Microbial Life Detection with Minimal Assumptions

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ABSTRACT

To produce definitive and unambiguous results, any life detection experiment must make minimal assumptions about the nature of extraterrestrial life. The only criteria that fits this definition is the ability to reproduce and in the process create a disequilibrium in the chemical and redox environment. The *Life Detection Array* (LIDA), an instrument proposed for the 2007 NASA Mars Scout Mission, and in the future for the Jovian moons, enables such an experiment. LIDA responds to minute biogenic chemical and physical changes in two identical "growth" chambers. The sensitivity is provided by two differentially monitored electrochemical sensor arrays. Growth in one of the chambers alters the chemistry and ionic properties and results in a signal. This life detection system makes *minimal* assumptions; that after addition of water the microorganism replicates and in the process will produce small changes in its immediate surroundings by consuming, metabolizing, and excreting a number of molecules and/or ionic species. The experiment begins by placing an homogenized split-sample of soil or water into each chamber, adding water if soil, sterilizing via high temperature, and equilibrating. In the absence of any microorganism in either chamber, no signal will be detected. The "inoculation" of one chamber with even a few microorganisms which reproduce, will create a sufficient disequilibrium in the system (compared to the control) to be detectable. Replication of the experiment and positive results would lead to a definitive conclusion of biologically induced changes. The split sample and the "nanogram" inoculation eliminates chemistry as a causal agent.

Keywords: Mars, Astrobiology, Life Detection, Microbial Growth

1. INTRODUCTION

> *"The minimum requirements for us to recognize an object as living is that it…. or some members of its kind should… reproduce."* (Richard Dawkins)

 The modern debate as to what characteristics to look for in attempting to identify extraterrestrial microbial life has been ongoing since the pre-launch days of the Viking Landers. Because the question of extraterrestrial life has such profound scientific and philosophical implications, the evidence must be definitive and unequivocal. Anything less than that will be unacceptable to a large segment of both the public and scientific communities. Even though both the Viking and the more recent martian meteorite evidence appears strong, both present us with equivocal results. In both cases the possible presence of abiotic chemical artifacts has diminished the reliability of the evidence.

Proposals and implementations for detection of extant organisms have included a variety of instruments and methods, but can generally be divided into three categories. The most unambiguous would be direct microscopic observation with subsequent culturing and chemical identification. Such a scheme requires sample returns or human landed missions, neither of which appear likely this decade. The second, and more easily implemented with robotic missions, includes techniques which attempt to detect and identify biomarkers, compounds that result from biological activity. These compounds may include amino acids, DNA/RNA, PAHs, polycyclics, and lipids. There are however major drawbacks in using such compounds as definitive biomarkers since they may also be formed abiotically (as with amino acids and PAHs) or because we must make untenable assumptions (such as DNA/RNA being a required constituent of life). The third category consists of techniques which attempt to indirectly detect life by monitoring parameters which indicate its presence. Attempts in this area have focused on culturing the organisms and detecting the release of a specific substance.

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The first (and only) attempts at extraterrestrial life detection using culturing techniques were made on Mars by the Viking 1/2 Lander missions in 1976. These landers incorporated three different biological experiments designated as: Labeled Release (LR), Gas Exchange (GEX), and Pyrolytic Release (PR). Both Viking landers also included a Gas Chromatograph/Mass Spectrometer (GCMS) to detect any organic compounds in the martian surface soil (regolith).[1-5] The procedure for the LR experiment consisted of moistening a soil sample with a "nutrient" consisting of water and ^{14}C-labled organic compounds. The sample was then to incubate for at least 10 days, during which time any microorganisms would consume the nutrient and produce gases containing the ^{14}C that would eventually be detected via its radioactivity. There were several assumption made in this experiment about the organism. For example, that it would consume the organic compounds supplied and that it would produce carbon containing gases. Both of these assumptions are unsupportable in that they presuppose that martian organisms would possess several metabolic similarities to Earth organisms. Another assumption is that no unpredictable chemical reactions would occur with the substances added. Since the reactivity and chemical composition of the martian soil was unknown (and still is) this assumption is also untenable.

