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Habitability, and Evolution of Microorganisms under Extreme Conditions

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Habitability, and Evolution of Microorganisms under Extreme Conditions

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by

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Abstract

The choice of a solvent determines the possible biochemistry of life. Life on Earth is based on carbon biochemistry and has evolved in an environment with water as a solvent. As a polar solvent abundant on Earth, water has unique physical properties, including a large range of liquidity and low viscosity, making it a very good solvent for terrestrial life. Liquids other than water are abundant in the universe, and the chemical nature of these liquids might lead to different chemistries of life. In the first chapter, we review the main characteristics of a good solvent, and then we use this knowledge to examine the similarities and differences between water and cryogenic liquid hydrocarbon, methane, and ethane, as potential solvents for life. We argue that at cryogenic temperatures, mobility, and the reaction rate slow down. We discuss that Titan might not be habitable for terrestrial life but having a rich atmosphere and surface lakes of methane and ethane, it might be a habitat for exotic living systems. We then review multiple investigations on two proposed alternative chemistries for life on Titan, Azotosomes and Silanes, as terrestrial cell membranes cannot form in cryogenic organic solvents. We conclude that there is a need for directing future investigations to planetary bodies that support solvents other than water.

As we discuss in the first chapter, life on Earth has evolved around liquid water. Therefore, the presence of liquid water on a planetary body might make it a potential habitat for life. Jupiter's moon, Europa, is one of the best candidates in the solar system due to the presence of a global saline ocean beneath its icy surface. In the second chapter, we argue that although the extreme conditions of Europa's ocean (high pressure, low temperature, and high salinity) are not optimal for terrestrial life, microorganisms such as bacteria have shown extraordinary abilities to survive and occupy extreme habitats. Cells constantly adapt themselves to changes in the internal and

external environments. Studying the adaptive evolution of bacteria and investigating the signatures of the adaptation under specified simulated conditions in the laboratory can provide a better understanding of the habitability of extreme terrestrial and extraterrestrial environments. We then review a research study done by Yazdani et al. (2019) at the University of Arkansas with an objective to investigate the growth, gene expression, and general strategies used by a mesophilic bacterium, *Escherichia coli*, to survive and adapt to high concentrations of magnesium sulfate, the proposed dominant salt in Europa's ocean. We argue that although adaptation to a new environment might take a long time, the adaptive evolution experiments were feasible in laboratory time scales. We also discuss that the bacteria from the laboratory adaptive evolution experiments (called the "adapted sample") were capable of growing in high concentrations (20% (w/v)) of magnesium sulfate in which the control population of cells could not grow. We then discuss that a strategy used by bacteria to overcome the osmotic stress was to balance the intake of sulfate and magnesium and prevent water loss based on the study of the regulation of gene expression of adapted and control samples.

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1. Introduction

Life thrives in most known habitats on Earth, and different organisms have shown the ability to adapt to different physicochemical conditions (Schulze-Makuch et al., 2015; Bains, 2004). Although terrestrial life has adapted to different conditions, the limits of adaptability are to be investigated in extreme conditions. Some of the physicochemical extremes, such as high temperature, might destroy life. In contrast, some organisms might be capable of surviving at low temperatures, but the biochemical activities will be extremely slow. Traditionally, we assume that these extremes are only for terrestrial life.

Terrestrial life is based on carbon as a major building block, and it uses water as a solvent for biochemical activities. As the most abundant molecule on Earth, water can be found in the liquid phase in a wide range of Earth-like temperatures, under a pressure of 1 bar. Water has major advantages and some disadvantages for life. The presence of water is critical for terrestrial life because it provides an environment for the feasibility of biochemical processes and assists in transferring essential compounds in and out of the cells. On the other hand, water degrades some biomolecules and damages cell membranes when it freezes (Benner et al., 2004; Steponkus, et al., 1995).

A wide range of environmental conditions has been observed on other planetary bodies. A few natural compounds might be found in a liquid state for most planetary conditions. Thus, a liquid environment might be relatively common in the universe (Schulze-Makuch & Irwin, 2006). The search for extraterrestrial life raises important questions including: Is water essential for life? Does life use other solvents for its survival? Would that life be similar to terrestrial life, and if it is different, how different might it be? Could life emerge in organic solvents such as liquid methane and ethane? What is the temperature limit for the possibility of life?

2. Life and Its Requirements

Until we find life on another planetary body, our knowledge of life is limited to terrestrial life as the only form of life we know. Recent advances in genomics have provided us with a wealth of data that shed light on the characteristics of existing organisms and how different forms of life have branched out from each other. While we have come to learn a great deal about cellular processes and their adaptation to different environments, it only provides limited knowledge about their adaptation to these environmental conditions. To identify the habitability of an environment, we need to define what life is and what its requirements are.

One of the definitions of life was proposed by a panel assembled by NASA in 1994, according to which *life is a chemical system capable of Darwinian evolution* (Joyce, 1994). Life can also be defined by the essential thermodynamic and kinetic principles by which it operates. The system is surrounded by a membrane that keeps the ingredients in a defined volume and protects the system from the outside environment. The cellular functions of living systems on Earth are determined by Deoxyribonucleic acid (DNA) polymer, which is replicated from one generation to another generation. The DNA codes for different proteins and nucleic acids that carry out different functions in the living systems (Bains, 2004; Koshland, 2002).

Living systems have some basic requirements (Benner et al., 2004). The first requirement for life is thermodynamic disequilibrium. Disequilibrium provides an environment for organisms to adapt to different conditions and evolve. This requirement arises due to the need for a source of energy. Another requirement for life is the stability of information coding machinery and biomolecules that carry out cellular functions. The stability of carbon-carbon covalent bonds under

Earth-like conditions has made it an ideal choice element for terrestrial biochemistry. The final and most critical requirement is the presence of a liquid solvent that dissolves different compounds and provides a stable environment for biochemical reactions. In the following sections, we investigate the properties of various solvents and their effects on the biochemistry of life.

3. A Good Solvent for Life

The choice of the solvent determines the possible biochemistry of life. Compounds are liquid only over a specific range of temperatures and pressures. Theoretical studies suggest that liquid environments can be common in the universe, and many planetary bodies can support at least one liquid on or beneath their surfaces (Bains, 2004). Figure 1 shows the temperature ranges for twelve different solvents to be in the liquid state at the pressure of 1 bar. It is noticeable that liquid methane and ethane, the main components of Titan's surface lakes, can be found in a significant range of cryogenic temperatures. Such implies that on planetary bodies with colder environments, where liquid water cannot be present on the surface, these liquid hydrocarbons can be considered as alternative solvents that might support a habitat for life. Hence, there is a need to study the characteristics of a good solvent for life to gain a better understanding of the habitability of solvents other than water.

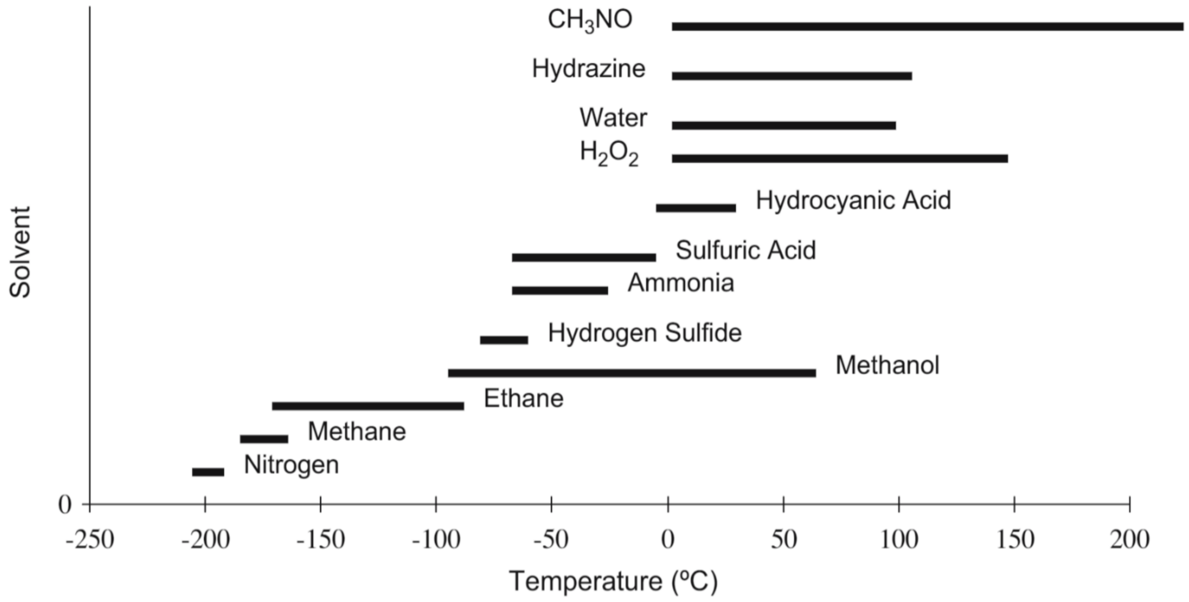

Figure 1. Temperature ranges for some solvent candidates to occur in the liquid state (at 1 bar). Figure obtained from Schulze-Makuch & Irwin (2006).

