Methodology article

Measurement of microbial activity in soil by colorimetric observation of *in situ* dye reduction: an approach to detection of extraterrestrial life

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Abstract

Background: Detecting microbial life in extraterrestrial locations is a goal of space exploration because of ecological and health concerns about possible contamination of other planets with earthly organisms, and vice versa. Previously we suggested a method for life detection based on the fact that living entities require a continual input of energy accessed through coupled oxidations and reductions (an electron transport chain). We demonstrated using earthly soils that the identification of extracted components of electron transport chains is useful for remote detection of a chemical signature of life. The instrument package developed used supercritical carbon dioxide for soil extraction, followed by chromatography or electrophoresis to separate extracted compounds, with final detection by voltammetry and tandem mass-spectrometry.

Results: Here we used Earth-derived soils to develop a related life detection system based on direct observation of a biological redox signature. We measured the ability of soil microbial communities to reduce artificial electron acceptors. Living organisms in pure culture and those naturally found in soil were shown to reduce 2,3-dichlorophenol indophenol (DCIP) and the tetrazolium dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT). Uninoculated or sterilized controls did not reduce the dyes. A soil from Antarctica that was determined by chemical signature and DNA analysis to be sterile also did not reduce the dyes.

Conclusion: Observation of dye reduction, supplemented with extraction and identification of only a few specific signature redox-active biochemicals such as porphyrins or quinones, provides a simplified means to detect a signature of life in the soils of other planets or their moons.

Background

The detection of microbial life in extraterrestrial locations is an important goal of human exploration of space because of ecological and health concerns about possible contamination of other planets with earthly organisms, and *vice versa* [1,2]. Several indirect methods for detecting extraterrestrial life have been proposed in the prior literature. These include (a) examining the ratios of stable isotopes in important elements such as carbon and sulphur for discrimination against heavy isotopes and selective use of the lighter isotopes, (b) microscopic observations of highly organized or specifically-shaped structures [3,4], (c) examination of soils for the presence of specific types of organic moieties [5], and (d) the detection of chiral molecules in extraterrestrial samples [6–12].

Previously we suggested a thermodynamic approach to the detection of life. Since living entities require a continual source of usable energy, we should be able to detect a chemical signature of life in the form of mixtures of redox molecules such as porphyrins, quinones, and flavins that transport electrons along redox gradients between electron donors and acceptors [13]. We examined methods to extract and detect the components of electron transport chains that might be used to indicate the presence of life on site in extraterrestrial locations. Our experiments showed this to be a promising approach, though instrument development and miniaturization for this purpose will be challenging [14].

Here we discuss the possibility of a related, but simpler approach for detecting life that will measure the actual existence of functioning electron transport chains. This can theoretically be done remotely and on site using robotically collected extraterrestrial soil samples. The method involves detection of actual activity of respiratory electron transport using artificial electron acceptors such as DCIP and tetrazolium dyes such as XTT. DCIP, for example, couples directly with NADH or NADPH to form $DCIPH_2$ and NAD^+ or $NADP^+$, making it an ideal electron transport chain indicator [15]. XTT has been used to measure microbial respiratory activity in complex natural environments [16]. Respiratory dyes have been used commercially for many years in the Biolog™ system to help identify pure cultures of microorganisms by their patterns of oxidation of multiple organic substrates [17]. Biolog™ also has been used to characterize oxidation capabilities of soil microbial communities [18]. Rodriquez, et al. [19] used the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for direct epifluorescent microscopic enumeration of respiring bacteria in environmental samples. The oxidized form of CTC is nearly colorless and non-fluorescent. Oxidized CTC was observed to be reduced by biological electron transport systems, forming a fluorescent, insoluble CTC-formazan. The formazan accumulated intracellu-

larly and could be visualized by epifluorescence microscopy in wet-mount preparations, on polycarbonate membrane filter surfaces, or directly in some biofilms. Thus, coupled with more limited analyses for specific electron transport molecules (e.g., porphyrins or quinones), the use of respiratory dyes for detection of active electron transport in the biological redox range could provide very convincing evidence for life in extraterrestrial locations. This approach would obviously only detect presently living entities, but these could include dormant life forms such as spores that might germinate and grow using added nutrients.