In the GEX experiment, a soil sample was partially submerged, under a simulated martian atmosphere, in a mixture of organic and inorganic compounds. The sealed chamber was then monitored for at least 12 days. Gases (CO_2, O_2, CH_4, H_2 and N_2) produced by organisms consuming the nutrients would then be detected by a gas chromatograph. The assumptions made here are the same as those made for the LR experiment. Even if no O_2 had been detected in the heat-sterilized control, the possibility of abiotic emission of O_2 would still have made the results non-definitive.

The PR experiment was to test for carbon assimilation. It did not use any added water or nutrients but instead incubated a soil sample in simulated martian atmosphere of ^{14}C-labeled CO_2 and CO, bathed in simulated sunlight provided by a xenon arc lamp. After five days, the atmosphere was flushed, the sample heated to 625°C, and any emitted gasses passed through a ^{14}C detector. The PR experiment resulted in carbon being detected in both samples and heat-sterilized controls. Even though in this case no assumptions were made about nutrients consumed or gases emitted by possible organisms, the question of abiotic chemistry and unknown metabolism, would also have rendered the PR results non-definitive.

Even though some of the biology experiments met the criteria for life detection, they did so ambiguously. Thus, opinions as to the significance of the biology results have ranged from those who feel the data rules out life [1,2] to those who are convinced the results leave no other conclusion but the presence of life,[3-5] and most recently to a study showing that the Viking GCMS would not have been able to detected the degradation products from several million bacterial cells per gram of martian soil at the part per billion level.[6] The most negative results for life detection though came from the GCMS which, to the lowest ppb detection limits, showed a total absence of organic material in the martian soil.[7] Many hypotheses have been advanced to account for this absence and also the possible chemicals and reactions that could account for the ambiguous biology experiments. These have included possible reactions involving oxidants such as hydrogen peroxide,[8,9] smectite clays,[10] superoxides,[11] and most recently superoxide radical ions.[12] Regardless of the numerous hypotheses, and our knowledge of the elemental composition, the bottom line is that we know very little about the chemical composition or reactivity of the martian surface.

The combination of the life detection and GCMS results forms the basis for the prevalent opinion among the scientific community that it is very unlikely that any life (that we would recognize as such) was detected on the surface of Mars, and the conditions are such that it makes unlikely that any organic-based life form could exist. It is against this backdrop that we must ask ourselves, what assumptions should be made, where should we look, and what type of instrumentation should be developed, that will enable us to unambiguously detect any microorganisms on Mars or the Jovian moons?

2. THE MINIMAL ASSUMPTIONS

The literature describing the possible forms that life may take on Mars goes back to the beginning of the 20th century and was bridled only by the author's imagination. More recent attempts, especially since the Viking and Pathfinder missions, have been limited to the microbial domain with severe constraints imposed by temperature, availability of water, and UV radiation. Even though such constraints may have limited the ecological and evolutionary niches where life may have developed and still flourish, there have been numerous attempts at defining these niches, their chemistry, and the possible martian life-forms that may inhabit them.[13-20]

It has been pointed out that if Mars and the Earth had similar environments during the first billion years after their accretion, similar events should have ensued and lead to the appearance of simple life-forms on both planets.[21] It would then seem reasonable that the first life-forms on Mars should be biochemically similar to those first appearing on Earth. In

terms of their composition though, the only reasonable assumptions that can be made is that they, like their Earth counterparts, most likely utilized water and carbon. These same minimal assumptions should also be valid for the Jovian moons that contain water. There is no evidence or reason that the similarity should extend beyond this level. We have only one type of life on Earth, all based on the use of the same 20 amino acids, 5 nucleotide bases, and 8 nucleotides. Since we have no other examples, it is impossible to separate the basic biochemical aspects of life from those that have been shaped by environmental contingencies and biological evolution on Earth. There is no a priori reason to assume that the same 20 of 70 known amino acids, or the same number, would be utilized in extraterrestrial organisms. There is no evidence that extraterrestrial life would be built on terrestrial biochemistry and thus it is untenable to assume any other commonality except water, carbon, an energy source, and reproduction.

