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ORIGIN OF LIFE: TESTING THE ISOTOPIC RESONANCE HYPOTHESIS

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Origin of Life: Testing the Isotopic Resonance Hypothesis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To My Family

致我的家人

ABSTRACT

The Miller-Urey (MU) experiment provided evidence supporting the abiogenesis theory, and is considered to be the seminal experiment in the context of origin of life. The MU mixture produced in the experiment is assumed to be an essential raw material for life emergence on the primitive Earth or beyond. However, there was no direct experimental evidence that this primordial soup supports life. In this thesis, we provided a proof that the abiotically produced MU mixture can support the growth of primitive living organisms, such as bacteria *Escherichia coli*.

The recent Isotopic Resonance hypothesis suggests that the rates of chemical and biochemical reactions are not monotonous upon the enrichment degree of isotopic composition of reactants. Instead, at some “resonance” isotopic conditions with certain compositions of CHON, the kinetics increases or decreases compared to the “off-resonance” conditions. To test the predictions of this hypothesis, we designed a precise (standard error $\pm 0.05\%$) method to explore the bacterial growth behaviour under different isotopic compositions. A number of predicted resonances including the terrestrial resonance and several other non-terrestrial resonances were tested, with significant enhancements in kinetics discovered at most of these conditions. The terrestrial resonance was intensively studied with multiple living organisms including prokaryotic bacteria *Escherichia coli*, eukaryotic yeast, mammalian RKO cells, grass seeds and shrimp. All obtained results strongly confirm the preference of living organisms for the terrestrial resonance and support the validity of isotopic resonance phenomena.

Our study confirmed that the MU-type process created hospitable environment for early life, which further benefited from the presence of the terrestrial isotopic resonance.

LIST OF SCIENTIFIC PAPERS

- I. **Xie, X.**, Backman, D., Lebedev, A. T., Artaev, V. B., Jiang, L., Ilag, L. L., Zubarev, R. A.
Primordial soup was edible: Abiotically produced Miller-Urey mixture supports bacterial growth. *Sci. Rep.* **5**, 14338; DOI: 10.1038/srep14338 (2015).
- II. **Xie, X.**, Zubarev, R. A.
Effects of low-level deuterium enrichment on bacterial growth. *PLoS One* **9**, e102071; DOI: 10.1371/journal.pone.0102071 (2014).
- III. **Xie, X.**, Zubarev, R. A.
Effects of low-level deuterium enrichment and depletion on yeast growth, seed germination and mammalian cell growth. Manuscript.
- IV. **Xie, X.**, Zubarev, R. A.
On the effect of planetary stable isotope compositions on growth and survival of terrestrial organisms. Manuscript accepted.
- V. **Xie, X.**, Zubarev, R. A.
Isotopic Resonance Hypothesis: Experimental verification by *Escherichia coli* growth measurements. *Sci. Rep.* **5**, 9215; DOI: 10.1038/srep09215 (2015).

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LIST OF ABBREVIATIONS

MU	Miller-Urey
HPLC	high performance liquid chromatography
MS	mass spectrometry
NIS	normalized isotopic shift
NMD	normalized monoisotopic defect
2D	2 dimensional
IsoRes	Isotopic Resonance
D	deuterium
E. coli	Escherichia coli
GC	gas chromatography
OD	optical density
BYOES	build-you-own-eco-system
NASA	National Aeronautics and Space Administration

1 INTRODUCTION

1.1 THE ORIGIN OF LIFE

The emergence of life on Earth is one of the greatest mysteries in science. Understanding the origin of life is fundamental to appreciating our place in the Universe.

Abiogenesis, proposed by Alexander Oparin, is one of the most prevailing theories today explaining life's beginning. This theory hypothesized that, under proper conditions, life can arise spontaneously from inanimate components, such as some simple chemicals. In Oparin's book, *The Origin of Life*, which was published in 1924 and soon became a worldwide bestseller, he postulated that life has risen through random processes in a primordial soup that existed in the ancient ocean¹. Based on his studies on the chemical composition of Earth's crust as well as other planets in the Solar System, Oparin believed that the primitive Earth atmosphere was a strongly reducing environment, rich in methane, water, and ammonia. Under certain proper conditions such as free energy flux provided by lightning and ultraviolet radiation from the sun, the mixture of simple non-living inorganic compounds in the early Earth atmosphere would form organic compounds, which would be brought into the primitive lakes and oceans via rain, where the free energy needed for the self-assembly of life was supplied by deep-sea hydrothermal vents, hot springs, volcanoes, solar and geothermal activities. Oparin's postulation played a key role in the formulation of our modern ideas on the emergence of life. Decades after his book was published, scientists such as Haldane², Bernal³, Calvin⁴ and Urey⁵, who were inspired by Oparin, tried to seek experimental evidences to support Oparin's hypothesis.

1.2 THE MILLER-UREY EXPERIMENT

The crucial breakthrough came in 1953⁶ from an experiment performed by Stanley Miller, a young graduate student working under the guidance of Nobel laureate Harold C. Urey at the University of Illinois in Chicago. The experiment, referred to as the Miller-Urey (MU)

experiment, confirmed the validity of Oparin's ideas and has later been widely recognized as a quintessential work in the context of origin of life⁷.

In this experiment, as shown in Figure 1, Miller and Urey tested Oparin's hypothesis by sending a continuous high-voltage electric discharge through an airtight glass flask containing a mixture of hydrogen, ammonia, methane and water. The reducing gaseous mixture was believed at that time to imitate the primitive Earth atmosphere^{8,9}. Heat was supplied to circulate the water in the apparatus to simulate evaporation of water from the ancient lakes and seas before moving into the atmosphere and condensing as rain. The products were allowed to condense and collected at the U-shape part of the apparatus. After continuous spark discharge and water circulation for a week, a substantial amount of organic compounds including aldehydes, cyanides and some amino acids were synthesized¹⁰. Although only five amino acids including glycine, D/L alanine, aspartic acid and alpha-amino-butyric acids, were reported in Miller's 1953 Science article⁶, recent investigations that utilized high performance liquid chromatography (HPLC) and time-of-flight (TOF) mass spectrometry (MS) to examine the preserved samples from Miller's original electric discharge experiments, revealed that more than 20 different amino acids, thirteen of which are necessary for life, were present in Miller's original mixture^{11,12}.

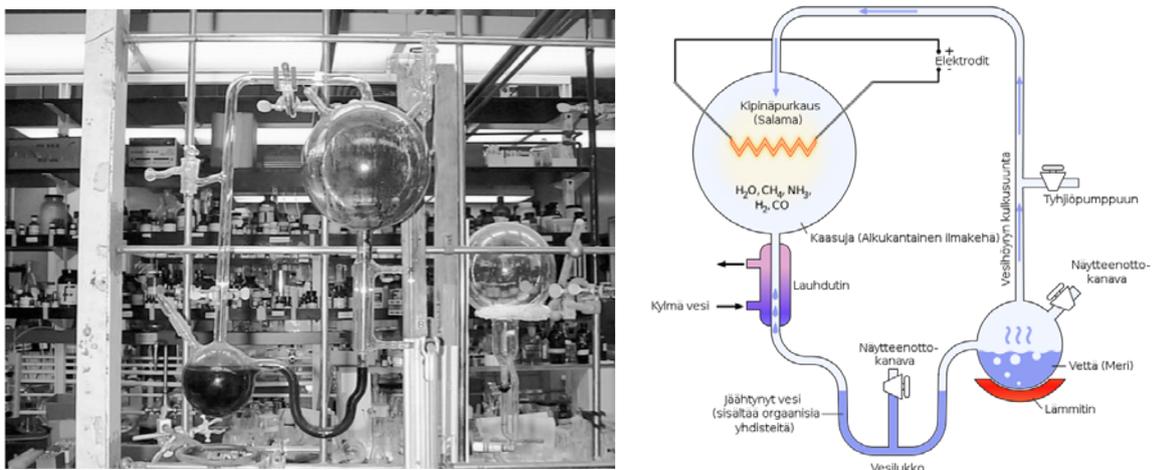


Figure 1. The Miller-Urey experiment.

The MU experiment marked the beginning of experimental investigation of prebiotic chemistry¹³, provided evidence supporting the abiogenesis theory¹⁴ and gave insights into the possibility of abiotic compound synthesis in various environments both on Earth and beyond. Although the abiotically produced MU mixture is assumed to have provided raw materials for life emergence on primitive Earth, there remained considerable uncertainty whether it represents a definite argument for spontaneous life emergence or even if the MU mixture can support life due to the toxicity of some of the produced compounds¹⁵⁻¹⁷. Direct experimental evidence whether the MU mixture supports life was still missing. In this thesis, we aimed to fill this gap.

1.3 DISCOVERY OF THE ISOTOPIC RESONANCE PHENOMENON

Different factors are believed to be crucial for the abiotic synthesis of ancient amino acids and emergence of early life, such as the reducing or oxidizing environment, chiral selection, presence of ionizing radiation, temperature, etc¹⁸. However, the effect of the isotopic composition in the ancient environment on life origin has not been yet considered and studied.