Results

Examination of the presence of a chemical signature of life in various soils

Figure 1 presents the results of a comparison of supercritical carbon dioxide extractions of control (heat treated at 600°C) and life-positive (unheated Idaho red sand) soils. We confirmed the structures of the compounds shown as marked peaks by their respective UV-VIS and mass spectra. The supercritical carbon dioxide ($SCCO_2$) extracts of Idaho red sand (Figure 1) and a Lake Boney Basin Antarctica soil, which contained relatively few bacteria (Table 1), showed detectable signature molecules in the extracted samples using either a diode array detector or an amperometric detector (Figure 1). The sterile soil, after heat treatment, did not yield significant amounts of our targeted redox signature molecules: nicotinamide, riboflavin, Q_0, FMN, FAD, and porphyrin (Figure 1). To confirm that soil samples yielding negative results and those yielding signatures of redox moieties were different because of the presence and absence of biological entities, we counted viable organisms and extracted DNA from the heated control sand, a Beacon Valley Antarctic soil, a Lake Boney Basin Antarctic soil, and the Idaho red sand (Table 1). There were small amounts of organic compounds in the extract of the Beacon Valley soil, but none matching our standards (no redox signature). At a detection limit of 1 ng per gram of soil, DNA was absent from the control and the Beacon Valley soil, as were cultivable bacteria. Soils that contained electron transport moieties (e.g., the Idaho red sand and Lake Boney Basin soil) also contained both DNA and cultivable bacteria.

Redox dye reduction by various soils

Figure 2 illustrates the color change seen when a soil microbial community reduces the dye XTT. Figure 3 shows results of measurement of DCIP reduction in an autoclaved control soil and an extraterrestrial surrogate soil (Idaho red sand). Figure 4 shows results of XTT reduction by the control soil and the life-positive Idaho red sand. Figure 5 shows results of XTT reduction by the control and the life-positive Boney Basin, Antarctica soil. The Beacon Valley, Antarctica soil did not reduce either dye. As anoth-
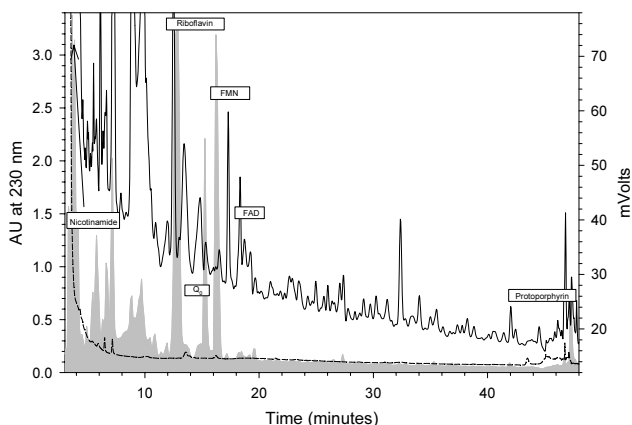
Figure 1
Supercritical carbon dioxide extract of unamended sandy soil (Idaho red sand) analyzed by ion pair HPLC For this separation we used a Supelco Discovery Amide C16 reverse phase column (15 cm × 4.6 mm, 5 μm). The elution method is described in the text; at the end of the run porphyrin was eluted with 100% acetonitrile. The filled chromatogram shows data from the optical detector (230 nm); the solid line chromatogram shows data from the electrochemical (amperometric) detector (ECD). For the ECD, the dual carbon electrode was set at two potentials: the first electrode was at -0.5 V and the second at +1.0 V. Signals from the second electrode were recorded. The ECD electrodes were polished at least once a day to keep the carbon electrode fresh for maximum sensitivity. The dashed line chromatogram shows optical data (230 nm) from extracted soil that had been heated at 600°C before extraction. Peaks for targeted redox signature compounds (nicotinamide, riboflavin, Q_0, FMN, FAD, and porphyrin) were not observed in this control. Compounds identified in the chromatogram peaks of extracts of living soil (filled chromatogram and solid line chromatogram) are marked with flags and names. AU = Absorbance Units

Figure 2
Color change resulting from the microbial reduction in soil of the respiratory dye XTT Bottle on the far left is a control of the oxidized dye in sterile water. The middle bottle shows lack of dye reduction by a sandy soil that was autoclaved. The bottle on the far right shows dye reduction (increase in orange color measurable at 465 nm) from a biologically active (contains living bacteria) sandy soil.

er control, Figure 6 shows the reduction of XTT by a pure culture of *Pseudomonas putida*.

