compression and water condensation designed by Meyer & McKay (1984) and improved by Clapp (1985). Note that energy used in this process would not be dedicated to water extraction, as atmospheric gas could be isolated using the same setup. Energy needs for water extraction from the atmosphere could be brought further down, as shown by a system referred to as the Water Vapour

Adsorption Reactor (WAVAR). In WAVAR, atmosphere is filtered and drawn through a regenerative adsorbent bed of zeolite molecular sieve, from which water is later desorbed using microwave radiation (Williams *et al.* 1995; Coons *et al.* 1997; Schneider & Bruckner 2003). Energy consumption has been assessed as ranging from approximately 3 to 30 kWh kg^{-1} of water, depending on time and location (Grover & Bruckner 1998). Additional water could be recycled from human metabolism (about 0.4 kg of transpiration water and respiration moisture a day per crewmember) and human waste (Meyer & McKay 1989; Tikhomirov *et al.* 2007). It will also be a by-product of physicochemical processes such as on-site production of biofuels and materials (e.g., Zubrin *et al.* 1997). The systems described above are still in their infancy; increasing knowledge of Mars's resources, coupled to engineering effort, will likely support the development of more efficient water mining technologies and processes.

Strategies can also be developed to minimize water needs for cyanobacterial culture systems, in addition to recycling water from culture effluents. For the production of some products where cells lysis is not needed, water requirements (as well as requirements for maintenance, nutrients and energy) could be reduced by immobilizing cyanobacteria within polymeric matrices. Such entrapment can preserve cyanobacteria's metabolic activities for prolonged periods of time, up to several years (Lukavský 1988; Hertzberg & Jensen 1989; Chen 2001). Water requirements could be further reduced by growing terrestrial cyanobacteria as biofilms, directly on the surface of Martian rocks, in a semi-closed environment where suitable temperature, pressure and moisture are provided. Such a growth system could be relatively close to the natural lifestyle of rock-dwelling cyanobacteria, noteworthy in terrestrial deserts (see, e.g., Friedmann & Ocampo 1976; de la Torre *et al.* 2003; Warren-Rhodes *et al.* 2006 and Billi *et al.* 2013) but with more favourable moisture, UV protection and temperatures.

Finally, a reduced pressure at growth-permissive temperatures, as envisioned in the culture system, will foster water evaporation. However, if pN2 sets the lower limit for atmospheric pressure and the culture system provides about a tenth of Earth's atmospheric pressure at sea level (see subsection 'Atmospheric pressure and composition'), evaporation can easily be reduced by saturating the incoming air with water vapour (Kirsi Lehto, personal communication). Besides, as the system needs to be air-tight (apart from controlled gas exchange), water loss by evaporation can be minimized.

Solar energy and harmful radiations

On Earth, the solar flux is in large excess compared with the needs of cyanobacteria and green plants, which cannot use more than about 10–20% of maximal sunlight on the surface (Way *et al.* 2011). Some cyanobacterium species such as *Arthrospira* spp. can utilize higher light densities but, at least for *A. platensis*, the radiation level for maximal photosynthesis activity is well below terrestrial mean day values – provided agitation intensity and cell density allow sufficient exposure (Hoshino *et al.* 1991). Even though Mars is on average 1.52

times farther from the Sun than Earth and consequently receives about 43% as much sunlight at comparable latitude and time of day, this is in excess compared with photosynthesis needs, even in the case of a dust storm (McKay *et al.* 1993). The light flux needed for optimal photosynthesis of many cyanobacterial species is about 3×10^{19} photons m^{-2} s^{-1}, which is only about 10% of Mars's average ambient light flux (Lehto *et al.* 2007). At lower light levels photosynthesis is still possible, albeit at lower efficiencies. It has for example been assessed that, at midday at vernal equinox, light levels on Mars are about 5000 times greater than the minimum required for photosynthesis (Cockell & Raven 2004).

It might be thought challenging to let photosynthetically active radiation (PAR light; 400–700 nm) reach cultures while protecting the latter from harsh radiation – namely UV radiation, solar energetic particles (SEP) and galactic cosmic rays (GCR). First, the fraction of biologically effective UV radiation reaching the surface of Mars is much greater than that reaching the surface of Earth and includes UV-C radiation (<280 nm). The models of Cockell *et al.* (2000) and Schuerger *et al.* (2003) predict a maximum flux of around 50 W m^{-2} of UV$_{200-400\,nm}$ irradiance on the equatorial Martian surface at the mean orbital distance. Recent data from the Curiosity rover suggest lower values, with a maximum UV$_{200-380\,nm}$ irradiance of about 20 W m^{-2} recorded at midday at Gale's crater (Gómez-Elvira *et al.* 2014). Despite being strongly germicidal on the surface, this radiation can be blocked by a few millimetres of Martian dust coverage (Mancinelli & Klovstad 2000; Dartnell & Patel 2014) or, to let PAR light in, by mere transparent covers such as glass filters.