If we accept the above set of conditions, then our first assumption is that we should find life where water, carbon and energy are available. This set of parameters though is trivial and not very constraining. Using these constraints excludes within our solar system only Mercury, Venus, and a few moons. Detecting organic substances such as; amino acids (or their chirality), polycyclics, DNA/RNA, polynucleotides, or inorganics such as carbonates or phosphates would indeed be valuable information, but not definitive as to the detection of extant (or extinct) life. The only property of life as we know it, that would identify it as such, is the ability to reproduce. If we take just a few "cells" of an apparent organism, provide them with water, their "native" growth media, and time, the most relevant observation would be the change in the immediate chemical environment caused by the reproduction and growth of the organism.

3. LIDA – AN ELECTROCHEMICALLY-BASED GROWTH SENSOR

In order to utilize growth as a method of detecting microbial life, the instrumentation and methodological issues must be addressed in keeping with the *minimal assumptions* philosophy. Growth of terrestrial bacteria in culture media has typically been monitored optically by measuring turbidity, or electrochemically by conductivity, pH, capacitance, or ion-selective electrodes.[22-28] Although never flown, several of these methods have been proposed for detection of extraterrestrial microbial life.[29-31] Of the above techniques, optical turbidity does not appear viable mainly because of the problems involved in running the analysis in a sample containing soil. Modifications have been proposed to resolve the dilemma[29] but very little is gained and the final results may be open to ambiguous interpretation. Even though more reliable, each of the electrochemical techniques individually may also be open to interferences or interpretation. However, we propose that integrating the conductivity, pH, and several ion-selective electrodes as a sensor array and incorporating them with an advanced sample handling micro-laboratory, can provide a reliable, robust, low-mass, low-power, device for monitoring microbial growth, or what we refer to as the *Life Detection Array* (LIDA). The heart of LIDA takes advantage of two chemical sensor systems recently developed for the Mars Exploration Program and the International Space Station.

3.1 The Mars Environmental Compatibility Assessment (MECA)

The MECA instrument was one of several payloads that was to be sent to Mars on NASA's now cancelled 2001 Mars lander. MECA was designed to provide knowledge about the physical and chemical properties of the martian soil. One of four MECA experiments, the Wet Chemistry Laboratory (WCL) was designed to provide the first chemical composition data in an aqueous environment on another planet. The MECA-WCL, shown in Figure 1, contained four individual 125-cm^3 analytical cells, and associated actuators for solid sample and reagent introduction, thermal control, and agitation. It incorporated fully flight-qualified analog, digital, and power management electronics. Each WCL cell was capped with a water reservoir/actuator assembly designed and fabricated by Starsys Research, Boulder CO. The actuator assembly mixed a soil sample with a leaching solution, provided agitation, and added calibration and reagent solutions.