Discussion

Energy use by living organisms directs our experimental design for seeking signs of extraterrestrial life. In terrestrial biological systems, energy is tapped via stepwise electron transport between electron donors and acceptors via an *electron transport chain*. Applying this principle universally, we should be able to detect the chemical components of electron transport chains or electron transport itself as a signature of life.

Our assumptions about the centrality of electron transport processes to life led us to a direct experimental approach for detection of life. We were able to measure actual electron transport in the redox range between -1.0

and +1.0 V in a microbe-poor soil, in the presence of air, and both with and without additions of an electron donor (dilute nutrient broth). The respiratory electron-accepting dyes DCIP and XTT were used to "short-circuit" microbial electron transport, and this was observed (dye reduction) by visible spectroscopy. This method might entail some site-specific challenges in environments that are different from those on Earth, especially in developing appropriate controls for abiotic processes. NASA learned this lesson from the Viking biology mission to Mars. In the Viking's gas exchange experiment, water vapor exposed to the Martian soil released O_2, but possibly only as a result of the activity of powerful soil oxidants that also might have been responsible for the chemical reactivity seen in other Viking biology experiments [20]. A molecule such as DCIP or XTT might be destroyed by oxidation in the presence of such soils. However, soil samples taken from borings beneath the surface, which is exposed to high energy, oxidant-generating ultraviolet radiation, or from other protected areas, might not be so destructive of organic compounds. Such stability problems should not be of such concern for extraterrestrial sites such as Europa or Titan. Thus, our ability, confirmed here, to detect actual electron transport within the redox potential range of life processes, and to observe simultaneously some of the signature chemical structures involved, offers a promising direct approach to detecting the capability of living entities in soil on Earth or elsewhere in our solar system to move electrons between universal donors and acceptors and obtain energy to support replication.
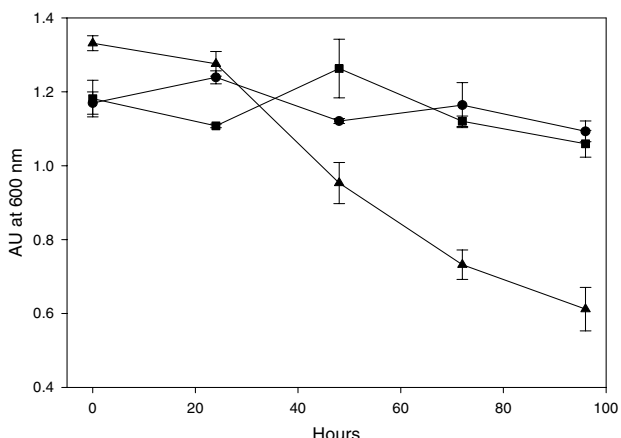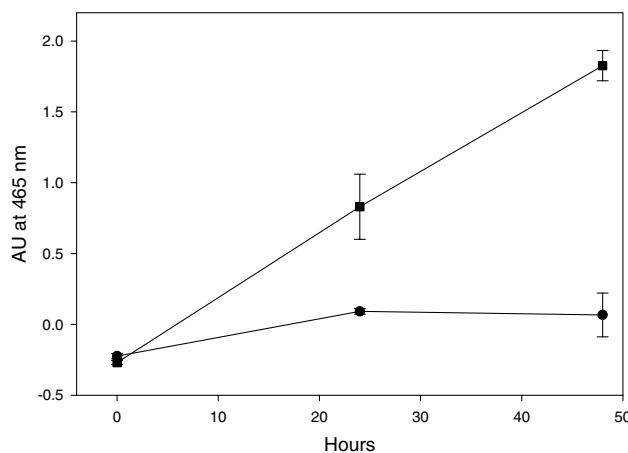
Figure 3
Reduction of DCIP dye by the heat-treated control soil and the extraterrestrial surrogate soil (Idaho red sand) Data points represent: (■), autoclaved (killed) control soil; (▲), live soil; (●) blank, no soil. Data points are means of 3 replicates. Error bars represent +/- standard deviation. Dye-only controls showed no reduction over the time course of the experiment. AU = Absorbance Units