Besides UV, ionising radiations of SEP and GCR can reach the Martian surface and subsurface with high energies due to Mars's lack of intrinsic magnetic field and thin atmosphere. Pavlov *et al.* (2012) and Dartnell *et al.* (2007) assessed absorbed dose rates at the surface to be in the range of 50–150 mGy yr^{-1}, and data from the Curiosity rover indicate an absorbed dose rate of 76 mGy yr^{-1} at Gale crater (Hassler *et al.* 2013). Could cells be protected from this radiation? Even though the primary cosmic radiation component decreases with shielding, secondary particles (lighter particles including neutrons and gamma-rays) form when radiation penetrates substrates and dose rates increase with shielding until the Pfotzer maximum, before decreasing due to energy loss, absorption and decay processes. From 76 mGy yr^{-1} at the surface, for instance, they reach 96 mGy yr^{-1} under 10 cm of Martian rock with a density of 2.8 g cm^{-3} (Hassler *et al.* 2013). The design of culture chambers and the choice of material used for shielding should take into account their interactions with primary radiations on the Martian surface (see Le Postollec *et al.* 2009).

Approximately 5 m of Mars dirt would confer a protection to radiation equivalent to Earth's atmosphere (McKay *et al.* 1993). However, bacterial cells are overall much more radiation-resistant than human cells and such a protection is unlikely to be needed. In addition, cyanobacteria are known to have high ploidy levels (Griese *et al.* 2011), pigments and specific morphological features which tend to increase radio-

resistance. The resistance of cyanobacteria to photon irradiation (gamma-rays and X-rays) has been studied as early as in 1951 (Bonham & Palumbo 1951), and the high resistance of some species was observed in the early 1960s (Shields *et al.* 1961; Godward 1962). In the following decade, the resistance of a wide range of species from various genera was tested. Resistance appeared to be highly variable among species, ranging from sensitive ones with D_{10} (dose required to reduce the viable number of cells by 90%) below 1 kGy to highly resistant ones with D_{10} above 10 kGy (e.g., Shields *et al.* 1961; Godward 1962; Bruce 1964; Kumar 1964; Kraus 1969; Asato 1971). These results were confirmed and deepened by recent studies. *Synechococcus* and *Synechocystis* spp. were shown to be relatively sensitive to gamma rays, with D_{10} of about 0.3 kGy (Domain *et al.* 2004; Agarwal *et al.* 2008) and 0.7 kGy (Domain *et al.* 2004), respectively. Two *Anabaena* strains were shown to tolerate a 5 kGy gamma-ray dose without loss of survival, to have a GI_{50} (dose where growth is inhibited by 50%) of 6–11 kGy and to survive doses of 15 kGy (Singh *et al.* 2010; Singh *et al.* 2013). *Arthrospira* sp. PCC 8005 cells were shown to be able to survive exposure to doses of at least 6.4 kGy of gamma irradiation, 1 kGy of He particle radiation and 2 kGy of Fe particle radiation (Badri *et al.* 2015). *Chroococcidiopsis* spp. cells were shown to withstand 2.5 kGy of X-ray irradiation in liquid culture with small to medium viability loss: 20–65%, depending on the strain, with a D_{10} of 2–5 kGy (Billi *et al.* 2000a). Viable *Chroococcidiopsis* spp. cells were also recovered after exposure to 15 kGy of X-rays (Billi *et al.* 2000a) and to 12 kGy of gamma-rays (Verseux *et al.* manuscript in preparation). Moreover, no significant DNA or membrane damage was detected after exposure to 1 kGy of He particle radiation, 2 kGy of Fe particle radiation or 1 kGy of Si particle radiation (Verseux *et al.* manuscript in preparation). A dose of 1 kGy roughly corresponds to a thousand years on the Martian surface, a timeframe way beyond that of division and repair of metabolically active cyanobacteria; cells are therefore not expected to be affected in BLSS cultures. The biological effect of ionizing radiation cannot be assessed based on the dose only: other parameters are to be taken into account – for instance, the composition of the radiation flux and its modification when interacting with the environment and the cells' properties – but the studies mentioned above show the orders of magnitude involved. Even if the effect of radiation on Mars was, at equivalent dose, more damaging by several orders of magnitude than the radiation flux used in these studies, the replication time and repair dynamics of even slow-growing strains would be way below the time needed to receive a sterilizing dose. Consistently, it has been estimated that even vegetative cells of the bacterium *E. coli* could survive the ionizing radiation dose that would be received after more than a thousand years on the surface of Mars (Dartnell *et al.* 2007). More generally, ionizing radiation on Mars is not expected to prevent microbial life (Dartnell *et al.* 2007; Horneck 2008).