There are some properties that a substance must have in order to be considered a good solvent for life (Millam & Klos, 2014). First, it must be present in sufficient quantities and be naturally available in the environment. If the substance is rare, it cannot sustain a habitat around it. Next, it must be a good solvent for both organic and inorganic compounds and have a reasonably large range of liquidity —the range of temperatures where solvent remains in the liquid state— so the organisms can survive and evolve in a wide range of temperatures and remain biochemically active. The liquidity range depends on two environmental factors—(a) the presence of impurities, such as salts, that can lower the melting point and extend the range of liquidity, and (b) the external pressure that has a direct effect on both the freezing and the boiling points of the liquid. It also needs to have the ability to encapsulate the living system by spontaneously developing boundaries around cell membranes.

A good solvent for life may also have other qualities. A large dielectric constant facilitates the dissolution of salts and allows large quantities of ions to be present in the solution. Being a good thermal moderator is another preferable quality. The large specific heat of a solvent allows large heat exchanges with the surroundings without changing the temperature a lot, giving thermal stability to organisms under the environmental changes. Finally, a low viscosity is preferable for a high degree of mobility, allowing biomolecules to interact efficiently (Schuzle-Makuch & Irwin, 2006; Freitas, 1979). Table 1 shows molecular weight, density, melting and boiling points, range of liquidity, dielectric constant, viscosity, and relative probability of occurrence (relative probability of liquids on bodies > 1000 km in diameter around any star in our stellar neighborhood) of water and other potential solvents including methane and ethane.

Property	Water (H_2O)	Methane (CH_4)	Ethane (C_2H_6)	Ammonia (NH_3)	Nitrogen (N_2)
Molecular weight	18.015	16.4	30.07	17.031	28.01
Density (g/ml)	0.997	0.426 (at -164 ºC)	0.572 (at -107 ºC)	0.696	0.85
Melting point (ºC at 1 bar)	0	-182	-172	-77.73	-210
Boiling point (ºC at 1 bar)	100	-161.5	-89	-33.33	-196
Range of liquidity (ºC at 1 bar)	100	20.5	83	44.4	14
Dielectric constant (ε)	80.1	1.7	1.9	16.6	1.45
Viscosity (10^{-3} Pa s)	9.6	0.0009 (at 20 ºC)	0.011(at 20 ºC)	0.27 (at -34 ºC)	0.204
Relative probability of occurrence	1	0.62	1.25	0.25	1.96

Table 1. Selected physical properties of water, methane, ethane, ammonia and nitrogen. Data obtained from Schulze-Makuch & Irwin (2006).

3.1 Water

On Earth, all the biochemical activities occur in liquid water. Water is unique among other solvents and possesses all the properties mentioned above. Water can be found in almost all terrestrial environments, and it is accessible to life. It forms a three-dimensional hydrogen-bonded network which enables it to participate in biochemical processes as a polar solvent. Polarity plays an important role in the stability of cell membranes and protein folding. Water has a large range of liquidity, and it can be found in a liquid state at most Earth-like temperatures. Pure water has liquidity of 100 ºC at 1 bar. Adding common salts to water lowers the freezing point. The application of an external pressure increases the boiling point and lowers the freezing point, resulting in a larger range of liquidity (Benner et al., 2004; Schulze-Makuch & Irwin, 2006).

Other beneficial properties of water include high heat capacity, low viscosity, and high dielectric constant. Besides, it moderates the global temperature and reduces climate fluctuations. Water can be found in all three phases (solid, liquid, and gas) within a range of temperatures and pressures that can create a variety of habitats and climates.

There are some disadvantages to water as a solvent. Water has a unique physical characteristic that allows it to expand while freezing at atmospheric pressures. While this expansion can be beneficial for aquatic life in lakes and oceans at low temperatures, it can damage the cell membrane (Steponkus, et al., 1995). Another property that can damage biomolecules is the water's reactivity. Water destroys unstable organic compounds and slowly degrades molecules such as DNA. As a result, DNA must be continuously repaired (Benner et al., 2004).

3.2 Cryogenic Organic Solvents

In the presence of hydrogen, which is an abundant element in the universe, carbon, nitrogen, and oxygen exist as hydrogenated compounds. When hydrogen bonds with oxygen, it produces water (H_2O), and in the case of nitrogen, the production will be ammonia (NH_3). When carbon is hydrogenated, it forms methane (CH_4). Figure 2 shows the distribution of occurrence of water/ammonia, methane/ethane, and nitrogen oceans as a function of distance from sun. The figure indicates that depending on the distance from the star we may find different solvents in liquid states. Nonpolar hydrocarbons such as methane and ethane are abundant in the solar system (Baines, 2004) and there is a high probability of the occurrence of surface lakes of methane and ethane in distances between 1 and 10 AU. The confirmation of the existence of stable surface lakes of a mixture of methane and ethane are confirmed on Titan (Brown et al., 2008). This raises the question of whether these liquids can create a habitat for life. To answer this question, we discuss some of the physicochemical characteristics of these solvents at cryogenic temperatures of Titan.

As mentioned, a good solvent for life should not be too resistant to flow. On Earth, when solvents become more viscous at low temperatures, the diffusion and the rate of metabolism slow down (Schulze-Makuch et al., 2015). If the same process applies to the cryogenic hydrocarbons, life on Titan might have a very slow metabolism (Gilliam & Lerman, 2016). The effect of temperature, particle size, and viscosity on the diffusivity can be found by the Stokes-Einstein's equation as given below:

$$D = \frac{RT}{6\pi\eta rN} \qquad (1)$$

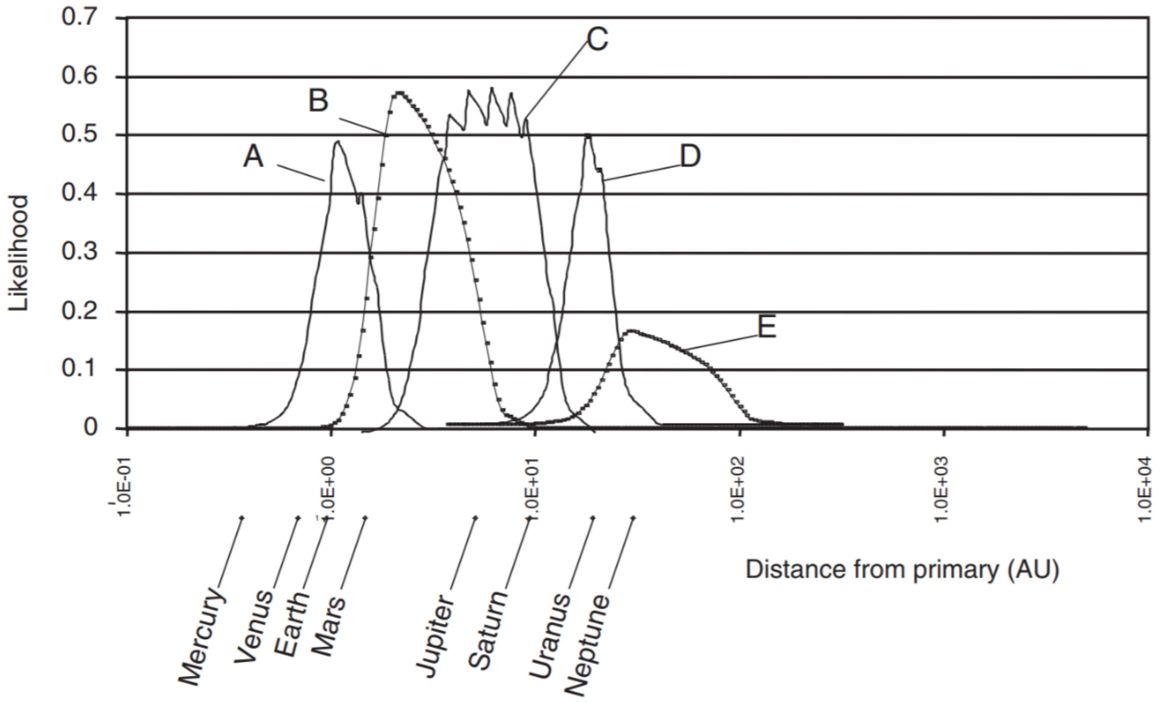

Figure 2. Expected distance distribution for water/ammonia, methane/ethane, and nitrogen "oceans" in our solar system: curve A, water/ammonia surface lakes; curve B, water/ammonia subsurface; curve C, methane/ethane surface lakes; curve D, surface nitrogen; and curve E, subsurface nitrogen. Figure obtained from Bains (2004).