Mounted on the inside wall of each cell was an array of 26 special sensors for redox, and conductivity, a variety of *Ion Selective Electrodes* (ISE) for anionic and cationic species, including; pH, Br^-, I^-, Cl^-, Cd^{2+}, NO_3^-, Na^+, Ca^{2+}, Mg^{2+}, K^+, NH_4^+ and two gases, CO_2 and dissolved O_2. The technique of cyclic voltammetry (CV) was used to evaluate reversible and irreversible oxidants present in the water/soil solution. *Anodic Stripping Voltammetry* (ASV) at an array of microfabricated gold ultramicroelectrodes was used to measure concentrations of heavy metals including Pb^{2+}, Cu^{2+}, Hg^{2+}, and Cd^{2+} at parts-per-billion levels. The WCL and support electronics were fully qualified with respect to shock and vibration, extreme freezing, thawing, vacuum, and electro-magnetic interference and compatibility. MECA demonstrated the features required to maintain sensor hydration for interplanetary travel, multiplexing of electronics to obtain a variety of measurements, and proper calibration procedures for a remote chemical analysis experiment.
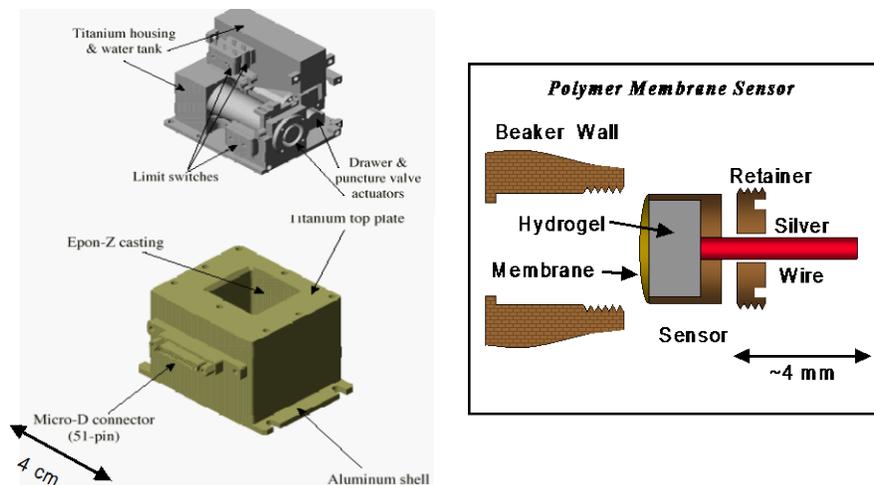
Figure 1. Schematic of the MECA sensor array embedded in an epoxy beaker (ThermoOrion) and the corresponding actuator assembly (Starsys). The actuator mixes the soil, water, and reagents. Inset shows construction of a typical polymer-membrane ISE .

The primary transducer for the MECA-WCL sensor array was the ISE. They are a well-characterized, readily available technology which has the potential to be made suitable for the space flight and habitat environment. The readout electronics consist of a high-impedance voltmeter and corresponding multiplexers and A/D converters. Since the ISEs draw no appreciable current, power usage is minimal. ISEs are compact and robust and are not subject to radiation damage. Most recently there have been several advances in ISE technology enabling a six-order improvement in the detection limits and allowing for detection or analysis of ionic species at levels down to 10^{-12} M.[32]

3.2 The Electronic Tongue

During the past year several members of our team have been involved in developing an electrochemical multisensor by planarizing the MECA-type ISE sensors to further reduce its size to the micrometer range and to provide an overall more robust sensor.[33] A diagram of these new ISE arrays is shown in Figure 2. A typical ISE is composed of an Ag electrode and an ionophore doped gel/polymer layer. The ionophore provides the ISE with selective sensitivity to a specific cationic or anionic chemical species.
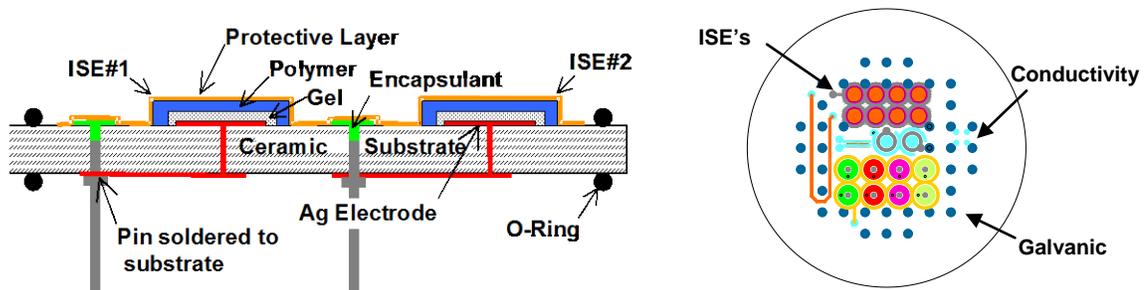

Figure 2. (Left) A cross-section showing the construction of two typical ISE elements. (Right) A top view of a 3cm diameter ceramic substrate showing the arrangement of the various E-tongue sensors, including; eight ISEs on the upper part, eight galvanic cells on the lower half, and across the middle are, left to right, a thermometer, two ASV sensors, and one conductivity sensor.