There are now reasons to believe that the isotopic composition played an important role in life emergence. The main reason is the discovery of Isotopic Resonance, which emerged from a method for mapping peptide masses for the purpose of visualization by Zubarev *et al*¹⁹. The isotopic resonance phenomenon is observed when the normalized isotopic shift of peptide molecular masses (NIS, the difference between the average and monoisotopic molecular masses normalized by the integer nominal mass) is plotted against their normalized monoisotopic defect (NMD, the difference between the monoisotopic and nominal masses of the molecule normalized by the integer nominal mass) on a 2D mass map²⁰. Instead of a smooth distribution of peptide “galaxy”, an obvious central line is observed crossing the “galaxy”.

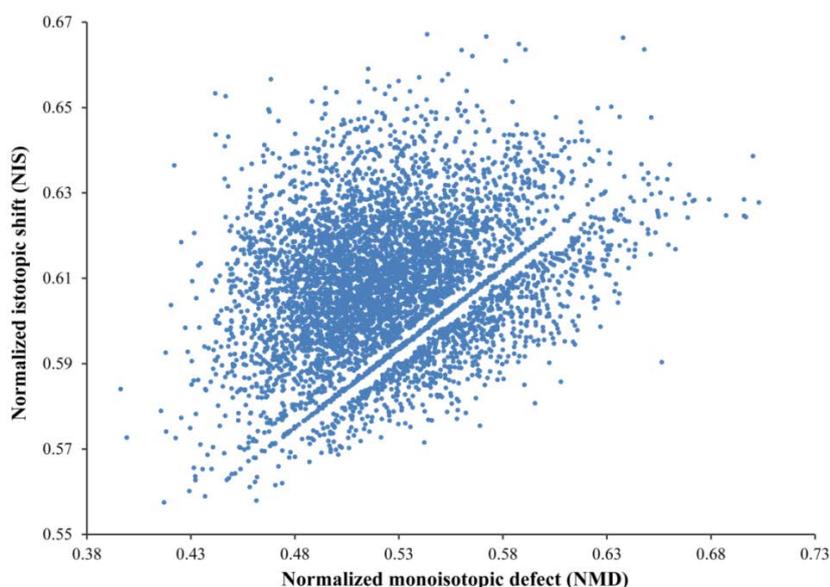


Figure 2. 2D mass map of 6,000 *E. coli* tryptic peptides. The axes represent: (x) normalized monoisotopic defect (NMD), and (y) normalized isotopic shift (NIS).

$$\text{NMD} = 1000 * (\text{Monoisotopic mass} - \text{integer nominal mass}) / (\text{integer nominal mass});$$

$$\text{NIS} = 1000 * (\text{Average isotopic mass} - \text{monoisotopic mass}) / (\text{integer nominal mass}).$$

This central line represents an “isotopic resonance” at terrestrial isotopic compositions. The line in the middle of the gap in Figure 2 arises due to the specific terrestrial isotopic compositions of the elements C, H, O and N (CHON). The appearance of the gap is associated with the discrete characters of molecular elemental compositions, which in turn determine the discrete characters of the monoisotopic defect and isotopic shift. Also, the molecules falling on the line at terrestrial compositions satisfy the condition: $Z = C - (N + H)/2 = 0^{19}$. Such a line can be destroyed by changing the isotopic composition of any of the four elements CHON. The *a priori* probability of such a resonance as in Figure 2 to appear at random choice of CHON isotopic compositions is ca. 1%¹⁹, so the resonance feature is unlikely to be a coincidence. It was essential to understand whether the existence of the isotopic resonance was significant for the emergence of life on Earth. It is remarkable that the molecules that appear on the straight line are peptides mostly composed of “ancient” amino acids (such as Gly, Ala, Ser, etc.), i.e. those that are thought to have appeared in the earliest forms of life¹⁹. It is known that these amino acids were overrepresented in ancient organisms,

and they were constantly replaced in the process of evolution by “new” amino acids (such as sulfur-containing Met and Cys, as well as aromatic Tyr, Phe, Trp, etc.)²¹. Thus the peptides from the most ancient organisms, when put on a 2D mass map, would give a much “thicker” central line and fewer masses distant from that line. For this reason, the line is a sort of a “relic” of early terrestrial life, a feature that used to be very prominent when life just emerged. It is now getting weaker (i.e. more points appear outside the line) as the evolution progresses¹⁹.

1.4 THE ISOTOPIC RESONANCE HYPOTHESIS

The Isotopic Resonance (IsoRes) hypothesis²² postulates that at certain “resonance” abundances of the stable isotopes of C, H, N and O, the rates of chemical and biochemical reactions of certain compound classes accelerate (or, less often, decelerate), affecting biological growth. The proposed mechanism relates to the overall reduction of the system’s complexity, which can be understood as a total number of distinct quantum mechanical states. At a resonance, the number of independent parameters to determine the average mass of the molecules is reduced. For instance, as shown in Figure 2, only six parameters including the four monoisotopic masses of CHON as well as the slope and intercept of the line are needed for all molecules on the line, while 14 parameters including the masses and compositions of all four stable isotopes of CHON are required for molecules off of the line¹⁹. The complexity reduction of the reacting system caused by the isotopic resonance could lead to a faster kinetics of chemical interconversion within the mix. As a result, production of abiotic amino acids and peptides, or organism growth, could proceed with a higher rate, and thus the emergence of life on our planet might have benefited from the terrestrial isotopic resonance¹⁹.

The isotopic resonance phenomenon can be observed at many different sets of isotopic abundances. The position and relative strength of a potential isotopic resonance are hard to determine precisely, especially for complex systems like living organisms, but can be

predicted semi-quantitatively by tuning the abundances of the four elements CHON on a 2D mass map as shown in Figure 2. A resonance is reached when a straight line is formed. In general, a resonance occurs when at least one of the following conditions is met: a) the width of the central line, as in Figure 2, is small; b) the slope of the central line becomes close to zero; c) the slope and intersection of the central line become commensurable (i.e., their ratio is an integer number). The number of dots and the abundances of the corresponding molecules falling on the line as well as their biological significance determine the relative strength of a resonance.

1.5 BIOLOGICAL EFFECTS OF STABLE ISOTOPES

Studies dealing with the biological effects of deuterium have been intensively carried out since the discovery of heavy water by Urey, Brickwede and Murphy in 1932^{23,24}. Excess of deuterium in water was found to have profound negative impact on the growth of many organisms, with negative effects such as reduction in protein and nucleic acids synthesis, disturbance in cell division, alternation in cellular morphology and suppression in metabolism^{25,26}. Strong biological effects are observed soon after the microorganism is exposed to highly isotopic substituted media²⁷, like an “isotopic shock”. Through a period of adaption after the “isotopic shock”, the growth of microorganism continues with changes in cellular morphology, usually at a slower rate compared to normal isotopic media²⁸. The changes in growth rate can be explained by the influence of heavy isotopes on the kinetics of enzymes²⁹. Although some bacteria strains can adapt to highly substituted media of deuterium, or even pure heavy water^{30,31}, high concentrations of deuterium were found to be generally toxic for higher organisms³². The cytotoxicity of heavy water is observed to increase rapidly after the body fluids become more than 20-30% deuterated³³. Mice are found not to be able to survive after drinking 10 g of 50% heavy water every day for one week, when 30-40% deuteration levels of body fluids were reached^{34,35}. The growth of mouse carcinoma cells is completely suppressed at 50% heavy water³⁵. The growth rate of tumor in

mice drinking 40% heavy water was half of its control, and the deuterated mice showed slower growth and earlier death³⁶.

Compared with deuterium, there have been fewer studies on the biological effects of other heavy isotopes, especially at low enrichment levels. Mice are found to be able to grow for several generations with a diet highly enriched with ¹³C³⁷, ¹⁵N³⁸ or ¹⁸O³⁹. In general, the heavy isotopes of C, N and O are usually considered to be safe³⁹, although systematic behavioral changes in mice grown on a ¹⁵N diet were recently observed by Turck *et al*³⁸. They also reported bacteria *Escherichia coli* (*E. coli*) growing slower in a media with high content of ¹⁵N⁴⁰.

1.6 EARLY STUDIES SUPPORTING THE ISOTOPIC RESONANCE HYPOTHESIS

The line in the 2D mass plot at standard terrestrial isotopic condition is not perfect, and can be tuned up to become mathematically thin to reach a perfect resonance by changing the isotopic composition of any element of CHON. For example, the perfect resonance near terrestrial condition, which we refer as “the terrestrial resonance”, can be achieved by raising the abundance of deuterium from a normal content of 156 ppm (0.0156%) to about 300 ppm²². As the Isotope Resonance hypothesis predicts the rates of biochemical reactions further increasing at such a perfect resonance compared to standard terrestrial conditions, the enrichment of deuterium to 300 ppm should affect the growth of living organisms. Actually, such prediction has been supported by a wealth of early studies that described the biological effects of slightly enriched deuterium (<0.1% D).