Cultures could be buried inside regolith or covered with regolith-based material: although not expected to be needed for radiation protection, regolith shielding could be relevant for temperature control (taking advantage of the regolith's thermal insulating properties) and protection from the wind and dust. Manufacturing processes could be simple and cost-effective, taking for instance advantage of: (i) the cement-like properties of unprocessed regolith mixed with water (McKay & Allen 1996); (ii) the ubiquity of clay-like materials on Mars; (iii) the large fraction (about 40% by weight of Viking 1 and 2 soil samples) of Martian soil represented by silicon dioxide (the basic constituent of glass); and (iv) the fact that plastics may be derived from local C and H (Zubrin & Wagner 2011). In this case, artificial lighting, mirrors or fibre optics could be used to bring PAR light to the cultures. Lighting could also be electrically powered, using for instance solar, wind or geothermal energy sources. Various studies have been performed regarding electrical lighting for plants in BLSS (easily adaptable to cyanobacterial culture), light-emitting diode (LED)-based systems being the most promising (Massa *et al.* 2007). However, even though electrical lighting would allow an accurate control on light intensity and photoperiods, energy and mass requirements could be greatly reduced – compared with electrical lighting – by using systems based on fibre-optics technology to harvest and transmit selected wavelengths from solar energy. Several of such systems have been developed in the context of space exploration, among others for the cultivation of microbial phototrophs (Mori *et al.* 1987) and plants (Jack *et al.* 2002; Nakamura *et al.* 2009). Equivalent system mass calculations showed a net benefit of using solar lighting rather than electric lighting in this context, under realistic mission assumptions (Drysdale *et al.* 2008). The cost effectiveness of such systems may be limited by temporary decreases in light availability (due to diurnal and seasonal light cycles, variable distance from the Sun and global dust storms), which drives a need for increasing collector size relative to culture areas, and may consequently be inadequate for growing plants in Mars BLSS (Massa *et al.* 2007). They could however represent a solution for growing cyanobacteria, which use light more efficiently and suffer less than plants from temporary reduction of light and from changes in illumination patterns. That being said, the simplest and most cost-effective way of providing PAR could be to directly expose cultures to solar light while protecting them from lethal levels of radiation. A transparent but UV-blocking and thermal insulating material, which should also be resistant to Mars surface conditions in the long term, could be used. Radiation coming from straight above could also be stopped by regolith-based materials, while allowing light to come from the sides at adequate intensities for photosynthesis to occur (de Vera *et al.* 2014).

Heating will be needed to maintain liquid water and allow metabolism. Heating systems could be either directly based on solar energy, or on electricity generated by solar energy (e.g., using solar panels) and/or based on other power sources used within Martian outposts (e.g., wind, geothermal activity or fuels produced on site). This issue is largely documented elsewhere, as it applies to other components of human colonies such as habitats, and will not be detailed here.

Gravity

Would Mars's lower-than-Earth gravity affect cyanobacteria? The earliest microbiology experiments in space, reported in the early 1960s, did not show any effect of microgravity on individual cells (Zhukov-Verezhnikov *et al.* 1962). Consistently, theoretical studies suggested that microgravity would not directly affect cells of a diameter below 10 μm, in part due to the density uniformity and smallness of intracellular components (Pollard 1965, 1967). Later, experiments on-board the orbital station Salyut 6 and biosatellites Kosmos 1887 and 2044 showed that unicellular algae were not affected in their development by microgravity (Sychev *et al.* 2001). However, a wide range of altered behaviour and growth properties such as increased virulence, reduced lag phase, increased final cell population, increased productivity of secondary metabolites and increased conjugation rates have been reported in later microbiology experiments in spaceflight and simulated microgravity (see, e.g., Nickerson *et al.* 2000; Benoit & Klaus 2007 and Wilson *et al.* 2007), in various prokaryotes including cyanobacteria (Wang *et al.* 2004, 2006; Xiao *et al.* 2010). A well-supported hypothesis suggests that these effects are motility-dependent, with non-motile cells being the most affected. This might be explained by the reduced flow of metabolites and nutrients and by the reduced exchanges between bacteria and the environment (e.g., because mass-driven convection does not occur), which result in a modified chemical environment around cells that alters biological responses (Horneck *et al.* 2010). However, microgravity was shown not to reduce N fixation abilities, photosynthetic O_2 production rates or growth in cyanobacteria (e.g., Wang *et al.* 2006). Mars's reduced gravity (0.38 g) is consequently not expected to be an obstacle to cyanobacterium-based processes, especially if stirring generates a non-limiting flux of nutrients and metabolites. Studies aimed at confirming that reduced gravity do not alter cell processes of interest would however be useful.

Engineering cyanobacteria

In addition to adapting the Martian environment to cyanobacteria, work can be done to adapt cyanobacteria to the Martian environment. Synthetic biology may provide the tools for this. This field, or set of fields, aroused NASA's interest due to its potential for engineering microorganisms with useful features for resource production in space (Cumbers & Rothschild 2010; Langhoff *et al.* 2011; Menezes *et al.* 2014; Verseux *et al.* 2016). In the present context, the use of synthetic biology tools and methods is investigated for optimizing the abilities of selected cyanobacteria to: (i) withstand environmental stresses faced during space exploration missions, and (ii) grow and perform biological functions of interest under on-site constraints (see Fig. 6).

Increasing cyanobacteria's abilities to withstand extreme conditions would allow failure risk and culture system-related costs to be minimized (Cockell 2010; Olsson-Francis & Cockell 2010). First, for storage during the journey: cells' tolerance to long periods of dehydration, possibly in a differentiated state (e.g., akinetes), would allow a storage free of risk of loss by freezing failure. Secondly, for growth on site: higher tolerance to Mars's environmental factors would reduce the required level of shielding. It would also increase safety in case of system malfunction during which cultures could be exposed to harsher conditions (e.g., desiccation, low pressure, high radiation levels, altered pH and sudden temperature shift), either when stored or grown. Thirdly, increasing the abilities of cyanobacteria to rely on on-site resources would allow productivity to be increased while relaxing constraints and supplementation needs. In particular, it would be highly beneficial to increase their abilities to leach Martian mineral resources and to get most of their nutrients from these, within a wide range of pH, and to fix N at a lower pN_2. Finally, new functions and nutritional properties could be engineered and optimized under conditions that would be provided within Mars bases.