Where D is the diffusion coefficient of a particle in the solution (m^2 s^{-1}), R is the gas constant (J mol^{-1} K^{-1}), η is the dynamic viscosity (Pa s), r is the particle's radius (m), and N is the Avogadro's number. For comparing the diffusion of two different solvents at different temperatures, we can rewrite the equation (1) as:

$$\frac{D_2}{D_1} = \left(\frac{T_2}{\eta_2 r_2}\right) / \left(\frac{T_1}{\eta_1 r_1}\right) \qquad (2)$$

As an example, the diffusions of liquid ethane and seawater were calculated (Gilliam & Lerman, 2016), using the values of viscosity of water, 1.791×10^{-3} Pa.s at 273 K (Sharqawy et al., 2010), and the viscosity of liquid ethane, 1.260×10^{-3} Pa.s at 90.4 K (Younglove & Ely, 1987). The diffusion coefficient was found to be a fraction of 0.47 for molecules of comparable size in ethane and water. The reduction of the diffusion coefficient in liquid ethane will be larger for polymers with a larger size (Gilliam & Lerman, 2016). Similarly, we can estimate the diffusion coefficient for methane and other solvents.

Aside from the diffusion coefficients and mobility of molecules, it is important to investigate how fast the biochemical processes occur at cryogenic temperatures. The rate of chemical reactions in these environments is estimated by using the Arrhenius equation:

$$k = Ae^{-\frac{E_a}{RT}} \qquad (3)$$

Where k is the reaction rate, A is pre-exponential factor, E_a is activation energy (J/mol), R is the gas constant (J mol^{-1} K^{-1}) and T is temperature (K). To compare the rate of reaction at a given temperature T_{low} with the reaction rate at T=273K, we can rewrite equation (3):

$$ln\left(\frac{k_{low}}{k_{273}}\right) = -\frac{E_a}{R}\left(\frac{1}{T_{low}} - \frac{1}{273}\right) \qquad (4)$$

The mean of several activation energy values for different polymerization reactions is about $130 kJ/mol$ at 337 K (Gilliam & Lerman, 2016). Considering that the activation energy remains the same and using 90.4 K as T_{low}, a fraction of 5.7×10^{-51} was calculated for the activation rate. As discussed, the reaction in the cold environments is slower and depends on the presence of catalysts. Enzymes can catalyze reactions efficiently so the reaction rate can approach the diffusion-limited maximum. It is notable to mention that the growth rate of living systems is also dependent on the competition for nutrients and not only the temperature and how fast the chemical reactions occur. Table 2 shows the diffusion-limited reaction rates in different solvents.

Another characteristic that needs to be considered is the polarity of the solvent and its effects on biochemical processes. The biochemical evolution of terrestrial life has evolved around water. As a polar solvent, water dissolves polar compounds such as salts and sugars. Unlike oxygen which has a high electronegativity, carbon is only slightly more electronegative than hydrogen, therefore the atoms share electrons almost equally in a methane molecule. Similarly, the electric charges in ethane molecules are symmetrically distributed, making the ethane nonpolar. Nonpolar solvents tend to dissolve nonpolar substances. The lipid membranes surrounding the cells create flexible and semipermeable containers. Terrestrial organisms have cell membranes composed of polar (hydrophilic) heads and nonpolar (hydrophobic) tails. These characteristics lead to a tendency for a spontaneous assembly in such a way that the hydrophilic heads orient in water and hydrophobic ends stay away from the water forming a stable bilayer membrane. Figure 3 shows the schematic of the lipid bilayer orientation in water. Vesicles made from these membranes are called liposomes. Lipid bilayers that are strong and elastic in water do not form in cryogenic temperatures of liquid methane and ethane. It has been proposed that life in a nonpolar solvent might have a reversed chemical orientation (Schulze-Makuch & Irwin, 2006).

Liquid	Freezing point (K)	Viscosity at freezing point (Mη)	Relative diffusion- limited reaction rate
Water	237.16	317	1
Methane	90.7	102.8	0.76
Nitrogen	63.2	152	0.35

Table 2. Comparative rates of diffusion-limited reaction rates in different liquids at their freezing points. Data obtained from Bains (2004).

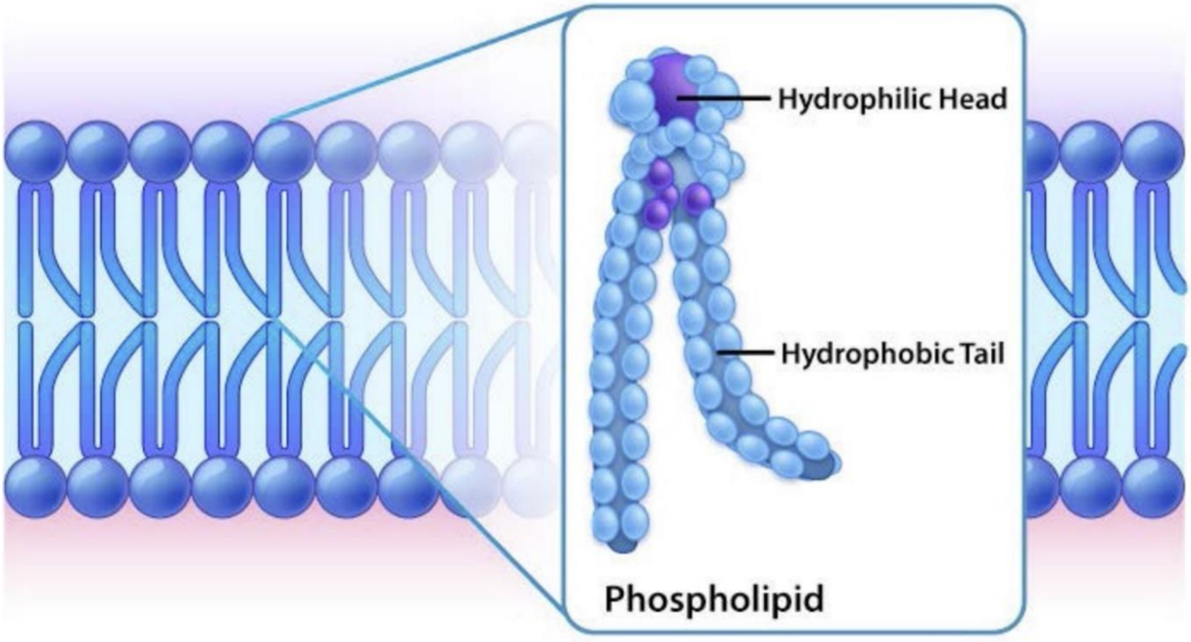

Figure 3. Lipid bilayer closeup. Image by Aleia Kim; Figure obtained from Ahern et al. (2016).

4. Biochemistry of Life in Cryogenic Organic Solvents

The availability of hydrocarbon liquids on many planetary bodies has broadened our search for life in the universe. Biochemistry of the living systems in cryogenic organic solvents might be different from the biochemistry of the terrestrial life. Azotosomes and silanes are biomolecules that are proposed to form the building blocks of living systems in Titan-like environments.

4.1 Azotosomes

Stevenson, et al. (2015) proposed a new type of membrane composed of small organic nitrogen compounds that could function in liquid methane at cryogenic temperatures. These membranes, called azotosomes, can arise from the compounds observed in Titan’s atmosphere. Azotosomes are held together by the polarity of their nitrogen-containing groups (azoto groups), similar to liposomes that rely on the alkyl group's polarity. Figure 4 shows a comparison between the azotosomes and liposomes’ structures. To better understand how azotosomes might be analogues to the lipid bilayers, two requirements, flexibility and stability, are compared. A common measure of flexibility is the area expansion modulus, K_a. The area expansion modulus of terrestrial cell membranes is between $0.24\, J/m^2$ and $0.5\, J/m^2$ at room temperature (Hofsäß et al., 2003). The area expansion modulus measured for several azotosomes was also within this range (Stevenson et al., 2015). Stability is commonly investigated through decomposition energy (Ayton, 2002). While lipid bilayers are generally metastable (Lasic, 1990), azotosomes have high decomposition energies relative to cryogenic environments, making them remain stable over a long period (Stevenson et al., 2015). Although the ability of azotosomes to form stable and flexible membranes does not demonstrate the possibility of life, it directs our search for biochemistries that would be compatible under cryogenic conditions.