The first report regarding the unusual biological effects of low concentration of deuterium came from Barnes *et al.*, who studied the physiological effect of diluted heavy water on the growth of *Spirogyra*⁴¹. In that study, they observed greater longevity and less abscission (cell- disjunction) for *Spirogyra* filaments when cultured in 0.06% D water compared to ordinary water⁴¹. The experiment was repeated and the effect of increased longevity for *Spirogyra* in 0.06% D water was confirmed⁴². They further demonstrated this

positive effect of diluted heavy water with *Euglena* and *Planaria* and found an increase in cell division for *Euglena* in 0.06 % heavy water⁴³ and greater longevity for *Planaria* in 0.06% and 0.07% heavy water media⁴⁴ compared to normal water. By growing yeast in diluted heavy water, Richards confirmed Barnes' observations and found a 26% increase in the dry weight of yeast grown in 0.06% D^{45,46}. The study from Lockemann and Leunig on the effect of water with less than 0.54% D showed that *E. coli* and *Pseudomonasa eruginosa* exhibited better growth at the deuterium level as low as 0.04%⁴⁷. Meyer also confirmed that diluted heavy water (0.06%D) affects the dry weight of *Aspergillus*⁴⁸; Curry's work revealed about a 10% faster growth for *Aspergillus* at 0.05% D compared with ordinary water, although the difference was not statistically significant⁴⁹.

Lobyshev *et al.* in the 1970s applied robust methods to investigate the biological effects of low deuterium enrichment on single enzymatic reactions as well as living organisms. More specifically, they studied the activity of Na, K-ATPase and the regeneration of hydroid polyps *Obelia geniculata* at different deuterium concentrations. They observed about 50% increase in the activity for Na, K-ATPase at 0.04-0.05% D^{50,51} and an inhibitory effect at high deuterium concentrations, and acceleration in regeneration of *hydroid polyps Obelia geniculata* at low deuterium concentrations of $\leq 0.1\%$ ⁵². The study from Somlyai *et al.* in the 1990s revealed that 0.06% deuterium in tissue culture activated the growth of L₉₂₉ fibroblast cell lines, while deuterium depleted water (20-120 ppm) strongly suppressed the growth rate of the same cell line and the growth of tumor in xenotransplanted mice⁵³⁻⁵⁵.

2 PRESENT INVESTIGATIONS

2.1 AIMS OF THE THESIS

The aims of this thesis are to test experimentally the predictions of the Isotopic Resonance hypothesis and, if confirmed, to investigate the implications of the hypothesis for the understanding of the origin of life. The specific aims for each paper are as follows:

Paper 1. To test the hypothesis that the primordial mixture produced in the Miller-Urey experiment, which is widely interpreted as providing building blocks essential for life emergence, will support the growth of primitive terrestrial organisms such as bacteria.

Paper II. To study the effects of the “perfect” terrestrial resonance (300 ppm D) on the growth of prokaryotic organisms, such as bacteria.

Paper III. To investigate the effects of terrestrial resonance on the growth of higher living organisms such as yeast, mammalian cells as well as germination of grass seeds.

Paper IV. To demonstrate the effects of planetary stable isotope compositions on the growth and survival of terrestrial organisms (bacteria and shrimp), and to provide evidence that terrestrial isotope conditions are better for the survival of living organisms.

Paper V. To explore the biological effects of a number of non-terrestrial resonances predicted by the Isotopic Resonance hypothesis, which appear at various isotopic abundances of ^{15}N , ^{13}C and ^{18}O , including the “super-resonance” (see below) on bacterial growth.

2.2 METHODOLOGY

2.2.1 Growth of bacteria on material produced from Miller-Urey experiment (Paper I)

The Miller-Urey apparatus was built (Figure 1) according to literature⁵⁶. The MU experiment was carried out continuously for one week using with methane (CH₄), ammonia (NH₃), hydrogen (H₂), and water (H₂O) as starting materials. Samples of MU mixtures were extracted from the U-shape section of the apparatus every one or two days during the experiment. Both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analyses were performed to identify the molecular components in the MU mixture.

The growth of *E. coli* bacteria was found to be strongly inhibited when a small portion (3%) of MU mixture was directly added into the bacterial growth media (M9 minimal media), not only in the samples wells, but also the in control wells, to which no MU mixture was added. To solve this issue, two strategies were used: one was to adapt bacteria to grow in a solution containing glycine and a racemic alanine in 1:1 molar ratio (two most abundant amino acids in MU mixture), another was to remove the volatile lethal components by drying and reconstituting the MU mixture. For adaptation, *E. coli* was grown for about 800 generations in a control media (Gly+DL-Ala solution supplemented with some necessary inorganic salts, such as MgSO₄, CaCl₂, and NH₄Cl). During the adaptation process, which took three months, the media were changed and bacteria were reseeded every day. The MU mixture was dried with SpeedVac and reconstituted in the same volume of water. The adapted bacteria were then grown in either M9 minimal media with addition of the reconstituted MU mixture or pure reconstituted MU mixture supplemented with the necessary inorganic salts as in M9 media. A Bioscreen C automatic fermenter was used to monitor the growth of bacteria.

2.2.2 Investigation of isotopic resonance effects on living organisms

2.2.2.1 Resonance prediction

The 2D mass map²⁰ that plots NIS against NMD can be used to predict the locations of potential resonances. A straight line appears on the 2D mass map when an isotopic resonance occurs⁵⁷. The fraction of the molecules on the line determines the relative strength of the resonance²². Resonances can be observed at many isotopic composition sets. As mentioned before, the standard terrestrial isotopic composition (Figure 3a) is not perfect with a comparatively wide line (data spread) and many molecules located slightly off the line, which limits the strength of this resonance. To reach a perfect “terrestrial resonance”, the abundance of deuterium needs to be doubled to near 300 ppm, at which point the line becomes mathematically thin. The molecules that fall on the line in the terrestrial resonance condition fulfill the rule $Z = C - (N+H)/2 = 0$. This rule is followed by most amino acids and polypeptides, which indicates the significance of this resonance for living organisms. Another strategy is to change the slope of the line to zero by increasing the terrestrial ¹⁵N abundance (0.37%) to $\approx 3.5\%$, which gives the ¹⁵N $\approx 3.5\%$ resonance (Figure 3b). However, the strength of this resonance is not expected to be strong due to the diffused line. An alternative strategy is to deplete the terrestrial ¹³C abundance (1.1%) to 0.35% to reach the ¹³C $\approx 0.35\%$ resonance (Figure 3c), which gives not only a zero slope for molecules on the line but a near-zero slope for molecules off the line. Thus this resonance is expected to be stronger than the ¹⁵N $\approx 3.5\%$ resonance. At the resonance of ¹⁸O $\approx 6.6\%$, the average isotopic masses of hydrogen and oxygen become proportional to the nominal masses, which completely removes the monoisotopic masses from the average mass equation. The resonance at ¹⁸O $\approx 6.6\%$ affects mainly water molecules, which is the most abundant compound in living organisms, thus this should be a stronger resonance than the ¹⁵N and ¹³C resonances. An even stronger resonance, which we refer to as the “super resonance”, occurs at ¹³C $\approx 9.5\%$, ¹⁵N $\approx 10.9\%$ and ¹⁸O $\approx 6.6\%$ (Figure 3d). Under this condition, CHON average

isotopic masses become proportional to the nominal masses, completely excluding the monoisotopic masses from the average mass equation, and all CHON molecules fall on the same line.