To increase their resistance, cyanobacteria could be transformed with heterologous genes known to increase fitness under conditions found on the surface of Mars (Cumbers & Rothschild 2010). This approach has been successful in other contexts: for instance, *E. coli*'s resistance to gamma irradiation, to desiccation and to low temperatures has been increased by expressing, respectively, the PprI protein from *Deinococcus radiodurans* (Gao *et al.* 2003), a sucrose-6-phosphate synthase from *Synechocystis* sp. strain PCC 6803 (Billi *et al.* 2000b) and the chaperonin Cpn60 and co-chaperonin Cpn10 from the psychrophilic bacterium *Oleispira antarctica* (Ferrer *et al.* 2003). Once specific genes have been shown to confer an advantage to a targeted stress in a target organism, they could be improved using various computational and molecular biology tools and methods. These are becoming more and more efficient thanks to, among other factors, a sharp decrease in DNA synthesis costs, the improvement of automated gene assembly methods, and the development of biological computer-aided design (BioCAD) and other computational tools.

However, despite the fact that expressing heterologous genes (or overexpressing homologous genes) may confer a significant advantage in coping with some environmental stressors, this approach might be much more challenging for resistance features which are highly multifactorial, each individual factor having a relatively weak impact. There is not a single factor that confers on *D. radiodurans* its extreme radiation resistance, for instance, but a very wide combination of features, including efficient DNA repair mechanisms, anti-oxidation mechanisms and specific morphological features (see, for instance, Slade & Radman 2011). For this kind of feature, dramatic increases through rational design seem very challenging given bio-engineering's current state-of-the-art. Instead, directed evolution – iterations of mutagenesis and artificial selection – at the scale of the whole organism can allow complex modifications, affecting both known and unknown mechanisms. The dynamics of experimental evolution have been widely studied in recent decades and have been successfully used to increase organisms' specific properties (see, e.g., Elena & Lenski 2003 and Conrad 2011), including radiation resistance in bacteria
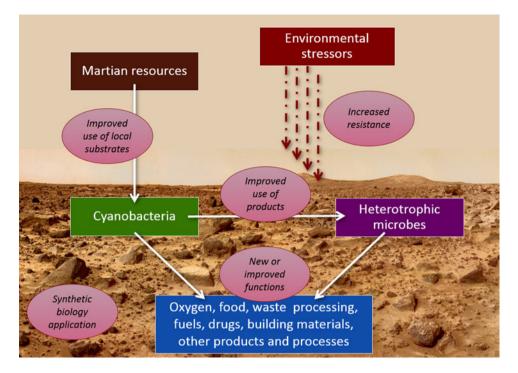
Fig. 6. Simplified overview of the potential roles of synthetic biology in the development of Mars-specific, cyanobacterium-based BLSS.

(Ewing 1995; Harris *et al.* 2009; Wassmann *et al.* 2010; Goldman & Travisano 2011).

For increasing the abilities of cyanobacteria to use resources found on-site, and for conferring on them abilities (and/or improving these abilities) to produce resources of interest from local resources, rational genetic engineering might be more efficient than it is for increasing resistance. Some clues have even been given regarding the engineering of microorganisms with increased bioleaching abilities (Cockell 2011). However, despite metabolomics's great advances in the last decade and the synthetic biology benefiting from it (see, for instance, Ellis & Goodacre 2012 and Lee 2012), the complex interactions occurring in cells are still hard to predict and whole-cell-scaled directed evolution will here again be very useful.

One of the main issues when designing an optimization process based on directed evolution is the need for either linking the optimized function (e.g., production of a compound of industrial interest) to organisms' fitness or selecting mutants after screening. When increasing resistance to environmental stressors (e.g., low pressure or radiation) or abilities to use a given nutrient source (e.g., Martian regolith or atmosphere), the process is more straightforward: selection can be done by applying increasing levels of the targeted stress (when improving resistance) or by conducting growth/re-inoculation cycles to let fast-growing mutants become dominant (when improving use of a given nutrient source). Directed evolution can be improved by automation (Dykhuizen 1993; de Crecy *et al.* 2009; Marlière *et al.* 2011; Grace *et al.* 2013; Toprak *et al.* 2013) and by recent methods such as, for instance, genome shuffling (Patnaik *et al.* 2002) and multiplex genome engineering (Wang *et al.* 2009). It might also be more efficiently performed on Mars, once a microbial production system is well

established and automated (Way *et al.* 2011): Earth-based simulations of some of the factors encountered on Mars (and their combinations) are difficult, expensive and cannot faithfully reproduce all of their effects. The most straightforward use of directed evolution would be to improve cyanobacterial cultures' productivity by, for example: (i) increasing the abilities of cyanobacteria to grow faster under low (and to grow at lower) pressures, including low pressures of Mars-like gas compositions; (ii) improving their efficiency to extract mineral nutrients from Martian regolith; and (iii) increasing their resistance to potentially toxic elements in the regolith (e.g., perchlorates). As selective pressure for some of these factors is low or inexistent on Earth, fitness under these conditions is likely far from its evolutionary potential and could be largely increased by directed evolution. The amounts of resources allocated to cyanobacterial cultures for producing a given amount of other resources could thereby be significantly reduced. Besides direct applications, performing directed evolution could give clues on whether and how much organisms can genetically adapt to the Martian environment, and therefore help assessing the risk of biological contamination in case of accidental release.