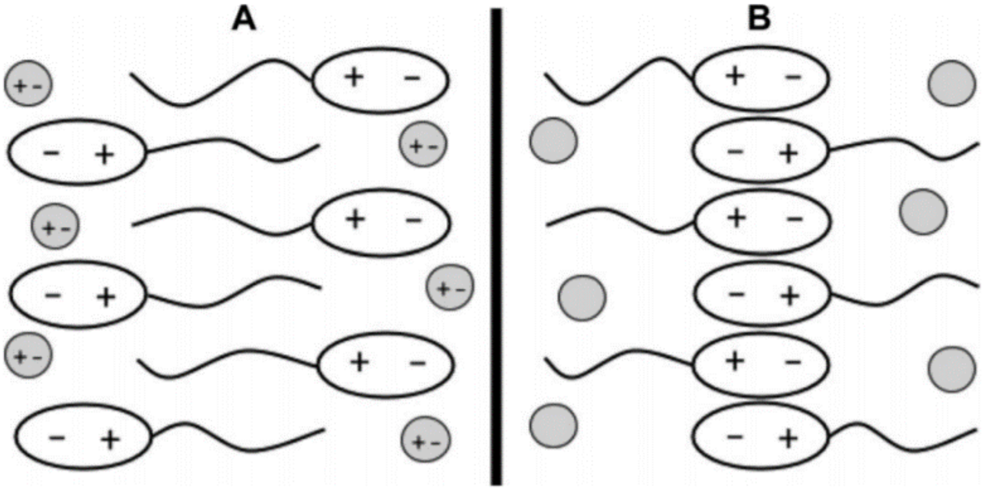

Figure 4. Liposomes and azotosomes; (A). Liposome in polar solvent. Polar heads are braced by nonpolar lipid tails. (B) Azotosome in nonpolar solvent. Nonpolar tails are braced by polar nitrogen-rich heads. Figure obtained from Stevenson et al. (2015).

4.2 Silanes

Another possible form of chemistry in Titan-like environments is silicon-based chemistry. Silicon chemistry, that supports biochemistry analogue to carbon-based chemistry, is flexible and might be common in the universe. Silicon can form stable bonds with itself, carbon, and some other elements. Compounds such silanes, silicon analogues of hydrocarbons, are more chemically reactive than their carbon-based counterparts, which can be an advantage in cryogenic environments. Furthermore, silicon can form stable silicon polymers, such as polysilanes, that can be found in solid state in cryogenic temperatures of Titan’s surface (Schulze-Makuch & Irwin, 2006; Petkowski et al., 2020). Figure 5 shows the structures of some of the known polysilanes. silanes can form flexible macromolecules with different assemblies similar to lipid bilayers (Schulze-Makuch & Irwin, 2006).

Although both azotosomes and silanes are proposed to survive and tolerate the extreme conditions of cryogenic organic solvents, neither of these compounds are confirmed to form spontaneously on Titan. A recent study on azotosomes (Sandström & Rahm, 2020) indicated that the compound was not viable for spontaneous self-assembly at cryogenic temperatures. Furthermore, silanes are less stable than their hydrocarbon counterparts. Most known polysilanes are monotonous making them to have more limited structures compared to biopolymers that contain variable and non-repeating sequences (Baines, 2004).

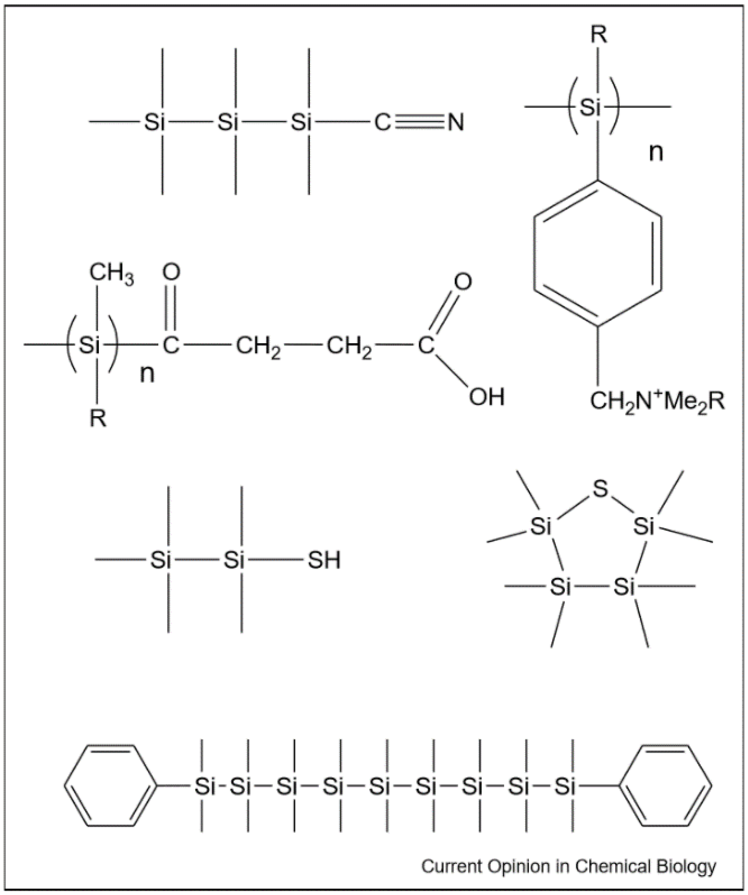

Figure 5. Structures of some synthetic polysilanes that have been described in the literature. There is a little question that polymetric diversity is possible using silicon, rather than carbon, as a scaffolding. Figure obtained from Benner et al. (2004).

5. Titan as a Potential Habitat for Life

Titan, the largest moon of Saturn, is one of the most studied celestial bodies and the only moon in the solar system that has a dense atmosphere and a cycle of methane similar to hydrological cycles on Earth (McKay, 2016; Lunine & Atreya, 2008). Titan has an active environment with an average surface temperature of 94 K and a surface pressure of around 1.5 bar (Raulin, 2008). Stable lakes of liquid methane and ethane have been identified on the surface of Titan (Brown, et al., 2008).

It has been proposed that the methane in the atmosphere might have biological origins (Fortes, 2000; McKay & Smith, 2005). In addition to methane/ethane lakes on the surface, it has been speculated that Titan might possess subsurface oceans of water and ammonia (Fortes, 2000). Photochemical processes generate a rich organic chemistry of hydrocarbons and nitriles in the atmosphere (Lorenz et al., 2008). Information about the composition of the atmosphere and surface materials has been obtained by various instruments aboard the Huygens and Cassini missions (Neiman et al., 2005). Ethane is the main product of methane photolysis in the atmosphere (Wilson & Atreya, 2004). Methane and ethane then react with nitrogen and produce various organic molecules. It has been speculated that the atmosphere is similar to the Earth’s primordial atmosphere (Raulin, 2008). Nitrogen which is the main component of the atmosphere dissolves in the methane/ethane lakes of Titan. The solubility of nitrogen in ethane is less than methane and it leaves the lakes in the form of a bubble (Fransworth et al., 2019).

In the presence of large lakes of methane and ethane and organic molecules, Titan meets almost all of life’s requirements. Previous studies proposed some reactions, including endogenic reactions of ethane and other organic molecules with the atmospheric hydrogen and catalytic hydrogenation of acetylene, that might produce enough energy for life on Titan (McKay & Smith, 2005; Schulze-Makuch & Irwin, 2006).

5. Habitable Worlds and Mission Design

Finding extraterrestrial life with similar or different biochemistries can change our view of the origin and evolution of life in the universe. Looking for biosignatures is an important goal of most space missions. Currently, the search is governed by our knowledge of terrestrial life. For this reason, we often focus on finding habitats similar the terrestrial environments. On Earth, wherever water is present, we can find life. If we only focus on finding water, our search will be limited to environments with special conditions. On the other hand, if we postulate that different solvents can be compatible with different chemistries of life, we can broaden our search. The Lakes of liquid methane and ethane on Titan are more accessible than the deep subsurface oceans of icy worlds and should be considered as potential candidates for future missions.

Furthermore, the current search for biosignatures is mainly focused on looking for the structures that resemble the terrestrial organisms, products of metabolic reactions, and tests for the amino acids and nucleotides similar to those found in terrestrial proteins and DNA (Committee on the limits of organic life in planetary systems, 2007). However, theoretical and experimental data suggested that under various environmental conditions, life might emerge based on different biochemistries (Schulze-Makuch & Irwin, 2006; Baines, 2004; Benner et al., 2004; Stevenson et al., 2015) that might not be detectable by the current *in situ* or remote sensing missions. Therefore, we suggest improving the design of life detection instruments and investigating the habitability of exotic environments of other planetary bodies.

6. Conclusions

The choice of solvent can determine the biochemistry of life. Terrestrial life has evolved based on carbon chemistry, and it uses water as a solvent for biochemical activities. Solvents other than liquid water exist on other planetary bodies and may support life. Among these bodies, Titan might be one of the best candidates for a habitable world. Titan has a rich atmosphere, and it is confirmed that lakes of liquid methane and ethane are present on the surface. As we discussed, although these cryogenic hydrocarbons have large ranges of liquidity and are abundant in the environment, terrestrial life may face many challenges on Titan. Hydrocarbons are non-polar and lipid membranes that are stable and flexible in water cannot form in these solvents. Besides, at cryogenic temperatures of Titan, diffusion and reaction rates become very slow. Thus, the chance of emergence and survival of terrestrial organisms in Titan-like environments is low. However, life on Titan might be based on a different biochemistry that cannot be detected by the current space missions. As a result, there is a need to investigate and study Titan to gain a better understanding of the habitability of the cryogenic organic solvents.