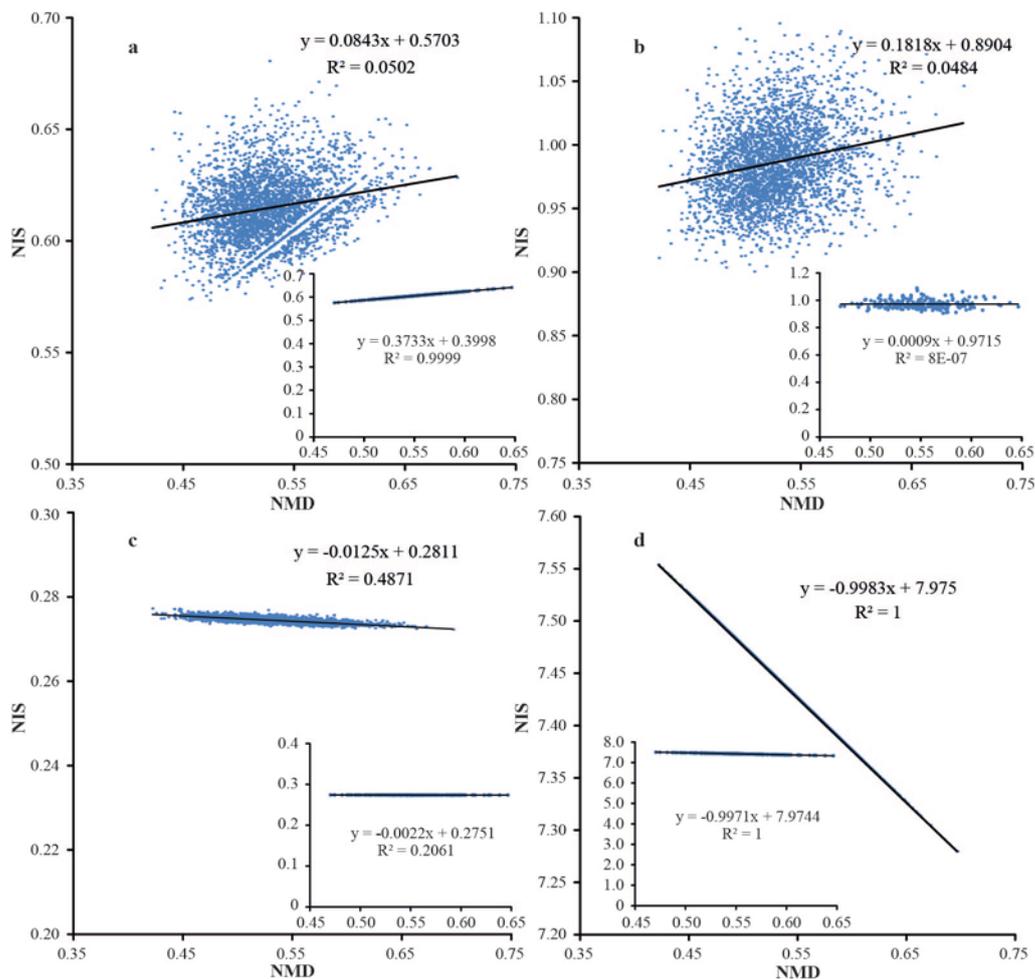


Figure 3. 2D mass plots of 3,000 *E. coli* tryptic peptides at different isotopic ratios of CHON. Insets show only peptides with Z=0. The axes represent: (x) normalized monoisotopic defect (NMD), and (y) normalized isotopic shift (NIS). (a) Terrestrial isotopic ratios; the gap with a central line correspond to the terrestrial isotopic resonance for Z=0 molecules. (b) Zero-slope resonance at $\approx 3.5\%$ ^{15}N for Z=0 molecules. (c) Zero-slope resonance at $^{13}\text{C} \approx 0.35\%$ for Z=0 molecules and a near-zero slope resonance for all molecules. (d) The “super-resonance” at $^{13}\text{C} \approx 9.5\%$, $^{15}\text{N} \approx 10.9\%$ and $^{18}\text{O} \approx 6.6\%$ for all molecules. Adapted from the reference⁵⁸.

2.2.2.2 Investigation of the isotopic resonance effects on multiple organisms

The effects of terrestrial resonance on bacterial growth were first investigated. We developed a very accurate method (standard error 0.05%) to monitor the growth of bacteria in M9 minimal media with various concentrations of deuterium, including the terrestrial resonance condition. This method utilizes a programmed Tecan robot for sample preparation and an automated fermenter Bioscreen C for data acquisition.

Nine independent experiments (all experimental materials such as streaked *E. coli* agar plate, growth media, etc. were freshly prepared) were performed with each experiment using ten different contents of deuterium, starting with 156 ppm and doubling the concentration of deuterium until 8% is reached. Five Bioscreen C runs were required to complete each experiment. For each run, bacteria were grown in two honeycomb well plates, each with two different contents of deuterium, one being always the normal deuterium content as control. On each plate, 32 replicate pairs of “sample” and “control” wells were prepared, and the outside wells were filled with pure media. In this way, 288 individual experiments were performed for each deuterium content data point. The optical density (OD) of bacteria in each well was monitored automatically by the Bioscreen C instrument. The OD was plotted on a logarithmic scale against the growth time to obtain a bacterial growth curve, from which three independent parameters can be extracted: the maximum growth rate, the lag phase duration and maximum density. The maximum growth rate was determined with the maximum value of the slopes for every 8-h interval. The extrapolation of the line with maximum slope gave the lag time, and the maximum turbidity for each well was taken as the maximum density. The data for each “sample” well was always normalized to the adjacent “control” well to minimize the systematic instrumental errors. The 32 replicates were divided into 4 groups according to the positions on the honeycomb plate (group 1: columns 1 and 2; ..., group 4: columns 7 and 8). To minimize the influence of non-statistical outliers, the median of the eight values from each group was taken. The average of the four medians was calculated to

determine the value (R) for the specific deuterium content. The average and the standard error of the nine values (R) from the nine experiments were calculated to give the final results. After normalizing the obtained values (R) to that of the terrestrial deuterium content in the same experiment, p-values for each non-terrestrial deuterium content were calculated using two-tailed, paired Student's t-test by comparing the R values to those obtained for terrestrial deuterium content of 156 ppm (also normalized by data from adjacent control wells with terrestrial deuterium content).

To validate the effect of ultralow enrichment of deuterium ($\leq 1\%$ D), we adapted bacteria *E. coli* for 400 generations in four isotopically different environments (including 156 ppm, 0.03%, 0.25% and 1%) followed by testing the growth of the adapted bacteria in these deuterium conditions compared to normal deuterium content (156 ppm D). Six independent experiments were performed.

The same methodology was applied to study the effects of terrestrial resonance on the growth of yeast and the effects of several non-terrestrial resonances, including $^{15}\text{N}\approx 3.5\%$ resonance, $^{13}\text{C}\approx 0.35\%$ resonance and the super-resonance, on the growth of *E. coli* bacteria.

To test the effect of terrestrial resonance on the germination of grass seeds, the seeds were germinated in water with different concentrations of deuterium (25 ppm, 105 ppm, 150 ppm, 300 ppm, 600 ppm, 1200 ppm, and 3%) with six replicates for each in a plant growth cabinet (Adaptis A1000, Conviron, Canada). The seeds in the cabinet were kept at room temperature without airflow and light for about one week. The seeds were collected and dried on air when yellowish spouts were observed, and the weight was measured after every two hours of drying.

To test the terrestrial resonance on the survival of shrimp, the BYOES ("build your own eco-system") kit developed originally by NASA was used. Each BYOES 300 contained 300 mL salted water with specific content of deuterium, two fibrous sticks, some ceramic beads, algae, microbes and two shrimp. Shrimp were grown in five different deuterium contents

including 120 ppm (D-depleted), 150 ppm (D-normal), 300 ppm, 600 ppm and 1200 ppm with five replicates on an office desk arranged in a randomized 5x5 matrix. The survival of the shrimp was investigated and recorded every 2-3 weeks for 20 months.

2.3 RESULTS AND DISCUSSION

2.3.1 Abiotically produced Miller-Urey mixture supports bacterial growth (Paper I)

Although a recent report suggests that the biologically relevant L-form amino acids may be slightly more abundant⁵⁹ in the MU mixture, analysis with LC-MS revealed that the MU mixture contained a roughly racemic mixture of amino acids. Addition of a small volume (3%) of MU mixture into the bacterial growth media was found to strongly suppress the growth of *E. coli*, with no growth of bacteria observed in both samples wells and control wells containing no MU mixture. Results from GC-MS analysis showed that the MU mixture contained highly toxic compounds including hydrogen cyanide, cyanic acid, allyl alcohol, pyridine, diethylamine, and other hazard components, such as methyl- and ethylamine, N,N-dimethylacetamide, formamide, butanedinitrile, and 1-methylpyrrolidinone-2. etc. These lethal compounds are volatile, thus can be removed by drying.

Adaption of *E. coli* for 800 generations in the control media (Gly+DL-Ala solution) resulted in a reduction of 16% for the lag time, an increase of 67% in the maximum growth rate and an increase of 63% for the maximum density. As shown in Figure 4, the adapted bacteria showed significantly better growth in terms of faster growth rate, higher maximum density and especially shorter (20%) lag time in the Control media with 3% of the dried and reconstituted MU mixture, compared with standard (with 3% of pure water) and even 100% Control media. It was found that the addition of 1 mg/L of L-Asn reduced the lag time of bacteria as much as 80 mg/L D-Asn, so the strongest effect on lag time reduction may come from the L-amino acids present in MU mixture. Most importantly, we found that the adapted bacteria were able to grow in the pure reconstituted MU mixture with addition of some simple inorganic salts (Figure 5), which means the reconstituted MU mixture can serve as the only carbon source for the growth of the adapted bacteria.