Since we are not yet able to test this approach extraterrestrially, we validated our proposed detection method using pure microbial cultures and microbe-poor soils collected on Earth. A select group of signature chemicals were extracted from an organic-poor soil (Figure 1) and identified with instruments that eventually can be miniaturized for use on space exploration missions [13,21,22]. Actual electron transport by living cells was confirmed by use of two specific dyes (DCIP and XTT) that short-circuit respiratory electron transport chains. This was demonstrated using a pure strain of the common earthly soil microorganism, *Pseudomonas putida* (Figure 6), and with native soil microorganisms within two nutrient-supplemented soil matrixes (Figures 3,4,5). We easily detected a signature of biological electron transport within a biologically poor, sandy soil containing about 30,000 cultivable cells per gram (Idaho red sand), and also from a soil from the extreme environment of Lake Boney Basin, Antarctica (Table 1). Though the Boney Basin soil readily reduced XTT, it only very slowly reduced DCIP (data not shown), and the rate of reduction was not significantly different between untreated soil and killed controls. Since DCIP and XTT have different redox potentials, and XTT in our work appears to generally be more easily reduced by soil microbial communities, this shows a need to use more than one respiratory dye in this type of experiment.

In this work, we emphasized that during validation on Earth of any life detection method there is a requirement for control measurements that corroborate earthly results

of assays that might be carried out remotely on other planets. We chose measurements of DNA (present in all earthly life) and readily cultivable bacteria (present in most earthly soils) for validating our specific redox chemistry based approach. In the future, as we and others work to increase the sensitivity of this life detection approach toward values needed for the extreme environments on Earth and places such as the regolith of Mars, the simple controls we used should be supplemented by a number of others. These could include controls such as direct microscopic counts of active and inactive bacteria [19], extraction and quantitation of ATP [23], extraction and analysis of fatty acids [24], real-time PCR of universal 16S rDNA markers [25], and/or use of a larger variety of culture media, including dilute organic media, in place of or in addition to plate count agar [26]. All of these approaches also have problems, such as the possible presence in soil of biological molecules that are not associated with living cells, the always-present inability of microbiologists to culture most of the bacteria present in soil, and the often unpredictable performance of PCR primers. However, the sum of such multiple controls would build toward ultimate confidence in the sensitivity of this or any other life detection technique.
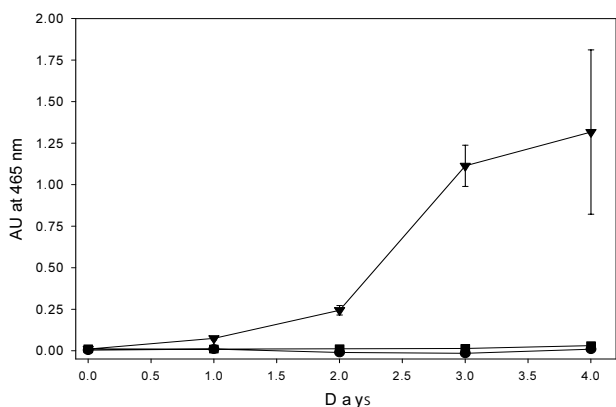
Our observations also indicate that in soils from extreme environments, one may expect differences in their patterns of dye reduction that probably reflect differences in their community phylogenetic compositions. A soil from Beacon Valley, Antarctica that showed no DNA at our detection limit also showed no dye reduction activity with
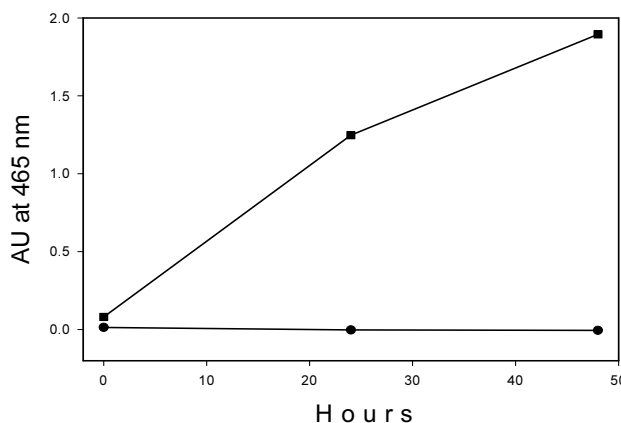