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Chapter 2. A Review of Adaptive Evolution of *Escherichia coli* to High Concentrations of Magnesium Sulfate: Implications for Habitability of Europa

1. Introduction

1.1 Europa, a Habitable Moon

Europa is the fourth largest satellite orbiting around Jupiter. The evidence for the existence of liquid water (Pappalardo et al., 1999), essential elements for life (Carlson et al., 2009; Kargel et al., 2000), and sources of energy (Chyba, 2000) imply that Europa is one of the best candidates for astrobiological explorations in the solar system. Europa is geologically active and slightly smaller than Earth's Moon (diameter ~3122 km). Figure 6 shows some of the proposed physicochemical processes on Europa. Conductive models showed that Europa possesses a uniform metallic core, possibly 50% of Europa's radius, a rocky silicate mantle, and a combined water ice/liquid shell ~80-170 km thick (Anderson et al., 1998; Pappalardo et al., 1999). Although not directly observed yet, the data from the Voyager and Galileo missions, the magnetic field studies, and the presence of smooth and young surface with few craters, the compression ridges, and the stretch features indicated that Europa has a global ocean, 2-3 times the volume of all liquid water on Earth (Pappalardo et al., 1999; Kivelson et al., 2000).

Data provided by Galileo's Near-Infrared Mapping Spectrometer (NIMS), observations, and laboratory experiments have demonstrated that a wide range of elements exists on the surface of Europa (Zolotov & Shock, 2001; McCord et al., 1998). Water ice exists in the form of pure ice and fine-grained frost combined with hydrated salts (such as magnesium & sodium sulfate and sodium & potassium chloride), sulfur compounds such as hydrated sulfuric acid (Dalton, 2003; Carlson et al., 1999; Kargel et al., 2000) and possibly small amount of carbonaceous materials that might have a biological origin (Dalton et al., 2003).

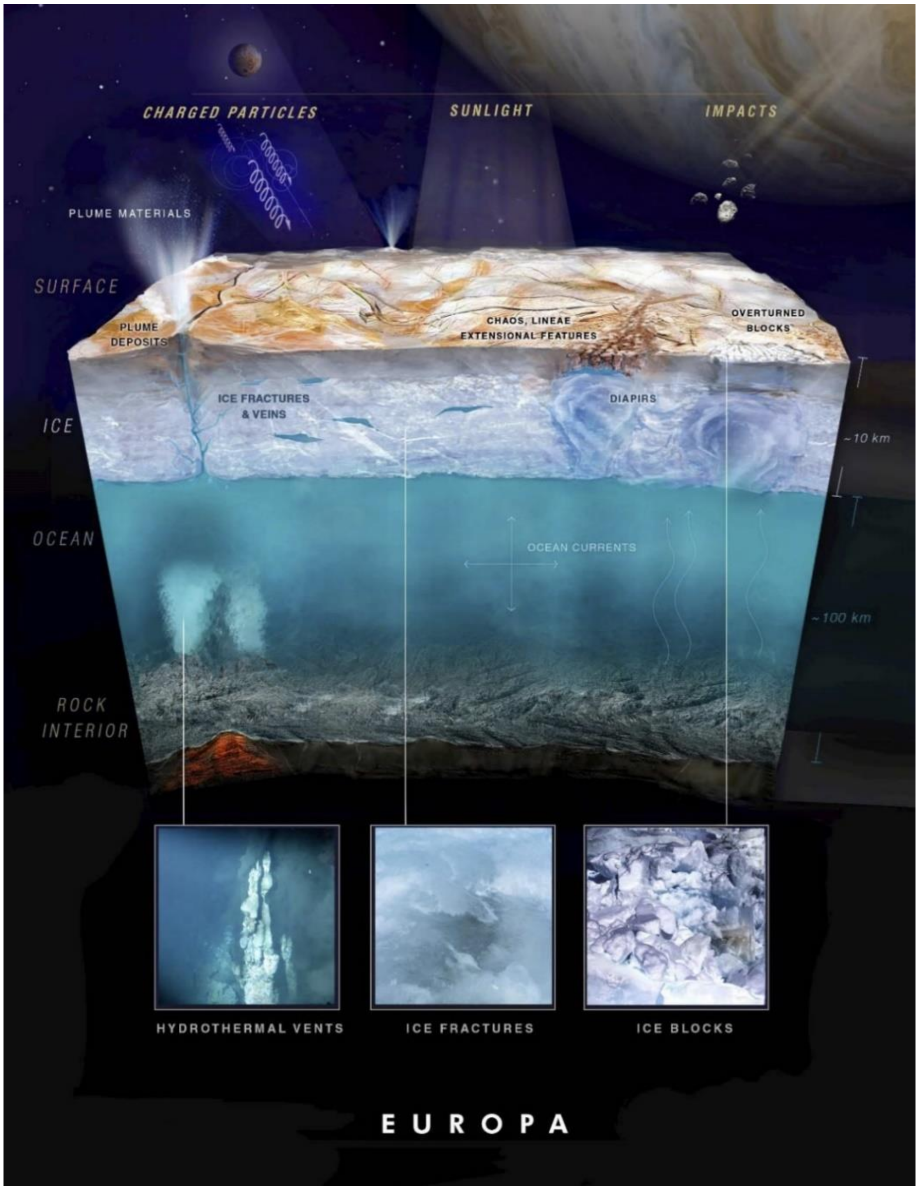

Figure 6. Artistic representation (not to scale) of Europa in cross-section showing processes from the seafloor to the surface. Boxes indicate potentially habitable sites such as hydrothermal vents, as well as regions on and within ice shells that could harbor biosignatures. This diagram shows an integrated perspective of how the seafloor, ocean, and ice shell could yield biosignatures detectable on the surface by a landed spacecraft. Figure obtained from Hand et al. (2022).

Experimental and theoretical simulations predict that the exchange between the seafloor, the ocean, and the surface occur through various mechanisms, resulting in an enrichment of the ocean with essential elements needed for life (Carlson et al., 1999; Vu et al., 2016). Figure 7 shows the composition of CI-chondrite-derived brine ocean on Europa at 1.01 and 1200 bars of pressures as temperature decreases to the eutectic. The figure represents a (neutral pH) Na-Mg-Ca-SO_4-Cl-HO_2 system. According to the model, the ocean is rich in magnesium and sulfate. As temperature decreases the salts precipitate and ice starts to form. At temperatures lower than eutectic, liquid water cannot exist in the ocean (Marion et al, 2003). Although the high salinity and the formation of ice at very low temperatures are limiting factors for life, the base of the ocean might have higher temperatures which can create a desirable environment for life (Kargel, 2000).

It was proposed that Europa's ocean is dominated by magnesium sulfate (Kargel et al., 2000). The compound might be a product of radiation (Brown & Hand, 2013) or have an endogenic origin (Kargel et al. 2000). The concentration of magnesium sulfate on Europa is predicted to be ~20 wt% in a model suggested by Kargel et al. (2000). Another necessity for life is energy. The energy of the ocean might be provided by physical and chemical sources (Chyba, 2000; Zolotov & Shock, 2003; Sparks et al., 2017).

Life cannot survive on or near the surface of Europa due to lethal radiation and low surface temperature (~ 86 - 132 K). However, it might find a potential habitat at the bottom of the icy crust where the temperature is higher (> 253 K), and the ice shell acts as a shield against radiation (Marion et al., 2003). Pressure is another factor that plays a critical role in the habitability of oceanic environments. It was suggested that the ocean has a hydrostatic pressure gradient of 12 bar/km, giving rise to a pressure as high as 1200 bar or higher at the base, which is within the range of pressure limits for biological activity (up to 1100 bar) on Earth (Marion et al., 2005).