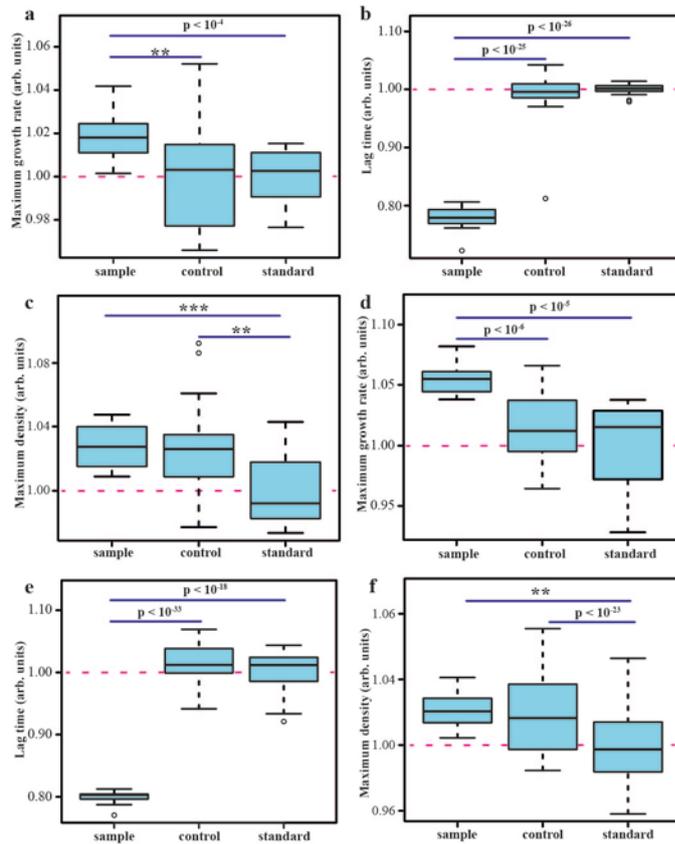


Figure 4. (a,b,c) Parameters of *E. coli* growth in a mixture of glycine and racemic alanine. Sample – 97% Control mix + 3% reconstituted MU solution; Control – 100% Control mix; Standard – 97% Control mix + 3% MiliQ water. (d,e,f) Results of a replicate experiment. Adapted from the reference⁶⁰.

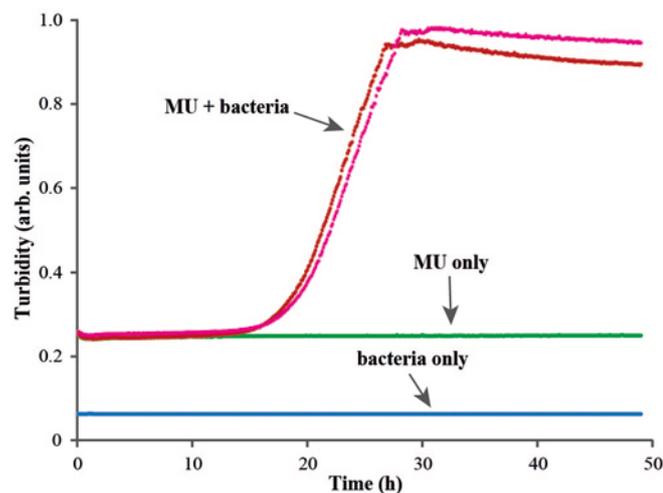


Figure 5. Growth curves for *E. coli* grown in dried and reconstituted in water MU mixture (red and pink), as well as controls: blue - bacteria in pure water and green - MU mixture without bacteria. Inorganic salts were added to all samples and controls. Adapted from the reference⁶⁰.

Bacterial adaptation and the drying and reconstitution of abiotic MU mixture in our experiment vividly mimic the natural process presumed to occur on primitive Earth. Our experiments provided evidence that the MU mixture could serve as food necessary for early life forms. Though it is hard to conclude that life must have originated from these abiotically synthesized organic compounds, the experiment here implies that our planet had a hospitable environment for early life forms, regardless whether they spontaneously emerged on Earth or arrived from beyond.

2.3.2 Isotopic resonance effects on living organisms

Although abundant phenomena regarding the unusual biological effect of low deuterium enrichment were reported, no convincing explanations were provided by early studies⁴¹⁻⁵⁵. Instead, these observations were considered to be ambiguous due to inaccurate measurement, lack of statistical experimental design and proper control. To overcome the limitations of these early studies⁴¹⁻⁵⁵, in this thesis we demonstrated the effect of low deuterium enrichment on bacterial growth by utilizing modern automatic fermenters and robust statistical experimental design. With this method, we probed the biological effects of both the terrestrial resonance with small concentration of deuterium and some non-terrestrial resonances related to other stable heavy isotopes (CON) on the growth of bacteria. In addition to prokaryotic bacteria *E. coli*, we studied exclusively the effects of terrestrial resonance on other organisms including eukaryotic yeast, mammalian RKO cells, and germination of grass seeds as well as the survival of shrimp.

2.3.2.1 Effects of terrestrial resonance on multiple organisms

As mentioned earlier, the Isotopic Resonance hypothesis predicts the “perfect” terrestrial resonance at ≈ 0.03 ppm D, thus one can reasonably assume that even low levels of deuterium enrichment may affect the growth of living organisms.

2.3.2.1.1 On the growth of prokaryotic bacteria *E. coli* (Paper II)

With our sensitive method to monitor growth behaviors of *E. coli*, the adverse effects (such as declined growth rate, prolonged lag time) prominent at high deuterium content (for example, 50%) were observed to be present even at 0.5% D. However, ultralow deuterium concentrations ($\leq 0.25\%$ D) showed signs of a reverse trend. Slightly shorter lag time (Figure 6B) and higher maximum density (Figure 6C) were observed in the region of deuterium $\leq 0.25\%$ with a significant increase ($p < 0.05$) in maximum density at 0.03% D (300 ppm D), which indicates a region of more comfortable growth condition.

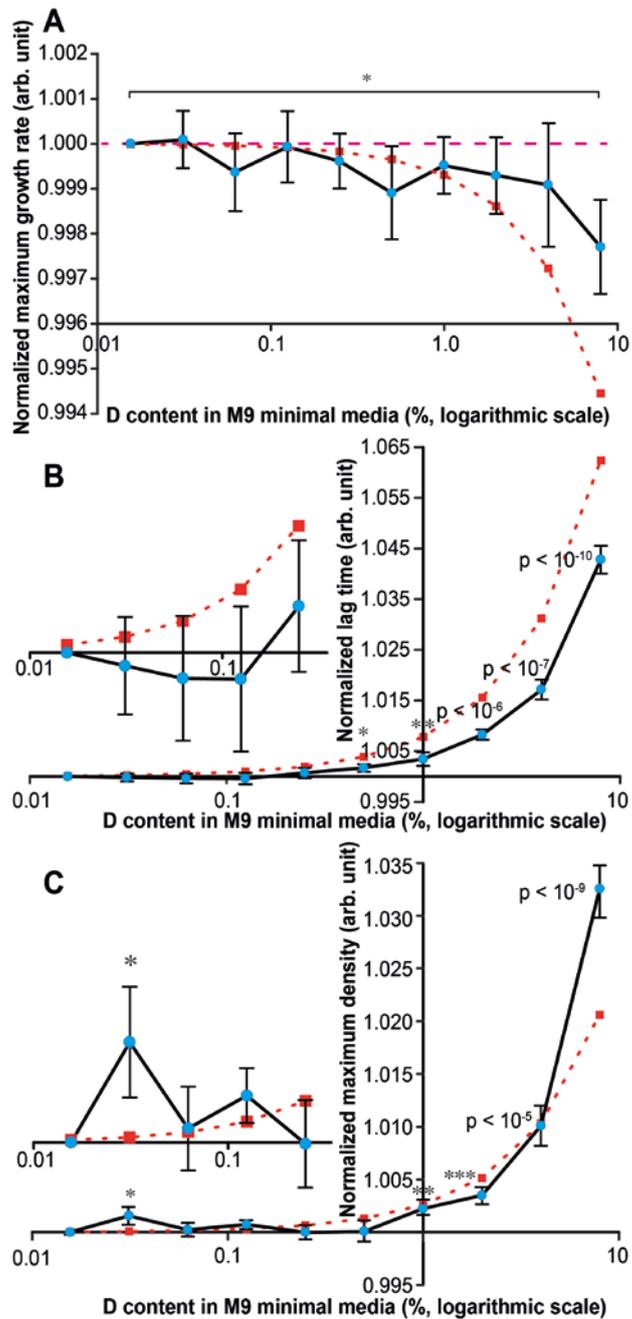


Figure 6. Maximum growth rate, lag time, maximum density of *E. coli* grown in minimal media. (A) Blue circles: maximum growth rate of *E. coli* grown in M9 minimal media normalized by that at normal deuterium content of 156 ppm. * denotes $p < 0.05$, ** - $p < 0.005$, etc. Brown squares: predicted maximum growth rate calculated according to the maximum growth rate of *E. coli* grown in 50% of deuterium. (B) Blue circles: lag time of *E. coli* grown in M9 minimal media normalized by that at terrestrial content of deuterium from 156 ppm (terrestrial value) to 8%. Inset shows a zoom-in of the ultralow enrichment region. Brown squares: predicted lag time calculated according to the lag time of *E. coli* grown in 50% of deuterium. (C) Blue circles: maximum density of *E. coli* grown in M9 minimal media normalized by that at terrestrial deuterium content from 156 ppm (terrestrial value) to 8%.