Rational genetic engineering and directed evolution are not mutually exclusive: they can be used in combination (Rothschild 2010). Rationally engineered organisms can be submitted to directed evolution for optimization and, conversely, data obtained from genome sequencing of evolved organisms (so as to understand which mutations are responsible for improved properties) can give gene targets for rational design.

Besides increased resistance and abilities to thrive on local substrates, synthetic biology could be used to confer on

cyanobacteria specific functions for Mars-specific BLSS. Numerous genetic engineering tools and methods have been developed for enhancing their metabolic capabilities. Various applications which are considered on Earth (see, e.g., Wang et al. 2012 and Berla et al. 2013) could be useful on Mars as well, but others might be developed specifically for this planet (Verseux et al. 2016). Some resources may be much easier to obtain using synthetic biology when on Mars, while being easier to obtain by other means (e.g., by chemical methods, using the natural host or by simply harvesting rather than producing them) when on Earth. Finally, metabolic pathways might differ: the set of potential starting compounds will be much reduced on Mars and some substrates that are cheap and abundant on Earth will be extremely hard to provide there, changing the cost-effectiveness of a given pathway.

A critical issue to be faced is genetic instability: artificial gene constructs might be lost. Plasmids are useful for screening and optimization steps, but are generally too unstable to be used on a large scale and in the long term. Exogenous genes should rather be irreversibly inserted in cyanobacteria's genomes, which leads to much higher stability and more control over expression levels (e.g., Herrero et al. 1990; Heap et al. 2012). Genome insertion in cyanobacteria is made harder than in most model bacteria by their polyploidy and the unviability of recA mutants (Murphy et al. 1990; Matsuoka et al. 2001), but specific methods are available to do so (e.g., Andersson et al. 2000; Takahama et al. 2004; Clerico et al. 2007; Chaurasia et al. 2008). For standard, organism-scaled directed evolution, genome integration will not be an issue as variability will mostly affect genomes. However, even when integrated into a genome, modifications may be lost. How commonly this phenomenon is encountered among teams performing cyanobacterium genetic engineering, and how limiting the issue is, is hard to assess due to a tendency not to document failures (Jones 2014). Many reports of successful and stable genetic constructs in cyanobacteria can be found (e.g., Guerrero et al. 2012; Bentley et al. 2014; Dienst et al. 2014), but a few others document instability (e.g., Takahama et al. 2003; Angermayr et al. 2012). In spite of the lack of available data, it is expected that modifications having a negative effect on growth (e.g., insertion of a system to produce a chemical of no use to the producing cyanobacteria) is more likely to be counter-selected than modifications conferring an advantage on cyanobacteria (e.g., a better ability to use provided substrates or to withstand environmental stress factors) under a given selection pressure, as far as this pressure is maintained. Studies are needed to determine how problematic genetic instability in cyanobacteria is and to develop appropriate solutions

Future research needs

What would be the efficiency of a cyanobacterium-based BLSS? This question can currently be answered only tentatively. There follows an example of a scenario, with some back-of-the-envelope calculations. Using a strain of wild-type *Anabaena* sp. and assuming that regolith on Mars is as suitable as a substrate as terrestrial basaltic analogues, obtaining a saturated culture from a tenfold dilution takes, roughly, 1 month (Olsson-Francis & Cockell 2010; Olsson-Francis et al. 2012; Verseux et al., unpublished data), without taking into account the growth-enhancing effects of a low-pressure/high-CO_2 atmosphere and of potential nutrients from recycled human waste products. Starting with a 1 litre culture (or from cyanobacteria concentrated from 1 litre, to further reduce volume and mass) and if the limiting factor was cyanobacterial growth, a 10^3 m³ (10^6 litre) saturated culture could be obtained within 6 months, and a 1 km³ (10^{12} litre) culture within 1 year. Assuming growth rates and final biomass yields as in Olsson-Francis & Cockell (2010)'s experiments with a basaltic regolith simulant, growth rates would be roughly 0.3 day^{-1} and biomass at saturation 100 mg l^{-1}. With a 10^3 m³ culture, 1.5×10^5 litres of culture (corresponding to 15 kg of dry biomass) could be harvested every day. How do these figures relate to needs in BLSS? Assuming energy contents similar to *A. platensis*'s (Tokusoglu & Unal 2003), about 500 g of dry biomass per crewmember would cover the crew's caloric needs (as mentioned above, covering caloric needs do not imply providing a healthy diet). If cells produce O_2 with yields per unit of dry biomass as *C. vulgaris* in BIOS-I (Kirensky et al. 1968; Gitelson 1992), the amount of culture used as food would also provide enough O_2 for crewmembers' respiration. Based on preliminary results, a 10 litre batch of lysed cyanobacteria could yield 10 ml of a culture medium for heterotrophic microorganisms in which *E. coli* could reach more than 10^9 cells ml^{-1} in 24 h; the amounts of cyanobacterial culture allocated to the growth of heterotrophic microorganisms would thus be negligible for most applications. Assessing the conversion efficiency of cyanobacterium-to-plant biomass would be too tentative without further experiment; however, given that more than 90% of plants' dry weight comes from C, O and H (see, for instance, Latshaw & Miller 1934), which can be obtained by plant photosynthesis from CO_2 and H_2O, it is reasonable to assume that the plant mass-to-cyanobacterium mass ratio can be much higher than 1, as with nutrients provided in commercial hydroponic systems.