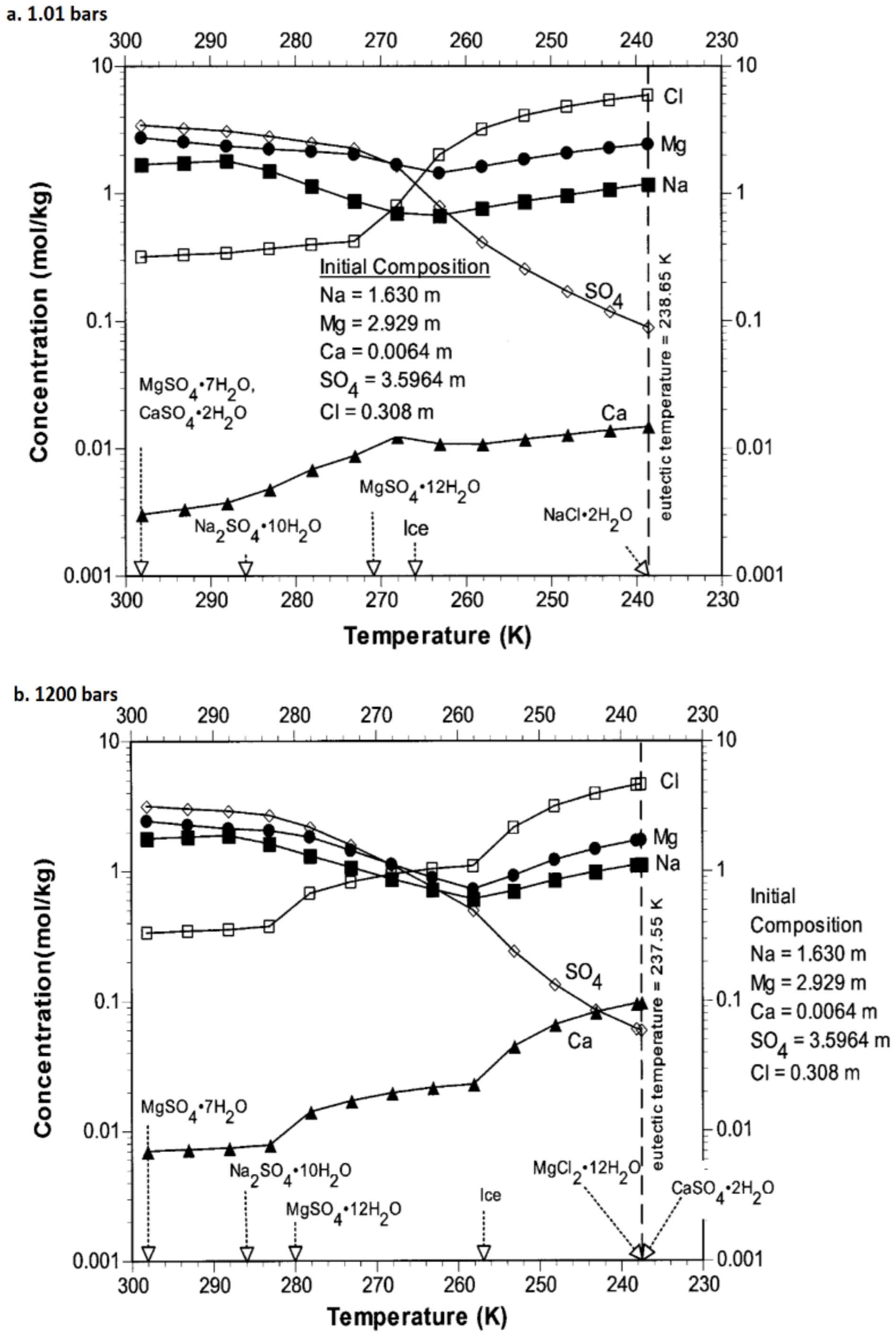

Figure 7. The evolution of a Na-Mg-Ca-SO_4-Cl brine at (a) 1.01 bars and (b) 1200 bars of pressure as temperature decreases to the eutectic. Figures obtained from Marion et al. (2003).

1.2 Terrestrial Analogues for Europa's Ocean

Investigating the terrestrial analogues for life on Europa is important because it can help develop and test models for the potential presence and evolution of life in Europa's environments. To find the best potential habitat analogues to Europa's ocean, we need to consider the limiting factors for life. Microorganisms are found to thrive in conditions from very acidic (pH=0) to very basic (pH 13), very cold (253 K) to very hot (394 K), the salinity range of $a_w = 0.6\text{-}1$ (where a_w is water activity), and pressures up to 1100 bar (Marion et al. 2003). Salinity, temperature and pressure are the main limiting factors in Europa's ocean and because we do not have direct evidence for the ocean's composition and geology, finding suitable analogues is difficult.

From the potential habitats in Antarctica, we can refer to subglacial lakes including Lake Vostok (Bulat et al., 2011), and cold hypersaline lakes, including Lake Vida (Murray et al., 2012), where the high salt concentrations can maintain the water liquid at very low temperatures. Subglacial hypersaline lakes in Arctic regions, including the lakes of Devon Ice Cap in Canada (Rutishauser et al., 2018), and magnesium and sulfate-rich lakes, including Basque Lakes (Crisler et al., 2019), are other proposed analogues for Europa. Considering the high pressures at the base of Europa's ocean, Mariana Trench (Marion et al., 2003), the deepest ocean basin on Earth, can be a suitable analogue to Europa.

1.3 Bacterial Life in Saline Environments

Salinity is a limiting factor for bacterial growth as the cells thrive to maintain an osmotic balance with the outside environment. External osmotic pressure affects the hydration and integrity of cell compartments (Altendorf et al., 2009). Large concentrations of ions in the environment can reduce the water activity and lead to osmotic stress which perturbs the cell's structure, stability, and functionality (Koch, 1984; Burg et al., 2007). Restriction of water availability in an environment causes water efflux (Wood, 2015) and plasmolysis (Nepal & Kumar, 2020; Rojas et al., 2014). A study investigating the effects of high osmotic pressure on *Escherichia coli* (*E. coli*) reported that cells maintained a lower growth rate compared to the cells cultured in optimal conditions (Cayley et al., 2000). Another study on the viability of *E coli* in minimal media containing high concentrations of sodium chloride (NaCl) argued that cells remained viable up to 7% NaCl concentration and the viability drops with further increase in the concentration of salt (Doudoroff, 1940).

Cells respond to high external osmolarities, by importing ions and compatible solutes (Imhoff, 1986; Epstein et al., 1965) and restricting water efflux. Some bacteria have developed strategies, such as regulating various gene expressions, to survive and adapt to a wide range of osmotic pressure (Barron et al. 1986). Previous studies investigated bacteria's response to osmotic stress and identified multiple genes, such as aqpZ, osmC, recA, and rpoH, that were involved in osmoregulation.

Many investigations were carried out on the microorganisms, called halophiles, that can grow optimally at elevated concentrations of salt. It was proposed that the main strategy that these organisms used to maintain an osmotic balance within their cells was the accumulation of salt concentrations and organic osmotic solutes (Siglioccolo et al., 2011). Halophiles can be found in all domains of life (bacteria, archaea, and eukarya) and are distinguished by their optimal growth at different salt concentrations. Extreme and borderline extreme halophiles can grow optimally between 2.5-5.2 M and 1.5-4 M salt, respectively. Moderate halophiles grow best in environments containing 0.5-2.5 M salt. Halotolerant organisms do not require high concentrations of salt for their growth but are able to grow in very high salt concentrations (Oren, 2008; Siglioccolo et al., 2011). Although the characteristics of halophiles and other microbial communities in saline environments were studied extensively, most studies focused on NaCl (Rodriguez-Valera, 1981; Gunasekera et al., 2008) and only a few studies addressed the effects of high concentrations of magnesium sulfate on bacteria.

To study the habitability of Europa, it is essential to investigate the effect of various salts, including magnesium sulfate, on cells. In a recent study Nepal and Kumar (2020) investigated the growth, cell division, and gene expression of *E. coli* at elevated concentrations of magnesium sulfate. *E. coli* cells were cultured in M9 minimal media containing 2mM, 0.41 M, 0.83 M, 1.25 M, 1.66 M, and 2.07, respectively, and different characteristics including cell growth, death, division, and gene expression were investigated. The study suggested that while the survival fraction of cells was not affected at concentrations 0-1.25 M, the fraction of dead cells increased at concentrations beyond 1.25 M. They observed a decrease in the growth rate of cells with increasing the salt concentration up to 1.25 M of magnesium sulfate. At 1.25 M, cells showed different populations: filamentous cells (due to lack of cell division), cells with the same size as the control cells and finally, smaller cells compared to the control cells. The heterogeneity might be due to stochasticity in cellular processes such as cell division and gene expression (Nepal & Kumar, 2020).

1.4 Adaptive Laboratory Evolution

Natural habitats are not steady and environmental fluctuations constantly affect microbial communities (Nguyen et al., 2021). Adaptive laboratory evolution experiments are a great tool to investigate the survival and adaptation of microorganisms under controlled scenarios and simulated conditions. Bacteria have an exceptional ability to adapt to environmental fluctuations and stresses. The ease of maintaining large populations, rapid generation times, and the ability to store the samples make the microbes suitable choices for these studies (Elena & Lenski, 2003).

The adaptation usually occurs in two main steps: a transient phase (shock response) where rapid responses to environmental changes initiate the adaptation process and a continuous phase (stress response), where responses support the exponential growth of cells and lead to a new growth rate (Gunasekera et al., 2008).

Fitness can be used as a measure of successful adaptation. During adaptive evolution, accumulating phenotypic and genetic differences increase the fitness of adapted cells (Dragosits & Mattanovich, 2013). The fitness can be measured by comparing a property such as the growth rates of the cells from the adaptive evolution experiments against their ancestor under the same condition. Also, previous studies suggest that adaptation to a particular condition may be associated with a decrease in fitness in other environments (Lenski & Burnham, 2018), so measuring the fitness in various environments can provide valuable information about the adaptation characteristics.

Long-term evolution experiments can provide insight into the trajectory of adaptation over time and the repeatability of the evolution (Lenski & Burnham, 2018). In a study started in 1988, 12 populations of a same strain of *E. coli* were cultured in media containing glucose and citrate. A small volume of cells from each sample, 1%, were transferred to fresh media every day and the process was repeated for more than 60000 generations. The study suggested that adaptation to new environments may take a long time and the evolution might or might not be repeatable (Lenski & Burnham, 2018). Several studies have investigated the adaptative evolution of bacteria to different environmental stresses, including osmotic stress due to elevated concentrations of NaCl (How, et al., 2013), change in temperature (Deatherage et al., 2017), high pressure (Marietou et al., 2015), and ultraviolet exposure (Alcántara-Díaz et al., 2004). However, little is known about the long-term effect of exposure to high concentrations of magnesium sulfate.