The current prototype contains eight ISE and galvanic substrates, a thermometer, two ASV sensors, and one conductivity sensor. The fabrication of the ISEs involves screen printing the gel, polymer and protective layers. This planarized ISE array is fabricated on a ceramic substrate using screen printing technology. The doping of gel and polymer layers is accomplished by mounting the substrate in a specially designed chamber where the ion selective ionophores in the electrolyte are then immobilized onto a particular electrode by applying the desired potential V_{cell}. After doping the gel, the first ionophore solution is removed and a different ionophore solution is introduced into the chamber and immobilized onto

a new electrode. This process allows the individual doping of each electrode in the array with a different ionophore. After the gel is doped, the polymer layer is screen printed and doped by the same procedure used to dope the gel. After polymer doping, a protective layer is screen printed over each electrode. This initial effort in developing an ISE array is pointing the way to higher-density multi-sensor arrays. It is envision that eventually the e-tongue will consist of an array of 25-30 ISEs with several other electrochemical sensors, all contained on a small centimeter-sized substrate.

3.3 An Automated Sample Handling and Growth Chamber System

As part of an ongoing effort to develop a second generation MECA instrument, Starsys Research has designed a wet chemistry laboratory which can be used on future Mars lander missions. An evaluation of different concepts and design approaches was conducted and an entirely new approach was developed, totally different from the original MECA/WCL system. The new approach resulted in the robotic chemical analysis laboratory (RCAL), shown in Figure 3, and which adds the capability to perform multiple experiments and multiple calibrations. It is designed based on a flexible modular approach that allows it to be used for a variety of scientific experiments. The RCAL is being adapted to function as an automated sample handling and growth chamber for LIDA.
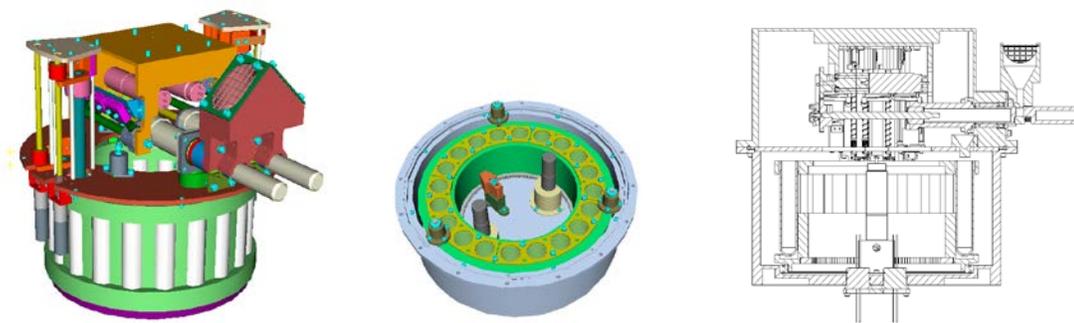

Figure 3. Several views of the Starsys robotic chemical analysis laboratory (RCAL) being used as the platform for the fabrication of the Life Detection Array. The carousel contains 10 pairs of growth chambers that can be rotated into position for each growth experiment.

The RCAL system for LIDA will consist of three primary subsystems: growth chambers with integrated sensor arrays, carousel and drive mechanism, and a soil delivery mechanism. These three subsystems provide the basic functions required by LIDA. In addition to these primary subsystems, several other components including pressure and temperature sensors, pressurization system, external housing and electrical connections make up the system. The growth chambers, carousel and drive mechanism take up a large portion of the lower half of the system. The twenty clear polypropylene or polycarbonate chambers with the integrated sensor arrays ride on a central carousel, each containing a small amount of moisture to maintain sensor hydration and heat sealed with an aluminum/polypropylene foil closure. Two redundant soil delivery mechanisms will deliver soil to the chambers after receiving it from an external lander robot arm or subsurface drill. The soil delivery system will split the homogenized sample and deliver it to two adjacent chambers. A water reservoir will add water if or as needed.