Inset shows a zoom-in of the ultralow enrichment region. Brown squares: predicted maximum density calculated according to maximum density of *E. coli* grown in 50% of deuterium. Adapted from the reference⁶¹.

Adaptation of *E. coli* for 400 generations to four different isotopic environments (0.0156% D, 0.03% D, 0.25% D, and 1% D) showed an enhancement in both growth rate and maximum density at ultralow deuterium concentrations ($\leq 0.25\%$ D), while exhibiting a reverse trend for 1% D (Figure 7), all of which support the existence of a preferred growth environment for bacteria at ultralow ($\leq 0.25\%$ D) enrichment.

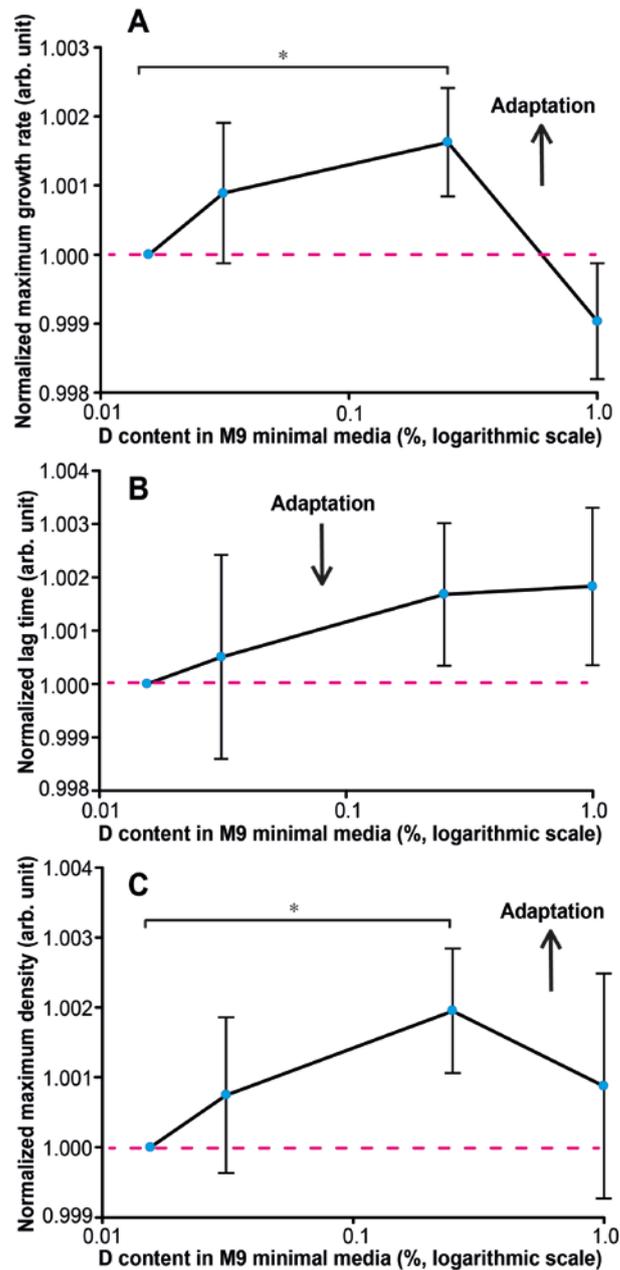


Figure 7. Maximum growth rate, lag time and maximum density of aged *E. coli*. (A) Maximum growth rate of aged *E. coli* grown in M9 minimal media with ultralow composition of deuterium. * denotes $p < 0.05$. (B) Lag time of aged *E. coli* grown in M9 minimal media with ultralow composition of deuterium. (C) Maximum density of aged *E. coli* grown in M9 minimal media with ultralow composition of deuterium. * denotes $p < 0.05$. Adapted from the reference⁶¹.

The observed effects of ultralow deuterium, especially at 300 ppm D, on the growth of bacteria is in agreement with previous findings^{41–55}, all supporting the presence of terrestrial resonance near 300 ppm D predicted by Isotopic Resonance hypothesis.

2.3.2.1.2 On the growth of eukaryotic yeast and mammalian RKO cells and germination of grass seeds (Paper III)

In this paper, we extended our study concerning about the effect of low-level deuterium enrichment to the growth of yeast, mammalian RKO cells as well as germination of grass seeds. The growth of yeast was accurately measured in the same way as in the experiments with *E. coli* bacteria growth. The growth of RKO cells was monitored by xCelligence fermenter. Multiple replicates and independent experiments were performed for grass seed germination. Combining precise measurement techniques and thorough statistical design, we found that the growth of yeast and RKO cells as well as germination of grass seeds was suppressed by 3% deuterium. At the same time, 300 ppm (0.03%) D accelerated the growth of yeast and RKO cells (but not the germination of grass seeds), which was manifested by a higher growth rate and/or higher cell density. Grass seeds were found to germinate best at conditions with moderate deuterium depletion of 50-80 ppm D. The effects observed at 300 ppm D are in line with early literature⁴¹⁻⁵⁵ and our study on bacteria⁶¹. These results can be explained by the IsoRes phenomenon, where the kinetics of biochemical reactions are changed at the resonance conditions, for example, near 300 ppm D.

2.3.2.1.3 On the survival of shrimp (Paper IV)

The survival of shrimp in 25 “build-your-own-ecosystem” (BYOES) vessels containing five different deuterium compositions (120 ppm, 150 ppm, 300 ppm, 600 ppm and 1200 ppm, five replicates each) was monitored. After 20 months of observation, out of the initial 50 shrimp in all BYOES vessels, only 16 shrimp remained alive (32%). Figure 8A shows the average number of survived shrimp over the observation period as well as the Student’s t-test (two-tailed, unpaired) results for each deuterium content. 120 ppm D water and 300 ppm D water gave statistically indistinguishable results from normal water, while 600 ppm and especially 1200 ppm enriched water showed statistically significant declines in survival probability.

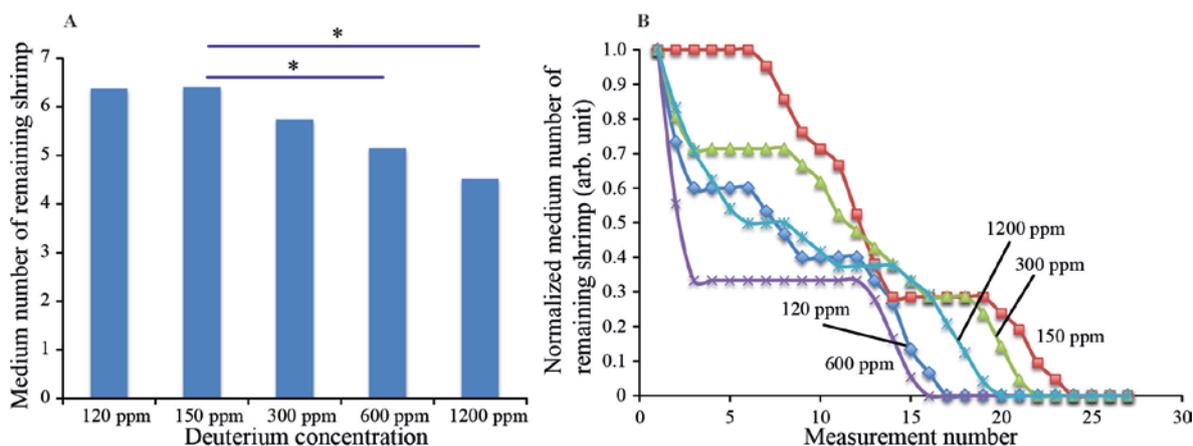


Figure 8. Shrimp survival in BTOES under the period of 20 months.

(A) The average number of shrimp in BYOES over the observation period of 20 months as well as the Student's t-test (two-tailed, unpaired) results for each deuterium content. (B) The survival plots (after 3-point smoothing) after the number of shrimp that lived after 20 months was subtracted for each D content, and the data were renormalized.

The results obtained the BYOES experiment can be explained by the isotopic resonance phenomenon: slight deuterium enrichment may not be toxic, but could instead lead to faster kinetics of most biochemical processes, resulting in a shorter lifespan. This phenomenon is a possible reason for shorter lifetime observed at 600 ppm, while the toxicity of deuterium may become more dominant for the lifespan decline of shrimp at 1200 ppm.

2.3.2.2 The effects of non-terrestrial resonance on bacterial growth (Paper V)

We continued to test the Isotopic Resonance hypothesis for other non-terrestrial resonances including $^{15}\text{N}\approx 3.5\%$ resonance, $^{13}\text{C}\approx 0.35\%$ resonance and the super-resonance, using the same method that was used to study the effects of the terrestrial resonance on the growth of bacteria (Paper II).