2. Review of Experimental Design

2.1 Objectives

We reviewed a study (Yazdani et al. 2019a) that intended to provide a better understanding of the habitability of Europa's subsurface ocean by investigating the strategies used by a mesophilic bacterium, *Escherichia coli*, to survive and adapt to high concentrations of magnesium sulfate, via long-term adaptive laboratory experiments. Investigating bacteria's response to fluctuations in their environment and studying their survival and adaptation mechanisms in different hostile environments provide insight into the physicochemical limits of life and the habitability of planetary bodies.

2.2 Technical Approach

Extreme conditions are non-optimal for cells and usually slow down the growth rate. Very high magnitudes of these conditions may lead to a total collapse of the population. Therefore, there is a need to find a region feasible for experiments in which the pressure is high enough for cells to feel the selective pressure from the environment but they are still capable of growing in the new environment and there is no collapse of the population. This region is called a tipping region. (Yazdani et al., 2019a; Osmond & Klausmeier, 2017).

The general strategy for the adaptive evolution experiment done by Yazdani et al., 2019, was to continuously culture the cells in the tipping region. First, a small volume of cells, that were not adapted to the extreme conditions, were cultured under selected osmotic pressure of the tipping region. The cells were then transferred to fresh media containing the same salt concentration and the experiment continued with successive growth passages until an improvement in the fitness was observed. The adaptive features of the new population were measured through comparative studies on the growth rate and gene expression of experimental and control samples. It was expected that although in the beginning there was large variation in the gene expression due to the osmotic stress, the selections of genes, that helped with better adaptation, occur over the time scale of the experiment and as a result the regulations became more stable.

2.3. Choice of Organism

The adaptive evolution experiments in high magnesium sulfate were performed using a mesophilic bacterium, *E. coli* (Yazdani et al., 2019). The *E. coli*'s capacity for adaptation allows the bacteria to survive under environmental fluctuations (Santos et al., 1999). Studies on the effects of exposure to high salinity, low temperature, and high pressure on the bacteria suggested that *E. coli* can thrive and survive under these conditions (Jones et al., 2004; Nepal & Kumar, 2018; Kumar & Libchaber, 2013), and therefore it is a good choice for this purpose.

E. coli is a Gram-negative bacterium with an optimal growth at 37°C under atmospheric pressure. It is important to consider that Europa's ocean temperature (Marion et al., 2003) is proposed to be far below the optimal growth temperature of *E. coli*, 37°C; therefore Yazdani et al., expected that *E. coli*'s growth would be very slow at low temperatures, making the experiments not feasible over laboratory time scales. Hence, the studies were performed at a temperature of 37°C and atmospheric pressure with the goal of only investigating the effect of salinity of the cells.

The studies were initiated with wild-type *Escherichia coli* strain MG1655 and cells were cultured in M9 minimal media containing 15% (w/v) $MgSO_4$ continuously for the span of the evolutionary experiments (Yazdani et al. 2019).

2.4 Experimental Protocol: Methods and Material

External osmotic pressure on cells causes water efflux and as a result, cells become dehydrated. For survival under a continuous high osmotic pressure, cells maintain high solute concentrations in their cytoplasm to gain an osmotic balance with the external environment (Wood, 2011). Investigating this osmoregulation is the key to understanding the adaptation to high salinity (Csonka, 1989). The choice of the maximum salt concentration for adaptive evolution experiments done by Yazdani et al. was based on the CI chondritic weathering with around 20 wt% $MgSO_4$ (Yazdani et al., 2019; Kargel et al., 2000; MacKinnon & Zolensky, 2004). For characterizing the tipping salt concentration, the viability and the growth curves at different salt concentrations were analyzed.

Once the tipping concentration of salt was characterized, adaptive evolution experiments were performed with this salt concentration (15% (w/v) $MgSO_4$) using the strategy described in the technical approach. The cells were cultured in tubes containing the respective media and were transferred into new tubes containing fresh media every day. This step was repeated for multiple passages maintaining constant selective pressure on the cells (Yazdani et al., 2019). Every few passages, a small volume of the cells was taken out to check the morphological characteristics and changes in the growth rates of bacterial of cells in comparison with control population of cells (cells that grew in optimal conditions and were not adapted to extreme conditions). After a significant change in the fitness was observed through the comparison of growth rates of the experimental samples over time, the expressions of different genes involved in a number of cellular processes, including osmoregulation, were checked by performing a one-step Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) (Yazdani et al., 2019).

2.5 Overview of Analytical Methods

Analytical methods include optical density measurements and RT-qPCR.

Optical density (OD) is an estimation of cells' density in liquid culture and can be measured using a spectrometer (Beal et al., 2020). OD_{600} refers to the optical density of samples measured in 600 nm wavelength and is commonly used to estimate the number of the cells in microbial samples. The measurement of optical density is based on the amount of light scattered by a suspension of bacterial cells (Sutton, 2011). The concentration of the cells is proportional to the amount of light scattered. As bacteria grow, more cells can be found in the sample, and more light is scattered. We can record the change in optical density over time and report the growth rate. Doubling time (τ_d) is the time that takes for a population of cells to double over one generation (Slonczewski & Foster, 2017) and can be measured by fitting an exponential form of $OD_{600}(t)=OD_{600}(t_0)2^{\frac{t}{\tau_d}}$ equation to the growth curves.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a highly accurate method for rapid and reliable quantification of target sequences of DNA and investigating RNA transcription and gene expression. In contrary to polymerase chain reaction, qPCR collects the data during the exponential phase of the reaction, in which the products are exactly doubled with high efficiency. In this technique, the starting material is the RNA extracted from the bacterial samples. The RNA then will be transcribed to complementary DNA, cDNA, using RNA transcriptase. By adding a fluorescent dye to a mixture of cDNA, primers and other reagents, we can monitor, and measure the amplification of the products in real-time. The process can be performed in one step where the reverse transcription and qPCR are combined in a single tube, or it can be done in two steps using separate tubes and different buffers and strategies (Adams, 2020). The mixture is transferred to the RT-qPCR system for amplification. During the amplification process, the dye binds to the products, making it possible to detect and quantify the amplicons. When the fluorescent signal is detectable, a quantification cycle value, C_q, is determined. The value can be used to evaluate the abundance of target genes in each sample. To perform a relative quantification analysis, the data is first normalized against a reference gene such as GAPDH. Then the relative expression of the of the target gene is measured by increase or decrease in the expression relative to an experimental control sample.

3. Investigations

To examine the adaptation of the samples, two sets of experiments were performed by Yazdani et al. (2019):

1. A small volume of cells from the adaptive evolution experiments (cells that were continuously grown in media containing 15% (w/v) $MgSO_4$; Adapted sample) was taken out in mid exponential phase and diluted to $OD_{600} \approx 0.1$ in M9 minimal media containing 20% (w/v) $MgSO_4$ (three replicates of the same bacterial sample). At the same time, the control population of cells were first grown (up to exponential phase) in M9 minimal media containing 2mM $MgSO_4$ and then were diluted to $OD_{600} \approx 0.1$ in M9 minimal media containing 20% (w/v) $MgSO_4$ (two replicates of the same bacterial sample). All samples (control and adapted) were then incubated at 37°C simultaneously and the optical density (OD_{600}) of the cells was measured every 30 minutes using a spectrophotometer. The data were then analyzed, and growth curves were generated (Yazdani et al., 2019).

2. Regulation of gene expression is a strategy used by bacteria that enables them to adapt to changes in their environment (Barron et al., 1986). To further understand the nature of

adaptation, the gene expression of bacteria under continuous osmotic shock was studied by performing a One-step RT-qPCR on three samples: cells that were continuously grown in M9 minimal media containing 15% (w/v) $MgSO_4$ (Adapted sample), control population in the same media (15% control sample), and control population in M9 minimal media with 2mM magnesium sulfate (0% control sample). Considering the presence of high concentrations of magnesium and sulfate in media, 14 genes were selected for the experiment including the genes that code for *CorA* (magnesium transporter), *CysP* (sulfate transporter), *AqpZ* (porin protein involved in osmotic transport of water), *FtsZ* and *MreB* (cell division proteins) and other genes including fabA, fabR and cysN, envZ, rpoH, groEL, recA, osmC that are commonly regulated under osmotic, heat and pressure stresses (Yazdani et al, 2019). *GAPDH* was chosen as the reference gene. The primers for these genes were designed using the NCBI Primer-Blast tool. Purity of the mRNA and negative control for water contamination was verified (Yazdani et al., 2019).