Figure 4
Reduction of XTT dye by the heat-treated control soil and the life-positive Idaho red sand Data points represent: (●), sterilized soil; and (■), live soil. Data points are means of three replicates, +/- standard deviation. Dye-only controls showed no reduction over the time course of the experiment. AU = Absorbance Units

either dye. This soil appears to be sterile by our method of life detection; certainly it contains fewer microbial cells than our detection limit. Further investigations of this soil are thus warranted. For the Idaho red sand, assuming 30,000 cells represent about 1% of actual biomass in the soil, the detection limit of our method should be about 3,000,000 cells/g. Though the amount of DNA observed in the Lake Boney Basin soil was actually higher than seen in the Idaho red sand, the microbial counts were lower (Table 1). This shows once again the limitation of viable counts, which only count organisms that grow on the specific medium employed. It is well known that most bacteria in nature cannot be cultured. Our work indicates there is probably a more dominant non-cultivable microbial community in the Antarctic soil compared to the Idaho soil, since DNA analysis indicated high numbers of microorganisms that were not reflected in viable count data.

We need to improve the current detection limit for organic moieties (Figure 1), estimated at 3×10^6 cells per gram of soil. Processing larger quantities of soil could easily increase sensitivity for detection of redox molecules. Supplementing soils with appropriate electron donors and acceptors could stimulate extensive growth of low numbers of indigenous organisms. In theory, even a few cells/ gram of soil might be detected using this approach. The former procedure might be accomplished by using a "flow-through" soil extractor that could receive multiple soil additions and concentrate the extracted residues prior

Figure 6
XTT reduction by a pure culture of *Pseudomonas putida*. Data points represent: (●), control without pseudomonad; (■), soil inoculated with *P. putida*. Data points are means of three replicates. AU = Absorbance Units

to downstream analysis. The latter procedure could be implemented by using a microtiter plate format similar that employed by Biolog™, where hundreds of potential electron donors could be examined simultaneously and the results (dye reduction) read by an on-board UV-visible spectral scanner designed for scanning microtiter plates. Examples of donor-acceptor combinations might include: simple sugars and/or amino acids or other organic molecules in the presence of oxygen or nitrate (organisms capable of heterotrophic respiration), hydrogen and ferric ions or oxidized iron minerals (chemoautotrophic hydrogen oxidizers), organic acids and ferric ions or oxidized iron minerals (heterotrophic iron reducers), bicarbonate/carbonate and trace minerals in the presence and absence of light (photosynthetic organisms), carbon dioxide and a reduced metal such as ferrous ions (chemoautotrophs), ammonium ion and bicarbonate/carbonate (ammonia oxidizers), etc. The combinations could be adapted to the environment under study, such as Mars (perhaps an iron-dominated energy system).

Figure 5
Reduction of XTT by the heat-treated control soil and the life-positive Lake Boney Basin, Antarctica soil Data points represent: (▼), Live Boney Basin Soil; (■), Killed Boney Basin Soil; (●), Negative Control (no soil); Lake Boney Basin soil only very slowly reduced DCIP (data not shown). Data points are means of three replicates, +/- one standard deviation. Dye-only controls showed no reduction over the time course of the experiment. AU = Absorbance Units

Because there is great diversity among habitats for microbial life on the Earth, and sterility of an earthly soil is rare, it would be desirable to examine as many as possible of these environments to determine the absolute reliability of our techniques for use extraterrestrially. Unfortunately, we cannot sample all possible terrestrial environments on Earth, or even a small fraction of them. We feel the diversity we have examined is sufficient to indicate the value of our approach, particularly since one of these extreme environments we did examine (Beacon Valley, Antarctica) appears to be sterile. We also cannot examine all known heterotrophic bacteria stored in culture collections to confirm that all or most of them effect dye reduction. Howev-

Table 1: Confirmation of the presence of bacterial life in terrestrial soil samples.