4. HOW LIDA WORKS

A schematic diagram of the growth chambers is shown in Figure 4. The are several crucial and necessary aspects of the LIDA system which will insure that the results are as unambiguous as possible. These include the use of two chambers, one as a control and the other for the inoculation, the sterilization and inoculation processes, the specialized sensor array, use of the local soil as a growth medium, and the ability to provide multiple replications of the growth experiment. The growth experiment starts as soon as a soil sample has been collected, homogenized, split, and delivered to two adjacent chambers. Both chambers are identical in all respects and the sensors are monitored at all times.

After equal amounts of soil sample are delivered, both chambers are filled with equal amounts of sterilized pure water to cover the sample. Once this has occurred, the stirrer and sterilizing heaters are turned on. The temperature is then increased to a predetermined point (120ºC would probably be more than sufficient) and maintained for a period of time (10-30 min.). The heaters are then adjusted to provide a constant temperature just above freezing (1-5ºC), and the system is allowed to equilibrate and the sensors to reach a steady state. The atmosphere above the solution is maintained at 6-10

mbar CO_2.   At the end of this procedure, the two chambers should be able to remain stable for prolonged periods of time with no changes in any of the measured parameters. Any global changes due to temperature, pressure, or soil chemistry, should affect both chambers identically.
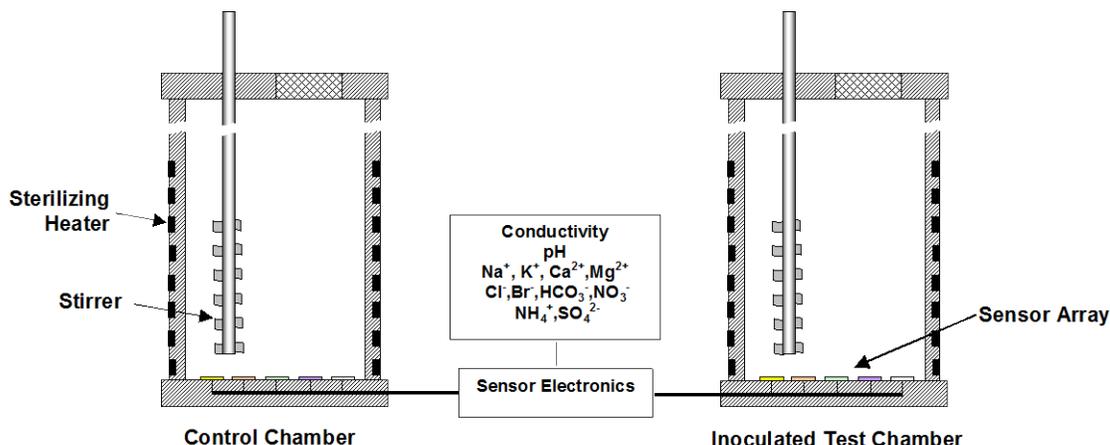

Figure 4. Schematic diagram of the basic components of the differential life detection chambers. Both chambers are identical in every respect until the unsterilized nanogram sample is added to the test chamber. (Not to scale.)

After the control and test chambers have been sterilized and all sensors have stabilized, the inoculation of the test chamber can be made. The inoculation is carried out by obtaining a nanogram quantity (or perhaps just a inoculation-loop swipe) and placing it in the test chamber. Introducing a nanogram quantity decreases the probability that a chemical reaction with the water will dominate or persist for any length of time. Soil reactions with the water would also have been observed with the original addition of the soil. Providing for replicate experiments and constant monitoring of the chemistry, should allow for the discrimination between an authentic growth response and a chemical contaminate. The chambers are monitored for at least several days, or longer if the mission permits. Microbial metabolism and excretion in the test chamber will alter many chemical parameters which can be detected by the various sensors in the array.

- Changes in cationic and anionic species concentrations will be detected by the array of ion selective electrodes (ISEs).
- Creation and/or removal of ionic species can be monitored by measuring conductivity.
- The surface of most of the sensors are extremely sensitive to "biofouling", a problem which is ever-present in terrestrial monitoring situations. This is especially true of the membrane-based ISEs. Even partial monolayer coverage can effect the transport and charge properties of ISE membranes.