4. Results and Discussion

Figure 8 shows the comparison between the growth curves of control and adapted cells. The doubling times can be calculated for the adapted and control cells by averaging over the replicate samples. It was observed that while the control populations of cells were unable to grow in 20% (w/v) $MgSO_4$, the cells that undergone adaptive evolution (Adapted cells) could grow significantly. The result implies the osmotolerance of adapted cells (Yazdani et al., 2019). Further investigation is needed to measure the fitness and confirm the adaptation.

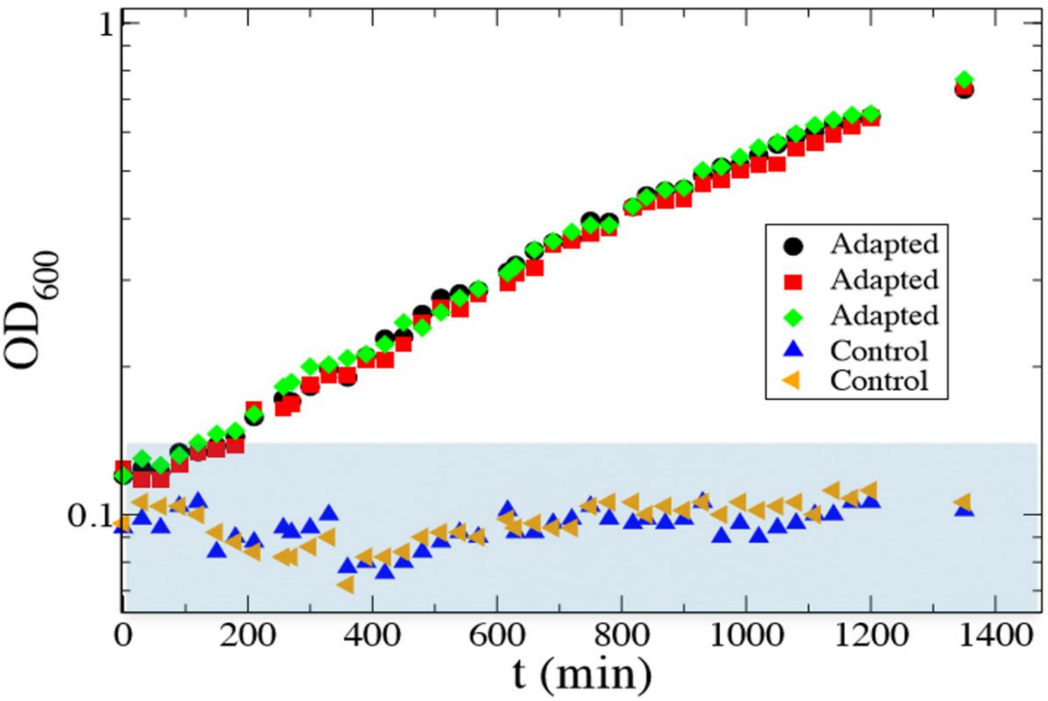

Figure 8. Comparison between the growth curves of the adapted bacteria and control population in 20% (w/v) $MgSO_4$. Figure obtained from Yazdani et al. (2019).

Figure 9 shows a comparison between the gene expression of adapted (in blue), 15% control (in red), 0% control (in green) samples. ΔC_q was calculated by normalizing the data against GAPDH and fold expression changes of genes, $2^{-\Delta Cq}$, were graphed for all samples. Based on the data a downregulation of cysP (involved in sulfate transport), aqpZ (involved in water transport) and corA (involved in magnesium transport) was reported. The result suggested that the strategy used by bacteria is to balance the intake of sulfate and magnesium restrict the water loss in the cells (Yazdani et al., 2019). More investigations are needed to study the nature of the adaptation.

To further investigate the regulation of the gene expression, the relative fold expressions of the genes, $2^{-\Delta\Delta Cq}$, were calculated by the increase or decrease in the expression data of adapted cells compared to 0% control samples, from figure 9. The fold expression greater than one would mean upregulation and fold expression less than one would mean downregulation of the gene. The error was estimated based on the standard error of ΔC_q. The result implied a downregulation of most genes but osmC and envZ (both involved in osmoregulation) that were upregulated. It was also observed that although the downregulation of cysP and aqpZ were noticeable, the change in the expression of other genes, including corA, was insignificant, in comparison to the expression data of the 0% control sample. Therefore, more experiments are needed to confirm these regulations. The result of fold expression changes of genes in 15% control cells implied upregulation of most genes possibly due to the stress from the environment.

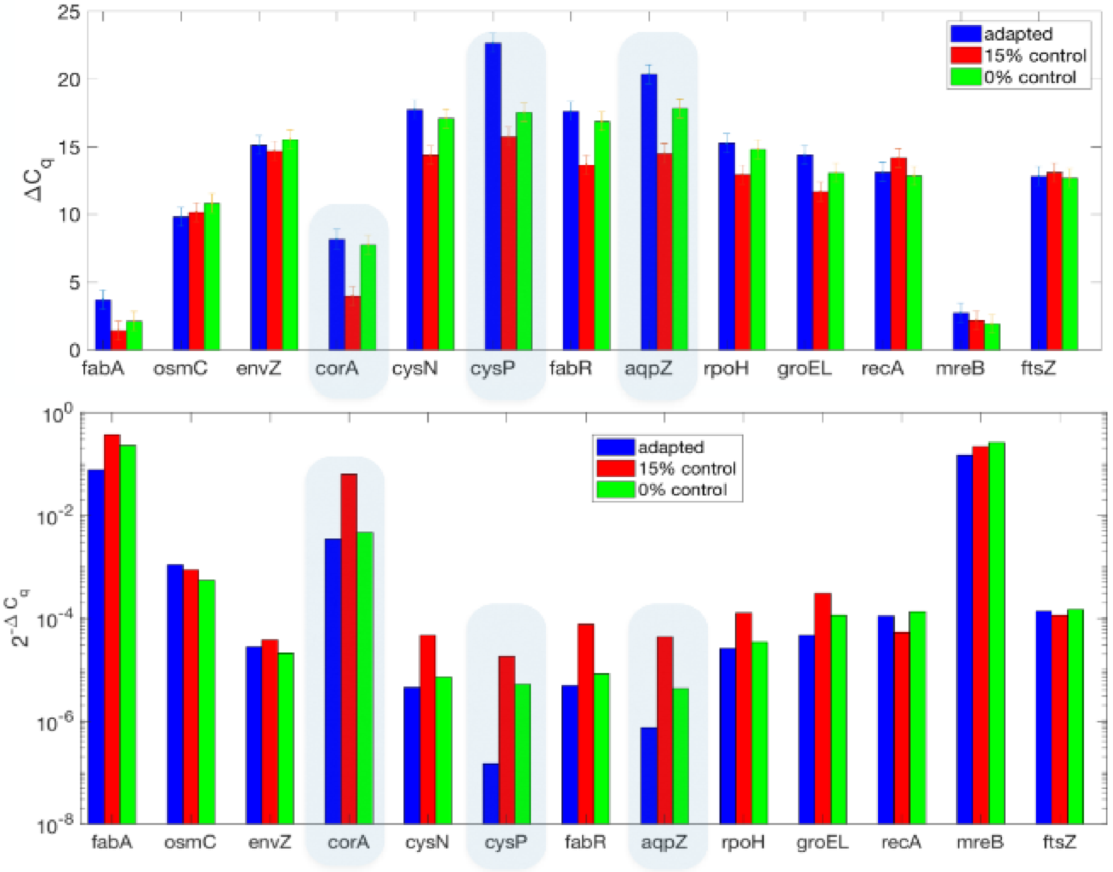

Figure 9. Comparison of gene expressions of adapted and control population of cells at 15% (w/v) $MgSO_4$. Figure obtained from Yazdani et al. (2019).

5. Conclusion

Europa is one of the best candidates for life due to the presence of a subsurface water ocean. Several studies addressed the potential habitability of Europa and investigated the survival of life in high pressure, low temperature, and high salinity. However, these studies only provide a limited quantitative perspective of the adaptation mechanisms. In order to gain a better understanding of the survival and adaptation to these extremes, it is essential to study the long-term effects of exposure to different magnitudes of these environmental conditions on the cellular processes. Furthermore, for adaptive evolution to be feasible in laboratory time scales, we need to study and characterize the growth, viability and regulations of gene expressions in the cells.

This review intended to fill some of these gaps by investigating the adaptation mechanisms of *E coli* to high salinity, under simulated conditions of Europa in the laboratory. The experimental results suggested that the adaptation of *E. coli* to high concentrations of magnesium sulfate was feasible at laboratory time scales and one of the adaptation strategies used by the bacteria was maintaining a balance between intercellular and external environments through regulation of expression of specific genes involved in transporting magnesium, sulfate, and water. Further studies on the survivability and adaptive evolution of bacteria in a combination of different salts, is suggested. Another interesting work would be addressing the cross-adaptation by investigating the growth, viability, and gene expression of the adapted cells at various temperatures and pressures. Finally, the effects of combinations of these extremes should be studied to gain a detailed comprehensive view of the habitability of Europa.

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