These estimations are far from accurate; firstly, because productivity will be highly dependent on choices which are still to be made and, secondly, because too much biological data are missing. In addition, these estimations are on the pessimistic side, as they are based on preliminary experiments performed in conditions which were not optimized. In this scenario, the time to develop large cultures is not an issue (crewmembers could rely on resources imported from Earth during the first months) but outputs per resource unit can be, and should be, extensively improved. Consequently, much work is still to be done before functional cyanobacterium-based BLSS are developed. Some of the needed tasks, mostly dealing with optimization and characterization, are briefly described below. This list is not exhaustive and the mentioned requirements have room for flexibility.

Firstly, the most promising strains should be selected according to their relevance for Mars BLSS. Selection criteria can include their abilities to grow under conditions easily

providable within Mars bases (mainly, to leach Martian regolith analogues, to efficiently fix N, and to be undemanding and flexible regarding nutrient inputs), their resistance to environmental conditions to be faced on Mars and during the journey, their growth rates, their suitability for genetic engineering, their abilities to perform specific tasks and/or their nutritional properties. The best option may be a combination of species rather than a single species, but versatility could reduce the number of strains and keep the system simple. Among interesting candidates are species from the genera *Arthrospira, Cyanothece, Chroococcidiopsis, Anabaena/Nostoc* and *Synechococcus/Synechocystis*. As described above, *Arthrospira* species are well studied in the context of life support because of the high productivity of their cultures, their edibility and their nutritional values. However, these species are demanding in terms of culture conditions, do not seem to efficiently leach regolith (Olsson-Francis & Cockell 2010) and do not fix N. *Cyanothece* sp. ATCC 51142 has been proposed as a complement to plants in BLSS due to its relatively high growth rates, N fixation abilities, versatile metabolism and probable edibility (Schneegurt *et al*. 1995, 1996; Schneegurt & Sherman 1996). *Chroococcidiopsis* species (see Fig. 7) have also been suggested as BLSS components (Billi *et al*. 2013), as they are dominant in terrestrial sites considered as Mars analogues due to radiation, rock composition, drought and extreme temperatures, and have been shown to be highly resistant to various simulated space and Martian conditions (Thomas *et al*. 2006; Cockell *et al*. 2011; Baqué *et al*. 2013, 2014). Another advantage comes from their resistance to long-term desiccation (Billi 2009), which is of strong interest for long-term storage: cells could be carried in a dry state and be rehydrated upon arrival. Among bacteria that can withstand long-term desiccation, the only species that can currently be genetically engineered belong to this genus (Billi 2010, 2012). Their main drawbacks are their slow growth rates (depending on the strain, their generation time ranges from about 4 to 16 days; see Billi 2010) – a common feature among rock-interacting extremophiles (Cockell 2011) – and, as in the case of *Arthrospira* spp., their need for fixed N supplementation (a few studies suggest that some strains could fix N under specific conditions [Boison & Mergel 2004; Banerjee & Verma 2009], but this is still to be confirmed). Then, species from the closely related *Anabaena* and *Nostoc* genera (see Fig. 7) seem very promising. In addition to their high growth rates (by cyanobacterial standards), they can fix N and some species have extensive bioleaching abilities (Arai *et al*. 2008; Olsson-Francis & Cockell 2010; Olsson-Francis *et al*. 2012). When cyanobacterium species from different genera were tested for their abilities to grow in distilled water containing only powdered analogues of Mars basaltic rocks, *A. cylindrica* had the maximum biomass production and the greatest specific growth rate under these conditions (Olsson-Francis & Cockell 2010). This species also forms akinetes, providing high resistance to simulated Martian surface conditions (Olsson-Francis & Cockell 2010), to simulated space conditions and to low Earth orbit conditions (Olsson-Francis *et al*. 2009). Some species are suitable for genetic engineering and *Anabaena* sp. PCC7120 has already
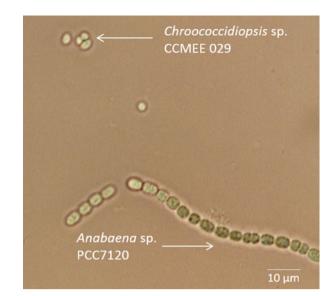

Fig. 7. Two examples of cyanobacteria: *Anabaena* sp. PCC7120 and *Chroococcidiopsis* sp. CCMEE 029.