For the $^{15}\text{N}\approx 3.5\%$ resonance, the growth behaviour of bacteria in M9 minimal media with various ^{15}N contents from 0.37% (normal condition) to 10% was investigated. As shown in Figure 9a, the maximum growth rate of bacteria was found to increase significantly ($p = 0.007$) by 0.5% at 3% ^{15}N compared with the normal nitrogen condition (0.37%). To further validate the effect around 3% ^{15}N , the growth of bacteria at a narrow ^{15}N composition range

between 2.0% and 4.1% with a 0.3% step was studied. Results confirmed the presence of a resonance around 3.5%-3.8%, with a significant increase ($p=0.006$) in the maximum growth rate (Figure 9b) and maximum density ($p<0.05$, Figure 9d) at 3.5% as well as a significant decrease ($p<0.05$) in the lag time (Figure 9c) at 3.2% compared to 2.0% ^{15}N , which is consistent with the broad-range experiment. The combined p-value for the effects of $^{15}\text{N}\approx 3.5\%$ resonance is <0.00005 .

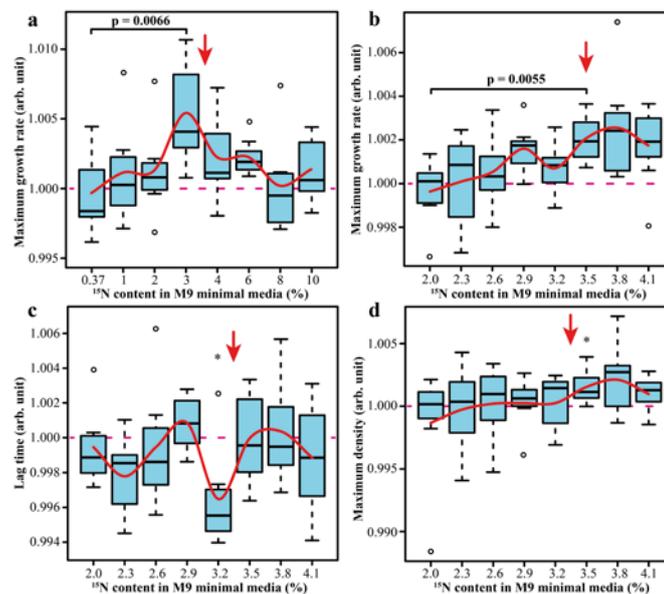


Figure 9. Growth parameters of *E. coli* grown in M9 minimal media with varying composition of ^{15}N : (a), (b) – maximum growth rate; (c) – lag time; (d) – maximum density. In the box plots, the box encompasses 50% of the data with a central bar corresponding to a median, while the “error bars” include the remaining 50% of data except for a few data points (outliers) represented by the open circles. The red line crosses the average value in each data set. Adapted from the reference⁵⁸.

Bacteria were grown in four different ^{13}C contents ranging from 0.2% to 1.1% (normal terrestrial value) to test the resonance of $^{13}\text{C}\approx 0.35\%$. An increase in both maximum growth rate (+0.7%) and maximum density (+1.3%) were observed at 0.35% ^{13}C , with a combined p-value as low as 10^{-6} (Figure 10), which supports the presence of a resonance. Meanwhile, the effect of temperature (15 °C to 41 °C) on the growth rate enhancement at 0.35% ^{13}C was studied. A higher maximum growth rate was observed under all temperatures compared with

the normal condition (1.1% ^{13}C) with the largest increase of $\geq 1\%$ found between 25 and 35 $^{\circ}\text{C}$ (Figure 11).

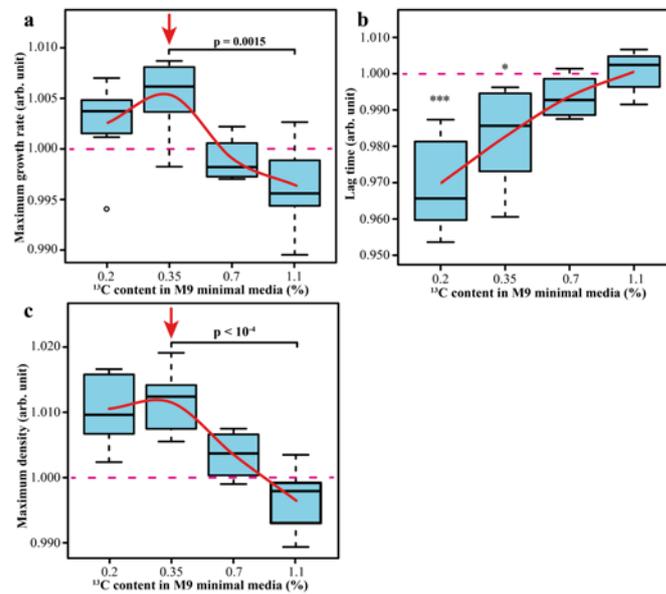


Figure 10. Growth parameters of *E. coli* grown in M9 minimal media with varying composition of ^{13}C : (a) – maximum growth rate; (b) – lag time; (c) – maximum density. Adapted from the reference⁵⁸.

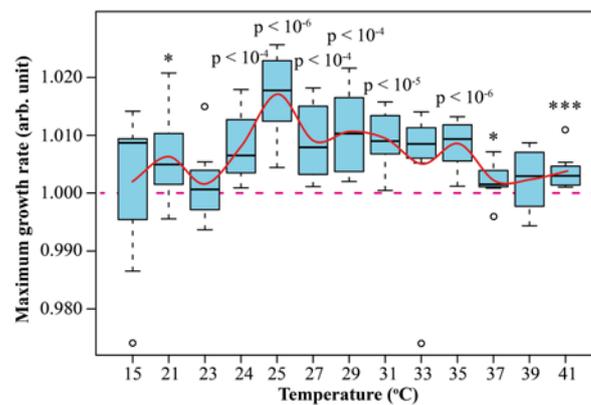


Figure 11. Maximum growth rate of *E. coli* grown in M9 minimal media with 0.35% of ^{13}C at different temperatures. Adapted from the reference⁵⁸.

A super-resonance was predicted at $^{13}\text{C}\approx 9.54\%$, $^{15}\text{N}\approx 10.89\%$ and $^{18}\text{O}\approx 6.6\%$. For this resonance, the three elemental enrichments were first studied separately, followed by pairwise enrichments and finally all enrichments combined. Results (Figure 12) revealed that $^{18}\text{O} = 6.6\%$ gives the strongest effect among the three individual enrichments, while $^{13}\text{C} =$

9.54% combined with $^{18}\text{O} = 6.6\%$ gives the strongest effect among all three possible pairwise enrichments, and that triple enrichment gave the largest effect than any combination of individual or pairwise enrichments.

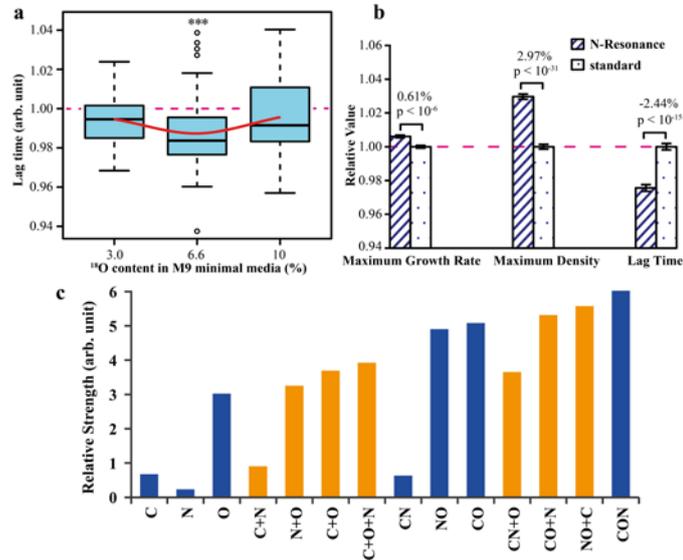


Figure 12. (a) Resonance in lag time at 6.6% ^{18}O . (b) Resonance at the “super-resonance”: at $^{13}\text{C}\approx 9.5\%$, $^{15}\text{N}\approx 10.9\%$ and $^{18}\text{O}\approx 6.6\%$. (c) Relative magnitudes of the effects of individual and combined isotope enrichment: blue columns – experimental results; orange columns – extrapolated data. Adapted from the reference⁵⁸.