Soil Type	Plate Count[1] (viable cells/g soil)	Fluorescence[2] (ng DNA/g soil)
Red sand	2.74×10^4	3.68
Silica sand	ND	ND
Beacon Valley, Antarctica	ND	ND
Lake Boney Basin, Antarctica	4.2×10^3	38.9
Blank (reagents only)	0	0

Data are means of three replicates. Standard deviations were less than 5% for all results. [1]Soil was suspended in distilled H_2O, diluted, and an aliquot spread onto Plate Count Agar plates for quantification of colonies. Counts were taken after 48 h. [2]Our detection limit was about 1 ng DNA/g soil. A 0.5 g amount of soil was used for each extraction. ND = not detected

er, the many years of experience using the Biolog™ system, which employs respiratory dyes to indicate microbial metabolism [17,18], is strong support for our hypothesis that respiratory dye reduction is a robust and conclusive assay for heterotrophic life in soil. Further validation efforts may show that observation of redox cycling of artificial electron acceptors of appropriate redox potentials may be sufficient in itself as a signature of life, greatly simplifying instrumentation requirements for extraterrestrial missions in our solar system and beyond.

Conclusions

Coupled with limited analyses for specific electron transport molecules (e.g., porphyrins and/or quinones), the use of respiratory dyes for detection of electron transport in the biological redox range could provide very convincing evidence for life in extraterrestrial locations.

Methods
Signature chemicals

As detailed in our prior work [13], the following compounds were used as representative examples of electron transport chain components. Protoporphyrin IX, hemin, hematin, and nicotinamide (vitamin B_3) were purchased from Aldrich (Milwaukee, WI). Nicotinamide-adenine dinucleotide (NAD), riboflavin (vitamin B_2), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and DCIP were purchased from Sigma (St. Louis, MO). 2,3-Dimethoxy-5-methyl-,4-benzoquinone (coenzyme Q_0) was purchased from Fluka (Buchs, Switzerland). XTT was purchased from TCI America, Inc. (Portland, OR). The other chemicals and solvents used were of the highest available purity.

Soils

Natural sand from dune areas of southern Idaho (Idaho red sand), poor in organic content and rich in oxidized iron minerals, was used as a life-positive soil and surrogate for extraterrestrial soils. As a control we used cleaned,

highly pure silica sand purchased from Fisher Scientific. These soils are described in our previous paper [13]. Other extraterrestrial surrogate soils were collected from Beacon Valley and Lake Boney Basin, Antarctica. The soil from Beacon Valley was from an area where higher organisms such as nematodes have never been observed.

Extraction of soil organic moieties

Soil samples of 5 to 15 g were used for supercritical fluid extraction (SFE) with supercritical CO_2 ($SCCO_2$). Samples were first hydrolyzed in 5 to 10 ml of acetone containing 2 M NH_4OH under ultrasound agitation (water bath at 160 watts) for 1 h at room temperature. Nitrogen blown over the sample then evaporated the solvents. The SFE extraction was performed with the dry sample within one day to avoid any degradation of redox compounds. A piece of Whatman glass fiber filter paper (1.0 μm pore size) was cut into a 0.8 cm diameter circle and placed at the outlet end of a 10-ml SFE extraction cell to retain large particles. A hydrolyzed sample (10 ml) was then packed into the vessel until it was full. Exactly 1.0 ml of 25% (v/v) trimethylamine in methanol as modifier was added to the sample, and the vessel was immediately connected to the SFE system. The extraction pressure was set at 300 atm, the oven temperature at 35°C, and the outlet valve temperature at 60°C. After a 15 min static extraction, a dynamic extraction was started until approximately 30 ml of liquid CO_2 passed through the SFE cell [27]. The flow rate was controlled at 5 to 10 ml/min by the outlet valve. In order to achieve maximum recovery, the SFE procedure was repeated again under identical conditions.