been modified to produce and secrete sucrose, which can be used to sustain the growth of heterotrophic organisms (Stanford-Brown 2011 iGEM team 2011). Several strains, notably *A. cylindrica*, are very efficient H_2 producers (Abed *et al*. 2009). Additionally, some strains are edible: *N. commune, N. flagelliforme* and *Anabaena* spp. in symbiosis with *Azolla* spp., for instance, are traditionally consumed. Finally, using model cyanobacteria such as *Synechocystis* spp. and *Synechococcus* spp. might also be a fruitful approach: their natural metabolic and resistance properties may be less appealing than those of the above-mentioned strains, but their high growth rates, well-characterized metabolism and availability of engineering tools (Berla *et al*. 2013) could make them relevant chassis organisms, provided extensive engineering is performed. Whether emphasis should be put on extremophile properties or metabolic functions of interest when selecting strains in this context has been questioned. The answer might vary according to the scenario, and stages, of colonization: if protection can be provided at low cost, for instance, the need for resistance is reduced and priority can be given to metabolic functions. It may also depend on the development of our abilities to confer new metabolic pathways on extremophiles and to increase resistance of species of metabolic interest. A summary of the main advantages of the genera mentioned in this paragraph is given in Table 2. Generally speaking, a relatively low number of cyanobacterium species and strains have been tested so far against factors relevant for BLSS. Systematic tests could point out new candidate species and strains. After and/or in parallel to the selection, genetic engineering can be used to improve the relevance of the strains of interest.

Next, a compromise should be identified between (i) conditions (radiation shielding, atmospheric composition and pressure, gravity, nutrient supply, etc.) optimizing growth and metabolism of the selected microorganisms, and (ii) conditions that depend as much as possible on resources available on site

Table 2. *Examples of cyanobacterial genera of relevance for Mars-specific BLSS*

Genera	Most relevant specific features
Arthrospira	Edible species with excellent nutritional properties; productive under adequate conditions; demanding in terms of culture conditions
Cyanothece	High growth rates; N fixation; suspected edibility
Chroococcidiopsis	Extremophile; can be stored desiccated for several years; can maintain exogenous plasmids
Anabaena/Nostoc	N fixation; symbioses with plants; edible species; efficient lithotrophic growth on regolith simulant; efficient molecular biology tools available; H_2 production; form highly resistant akinetes
Synechocystis/ Synechococcus	Fast-growing; well-studied metabolism; many genetic engineering tools available; efficient growth demonstrated in low pressure/high CO_2 atmospheres

and that minimize costs (e.g., low atmospheric pressure mainly composed of CO_2 and N_2). The minimal parameters needed for efficient metabolism of photosynthetic microorganisms remain poorly defined and, even more than extreme values of individual parameters, the effects of their combinations are poorly documented (Harrison *et al.* 2013). Extensive characterization work is thus needed. More specifically, conditions closest to Martian surface conditions while supporting growth, photosynthetic activity and, for some species, N fixation and H_2 production, should be defined. In particular, the minimal pressure of a gas mixture similar to the Martian atmosphere (possibly enriched in N_2, though) which is suitable should be identified. The use of Martian regolith as a nutrient source for all compounds except C and N should also be studied in this context, as microorganisms' interactions with minerals are poorly characterized, even in ambient terrestrial environments. It should be noted that suitable culture conditions are expected to be organism- and application-specific and, as described above, all these features could also be increased by genetic engineering. In any case, the genetic stability of the strains of interest under expected culture conditions should be tested to ensure that the efficiency of targeted processes and nutritional properties are not affected in the long term: targeted modifications may be lost (as described in section 'Engineering cyanobacteria'), but mutations can also appear at other genomic loci and spread, even in the absence of a strong selection pressure (e.g., Kanesaki *et al.* 2012; Trautmann *et al.* 2012).

Once a compromise between efficient growth and low cost has been identified, culture hardware that can provide the selected conditions directly on Mars's surface, from on-site resources, can be designed. Extensive on-ground testing should be performed to demonstrate operational capability of all the components of the system. A prototype could then be tested for its ability to support the growth of cyanobacteria in the long term in simulated Mars surface conditions

If edible cyanobacteria and plants are to be used as food during Mars missions, whether their nutraceutical properties are affected under the expected conditions should be investigated. A suitable diet could then be designed, which would cover nutritional needs while being psychologically satisfying in the long term. It might involve a combination of processed cyanobacteria and plants to balance protein, carbohydrate and lipid ratios while having a pleasant taste, although adequate processing and genetic modifications

of cyanobacteria may make the use of plants unnecessary in this context.