In summary, all experimental results obtained are consistent with the predictions of the Isotopic Resonance hypothesis. The $^{15}\text{N}\approx 3.5\%$ resonance was one of the weakest resonances tested, but it was supported by substantial amounts of data. The $^{13}\text{C}\approx 0.35\%$ resonance was verified by testing four different ^{13}C contents and an extensive temperature series. The $^{13}\text{C}\approx 0.35\%$ resonance has a maximum effect on bacterial growth at 25-35 °C, and a lower effect at 39 °C. This can be explained by an interpretation of the isotopic resonance phenomenon that suggests the complexity reduction leading to a lower density of quantum-mechanical states, hence an elevated internal temperature⁵⁷. The growth rate of bacteria at 25-35 °C is significantly lower than that of 39 °C, so the “temperature increase” achieved via isotopic resonance becomes more obvious in that temperature range. This effect becomes

weaker with rising temperature, and reaches a minimum when the growth rate reaches its maximum at 39 °C.

The effect for the $^{18}\text{O}\approx 6.6\%$ resonance was the strongest for any other individual element, which could be explained by the comparatively high enrichment level of this element and its possible biological effects on microorganisms⁶². However, even higher enrichment level of ^{13}C (to 9.5%) and ^{15}N (to 10.9%) did not produce such strong effect as ^{18}O enrichment to 6.6%. Triple enrichment of ^{13}C , ^{18}O , and ^{15}N to the super-resonance gave the strongest growth enhancement compared to all other individual or pairwise conditions, which indicated that the super-resonance conditions provide an extremely comfortable environment for bacterial growth, in agreement with the Isotopic Resonance hypothesis.

2.3.3 Effects of the planetary stable isotope composition on the growth of living organisms (Paper IV)

The isotopic environments of other planets in our solar system, such as Mars and Venus are very different from the terrestrial one, especially in the content of deuterium⁶³⁻⁶⁶. In this paper, Bacteria growth in a minimal media with the isotopic composition of the atmosphere of other planets in the solar system, such as Mars and Venus, was compared with that at Earth's isotopic composition. As shown in Figure 13, Both the Martian and Venusian isotopic conditions were observed to strongly suppress the growth of bacteria, with significantly slower growth rate and lower maximum density when compared with the terrestrial isotopic conditions. Moreover, in the BYOES experiment, we also found that the Martian deuterium content in water negatively affected the survival of shrimp. These results imply that the terrestrial isotopic conditions represent a better environment for the sustainability of life, compared to Mars and Venus.

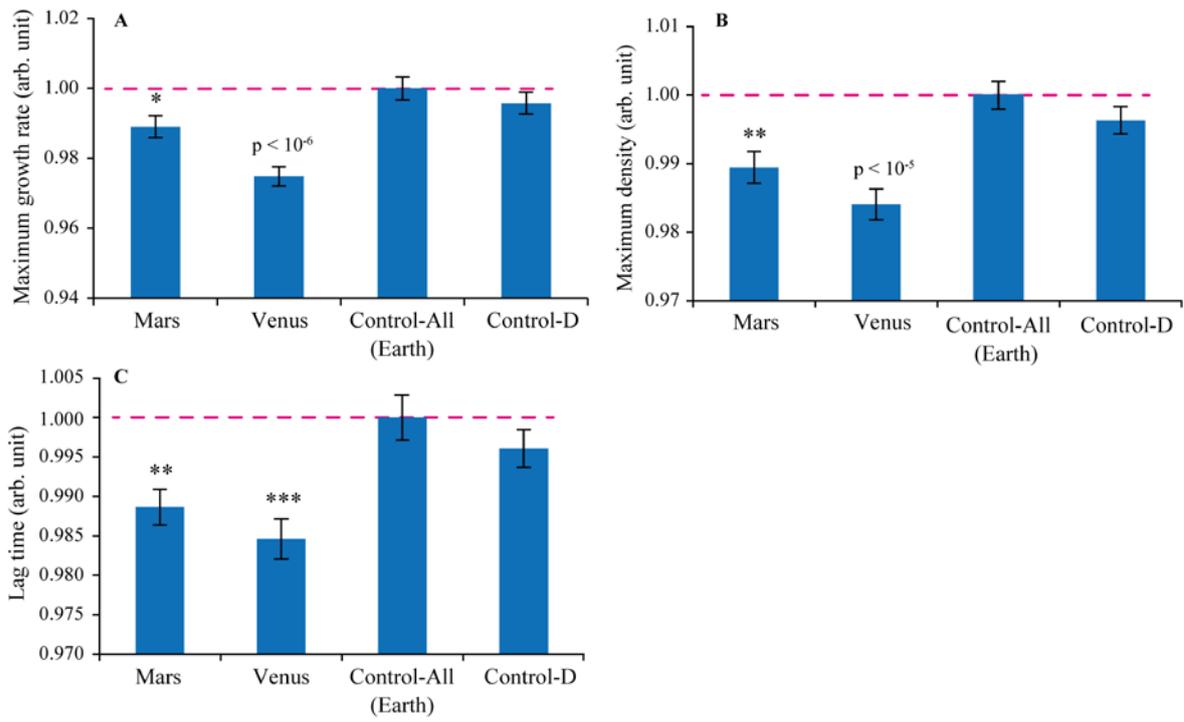


Figure 13. The results of *E. coli* growth parameter measurements at 25 °C summarizing two independent experiments (100-well plates).

(A) Maximum growth rate. (B) Maximum density. (C) Lag time.

3 CONCLUSION

We have provided experimental evidence that the primordial soup produced in the Miller-Urey experiment supports the growth of primitive living organism (*E. coli* bacteria), and have tested the validity of the Isotopic Resonance hypothesis, which predicted that life emergence or evolution on our planet might have benefited from terrestrial isotopic compositions of CHON. To validate the Isotopic Resonance hypothesis, we have intensively investigated the effects of terrestrial isotopic resonance on multiple organisms, including primitive prokaryotic bacteria, eukaryotic yeast, mammalian cells and shrimp as well as the effects of non-terrestrial resonances on the growth of bacteria.

A very precise method was established to monitor bacterial growth, and applied to test the effects of the “perfect” terrestrial resonance ($D \approx 300$ ppm) on bacterial growth. Bacteria’s preference for terrestrial resonance was indicated by their enhanced growth. We then extended this study to the growth of eukaryotic yeast and mammalian RKO cells as well as germination of grass seeds. Compared with normal terrestrial deuterium content (156 ppm D), 300 ppm D was found to enhance the proliferation for both yeast and mammalian cells but not the seed germination. Best seed germination was observed at moderate deuterium depletion conditions (50-80 ppm), away from the terrestrial resonance. We then investigated the effect of terrestrial resonance on the long-term survival of shrimp. Results revealed that shrimp survived equally well at the “perfect” terrestrial resonance ($D \approx 300$ ppm) as in average terrestrial conditions (156 ppm D), but died faster at Martian deuterium content. Most of these observations are in broad agreement with early findings⁴¹⁻⁵⁵. We have also investigated the effect of planetary stable isotopic compositions, including Martian, Venusian and terrestrial, on the growth of bacteria, and found that bacteria grew significantly slower at Martian isotopic conditions and even slower at Venusian isotopic conditions. This further provided evidence that Earth had a better environment for the emergence of life. The non-terrestrial resonance conditions, including $^{15}\text{N} \approx 3.5\%$ resonance, $^{13}\text{C} \approx 0.35\%$ resonance and the

super-resonance, on the growth of bacteria were investigated. Enhancement of bacterial proliferation was observed at all these non-terrestrial conditions, which is consistent with the predictions of the Isotopic Resonance hypothesis.

In summary, we have provided experimental evidence that a MU-type process creates beneficial environment for the origin of life, and that the terrestrial isotopic resonance could have added further advantage for the formation or evolution of early life.

4 FUTURE PERSPECTIVES

The responses of living organisms to isotopic resonance are usually observed via growth behavior changes, such as shorter lag phase, higher biomass growth or faster proliferation speed. However, the exact change of isotope composition inside the cell and the molecular mechanism of these observed growth behavior changes remain unknown. Proteomics and metabolomics analyses of *E. coli* and higher organisms (such as yeast) growing in both on- and off-resonance conditions can be performed to determine which proteins and metabolic pathways are involved in their cellular response to different isotopic environments.

Furthermore, we hypothesized that when organisms are grown in specific isotopic environment, their CHON-isotopic composition will be shifted towards the closest (or the biggest) isotopic resonance which deviates somewhat from the reference terrestrial isotopic abundances. This deviation is element-specific: in order to achieve exact resonance conditions starting from the reference terrestrial isotopic conditions, the abundance of D should be increased by 64%, or abundance of ^{18}O by 1%⁶⁷. Alternatively, the isotopic abundance of ^{13}C should decrease by 22%, or that of ^{15}N by 2%⁶⁷. Thus, the Isotopic Resonance hypothesis predicts that when the growth rate increases, the isotopic compositions should change roughly proportional to the numbers +64%, 1%, -2% and -22% for the elements H, O, N and C, respectively⁶⁷. To test this hypothesis, microorganisms can be grown in a fixed isotopic environment but using slow and fast-growing strains (chosen to be as genetically similar as possible). Their CHON-isotopic composition can be determined to test whether fast growth is associated with shifts towards the isotopic resonance.

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