If this experiment was performed in a totally sterile environment, there should be no difference between the two monitored chambers. Chemical reactions between the sample and the chamber walls, the water, the atmosphere, or the sensors, should be identical. Global changes of such parameters as temperature or pressure will affect both chambers. Any differential between the two chambers, that changes as a function of time, must necessarily be the result of the "substance" introduced into the test chamber. This substance and/or entity must thus be possess the ability to cause (reproducibly and exponentially) extensive slow changes in the chemistry of the sample even though it was introduced in nanogram quantities.

In the coming year we will be demonstrating the ability of LIDA to detect microbial growth in Earth samples. Using a variety of microorganisms and more advanced versions of the sensor array we will determine the levels and time necessary to obtain valid and reliable data. Using *Lactobacillus casei* and *Cyanobacterium cyanothece*, we have begun to investigate the responses expected from such cultures and the effect of growth on standard ISE and conductivity sensors. Figures 5 and 6 show typical examples of the preliminary results obtained. In both cases changes in conductivity and/or ionic species was detectable by the sensors after 6-12 hours. Application of the E-tongue technology should enable the detection of growth at even lower thresholds and thus after shorter time periods. Conversely, lower detection limits will also enable the detection of microorganisms with very slow growth rates.
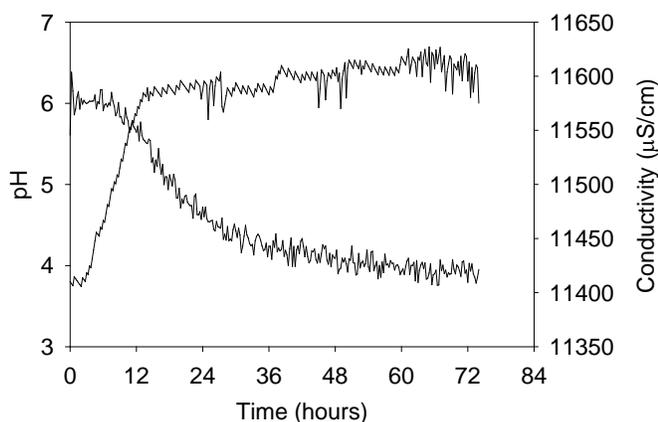
Figure 5. Conductivity and pH as a function of growth for test sample containing *L. casei*. The pH starts at 6 and decreases to 4 after about 48 hours. Conductivity increases approx. 200 μS/cm.
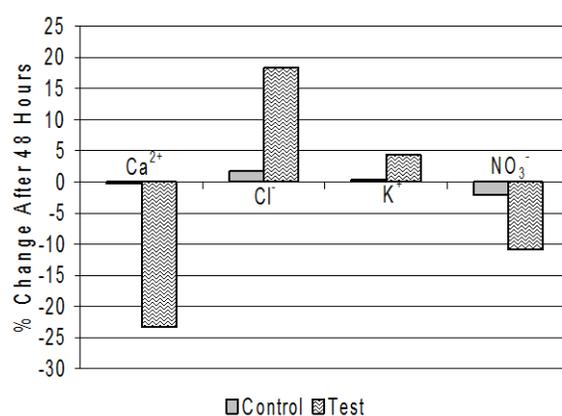
Figure 6. Typical response for an array of calcium, chloride, potassium, and nitrate ISEs as a function of growth for sterile control and test samples, the latter inoculated with *L. casei*.

5. CONCLUSIONS

We must strive to define life by its most general attributes and conscientiously avoid assigning to it any Earth-centric characteristics. Thus, we must research, design, and develop remote life detection methods and instruments which seek to identify extraterrestrial microorganisms based solely on simple chemistry and physics. Until the day we can show that Earth-like biochemistry is ubiquitous, the word "Biology" should really be translated as "Earth-biology". Our initial experiments and those to be undertaken with the integration of the E-tongue and RCAL to give an advanced version of LIDA, should enable remote planetary experiments which will provide reliable detection of microbial growth. Even though some will not be satisfied until a team of scientists, working in a laboratory on Mars, provide overwhelmingly undeniable evidence of extraterrestrial microorganisms, LIDA has the potential to provide significant and possibly unequivocal evidence.

6. ACKNOWLEDGEMENTS

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