Chromatography

Separations were performed using a Hewlett Packard HP-1090 HPLC (Avondale, PA). Samples of 20 to 50 μL were injected onto a Discovery Amide C16 or Discovery C18 reverse phase column (15 cm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA), and analytes were separated using the ion-pair chromatographic method. Solvent A was 5 mM

tetrabutylammonium phosphate, 30 mM KH_2PO_4, and 4% acetonitrile (V/V). Solvent B was 100% acetonitrile. Runs were performed over 35 min with gradient elution of 0 to 40% B, then with static elution over 35 to 40 min with 60% A/40% B, followed by 40 to 42 min with 100% A and a post-run wash of 5 min at a flow rate of 1.0 ml/min [28]. Detectors used were a tandem diode array detector (DAD) and dual carbon electrode amperometric detector (ECD).

Electrospray tandem mass-spectrometry

The identities of signature species present in extracts were confirmed by negative or positive electrospray ionization tandem mass spectrometry (Quattro II, Micromass Ltd., UK). Samples were delivered into the source at a flow rate of 5 µl/min using a syringe pump (Harvard Apparatus, South Natick, MA) or directly from a HPLC column, with effluent from the column split 1:100. A potential of 2.5 to 3 kV was applied to the electrospray needle. The sample cone was kept at an average of 15 V. The counter electrode, skimmer, and RF (radio frequency) lens potentials were tuned to maximize the ion beam for a given solvent. The resolution of the detector was 15,000, and the source temperature was kept constant at 80°C. The instrument was calibrated using a polyethylene glycol solution. All spectra were an average of 10 to 15 scans.

Biomass and DNA analysis in soil

The numbers of cultivable soil microorganisms were estimated by standard plate count and serial dilution methods. Standard plate count medium (PCM) was used to culture bacteria from soil samples [29]. DNA was extracted from different soils using the FastPrep DNA spin kit from Qbiogene, Inc. (Carlsbad, CA). The concentration of DNA was estimated by measuring fluorescence at 520 nm. Fluorescence was measured after staining the sample with Pico Green fluorescent dye (Molecular Probes, Eugene, OR). Our detection limit was about 1 ng of DNA in 1 g of soil.

DCIP and XTT reduction assays

Working in a sterile laminar flow hood, we added 2.0 g of soil, 16.0 mL of sterile 100 mM phosphate buffer (pH 7.5), 2 mL of 100X Nutrient Broth as a biological reductant (Difco), and 1.0 mL of 1 mM DCIP to a sterile 25 mL serum bottle. Bottles were plugged and capped with sterile closures and shaken at room temperature for required experimental time. Periodically, samples (1.0 mL) were removed aseptically and centrifuged to remove solids. The absorbance of the filtrate was measured at 600 nm. Concentrations of DCIP (oxidized) were calculated using a molar extinction coefficient of $\epsilon = 21.0 \times 10^3$ M^{-1} cm^{-1}. Reoxidation of $DCIPH_2$ was negligible under our experimental conditions. Samples were run in triplicate.

Reduction of XTT was monitored similarly as described for DCIP, except we measured the increase in absorbance at 465 nm resulting from reduction of the colorless oxidized dye to an orange product [16].

Authors' contributions

All authors worked within a multidisciplinary team. RLC conceived the study, and participated in its design and coordination. AP directed the analytical chemistry work reported and interpreted the analytical data. QL performed the $SCCO_2$ extractions. DPE and LBA performed the dye reduction experiments and provided ideas for improvements of methods. GC and TJA designed the $SCCO_2$ soil extraction cell. FC provided expertise on voltammetric detection of analytes. CW, BB, RW, TA, and MM provided input regarding data analyses and overall life detection system design for potential future use on extraterrestrial missions.

All authors read and approved the final manuscript.

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