Outputs (biomass, released metals, O_2, etc.) under the expected culture conditions should be accurately characterized for a clear vision of the scale needed and to assess the relevance of the system in various mission scenarios, including varying duration of mission and crew size. In particular, it would be useful to determine in which cases this system would be advantageous compared with stored supplies and resupply from Earth. How many resources could be produced, and how many crewmembers could be supported as a function of starting compounds (e.g., regolith amounts, atmospheric gas and culture supplements), initial payload mass, needed cultivation surface, crew-time and energy consumption? Extensive mathematical modelling, including results from laboratory simulations, would be useful. Some physical tests should be conducted on Earth (where the experiment can be stopped in case of a major problem), possibly with isolated crews in Mars analogue sites. However, some of the conditions that will be faced on-site (e.g., reduced gravity and space radiation) cannot be faithfully simulated. In particular, despite giving useful insights, ground-based reduced gravity simulators each have specific artefacts (e.g., centrifugal accelerations of clinostats) and only real spaceflight can be used for unambiguously asserting the effects of reduced gravity on a biological system (Herranz *et al.* 2013). Testing the components of the system in space, for instance on-board the International Space Station or within commercial satellites, would consequently be an important step in demonstrating the system's feasibility. Some useful regolith simulants are available, but given differences between these and real Martian regolith, validating some aspects of the system (e.g., growth rates while using it as a nutrient source, toxicity, etc.) would ideally be done using real Martian soil, provided sufficient amounts could be brought back by sample return missions.

The investigated processes should obviously not be developed in total isolation, but rather be grounded into broader Mars exploration plans and foreseen technologies. In particular, it seems critical to ensure its compatibility with currently developed life-support systems: since a cyanobacterium-based system could connect on-site resources and BLSS by turning the former into a substrate for the latter, it could be conjugated with advanced BLSS projects (e.g., MELiSSA), making these suitable for a Martian base. Technologies and processes could be developed and tested to make the link between the concepts

described in this paper and other BLSS projects. For instance, studies could point out the most efficient ways of processing cyanobacterial biomass and released nutrients to feed specific BLSS's plant and microbial modules.

As BLSS for human bases on Mars implies growing living organisms there, planetary protection is a critical concern. The Committee on Space Research (COSPAR)'s requirements for planetary protection need to be extended for manned Mars exploration (Horneck 2008), especially if human colonies rely on microbial cultures for life support and industrial processes. The planetary protection-associated risks of the concepts described above should be thoroughly investigated, and strategies to mitigate these risks developed. The main concern will be to bring contamination risks at regions of scientific interest down to close to zero.

Then, generated data can be gathered to propose an accurate and practical solution for the establishment of BLSS which are suitable for sustainable human outposts on Mars. The next steps will be to integrate this solution into mission plans, to test it on site while backup resources are available, and finally to implement it.

Conclusion

Advances in applied physics will allow us to step on Mars; advances in applied biology can allow us to settle there.

Landing humans on Mars in the 2020–2030s is targeted by several public and private institutions. NASA's Orion capsule successfully made its first in-space test last December. SpaceX's CEO Elon Musk will unveil his Mars colonization plans by the end of the year. Mars One is in the process of selecting crewmembers. Other institutions might enter this 'Mars race'. But whoever is the first to land, the same issue will have to be faced: if we are to stay on Mars in the long term to obtain valuable scientific data, we need to learn to be independent of Earth. However, while space travel technologies have made great steps forward in the last half century, current life-support technologies are not sufficiently developed for sustaining Mars manned exploratory missions (Horneck *et al.* 2006). Extensive efforts in this area should start now, so that life support does not become the limiting factor.

Living organisms, wild-type or modified using synthetic biology, are likely to become essential components of life-support systems. But in order to be independent of Earth, they should rely almost exclusively on local resources once set up from an initial set of components. Biological systems should be connected to Martian resources and cyanobacteria could provide this link. Selected – and possibly engineered – cyanobacteria and an appropriate culture system kept to a minimum thanks to cyanobacteria's adaptability to extreme environmental conditions.

The concepts presented in this paper are not intended as a full life-support system plan. They are rather intended as a proposed link between on-site resources and BLSS, through processing of local materials to make these suitable inputs for BLSS. This basis could be combined to currently developed BLSS, making these sustainable, autonomous and expandable within Mars bases. Efforts can thus be combined rather than duplicated. Basic requirements (e.g., food and O_2) could also be covered directly by cyanobacterial cultures. Some of these functions could be performed by other means (e.g., physico-chemical technologies and cultures of other organisms), but cyanobacteria would provide simple (and consequently characterized by a relatively low number of potential causes of failure) and flexible processes to complement other technologies and provide a safe redundancy. With the extremely fast progress which is being made in microbial engineering, more and more processes needed within Martian outposts could be performed by cyanobacteria. This versatility is a strong advantage when mass and resource use need to be kept to a strict minimum.

Even though the last decades have raised our knowledge in this field, much work is still needed to develop the concepts presented here. How challenging it will be to turn these concepts into reliable systems is currently hard to predict. Firstly, we are still learning about the Martian environment; several parts of this paper had to be modified during the writing process to integrate new inputs from the Curiosity rover. Discovering new factors can make things appear easier – for instance, if organic substrates are discovered to be widely available – or more challenging – for instance, with the discovery of toxic elements. Secondly, the pace at which biology engineering is progressing – not unlike microcomputers in the past 40 years – suggests that unexpected and highly efficient ways of modifying cyanobacteria and other organisms will become available. As we could once have scarcely imagined how much we rely on computers now, engineered biological systems on Mars may be more versatile and important in pioneers' everyday life than we can envisage at present. Our abilities to discover life on Mars might depend on our abilities to handle life on Earth.

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