

Glucose uptake rate of microorganisms living in hot springs above 70°C temperature: a study of Panamik and Puga hot springs in the Ladakh region, Jammu and Kashmir, India

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This study measures *in situ* microbial glucose uptake rate in two different hot springs in Ladakh, J&K, India with distinct temperatures >74°C and pH > 7.4. For this purpose, the slurry samples from each hot spring were incubated up to 4 h with ¹³C-labelled glucose in gas-tight incubation bottles at the respective hot-spring sites. The natural $\delta^{13}\text{C}$ particulate varies from -9.1‰ in Panamik hot spring to -11.7‰ in Puga hot spring. After incubation with ¹³C-labelled glucose, the $\delta^{13}\text{C}$ particulate reached a maximum 2472‰ in Panamik and 4365‰ in Puga hot-spring samples. The glucose uptake rate calculated from the final $\delta^{13}\text{C}$ particulate in the incubation bottles varied from 28 to 147 ng C g⁻¹ h⁻¹ in the Panamik and from 168 to 1196 ng C g⁻¹ h⁻¹ in the Puga samples. This reveals that even at >74°C temperature, thermophiles are capable of running their metabolic machinery, perhaps faster than the heterotrophic microbes/cells under normal temperature condition.

Keywords: Exogenous carbon, hot springs, thermophiles glucose uptake.

MOLECULAR phylogenetic studies have demonstrated that thermophiles are the most primitive organisms in our planet. They probably originated in hydrothermal springs¹. Similarly, microorganisms have also been recorded from other extreme environments, such as psychrophiles at low temperatures, thermophiles at high temperatures, acidophiles at low pH, alkaliphiles at high pH, piezophiles under intense pressure, xerophiles in desiccates, and halophiles in high-salinity environments^{2,3}. Organisms from all three domains, viz. Archaea, Eubacteria and Eukarya are found in extreme environments⁴. Therefore, extreme conditions are no longer considered uninhabitable; rather they act as potential sites to study the advent of life on Earth and other planets like Mars. As Ladakh hosts a pristine freezing environment with little precipitation and has hot-spring zones scattered close to the Indus Tsangpo Suture Zone (ITSZ), it can be used as a type

area for the study of life in extreme environments. The studies have implications for astrobiology.

Hot-spring outflows contain exogenous carbon (i.e. bicarbonate, formate, acetate and glucose) that potentially facilitate thermophilic microbial growth⁵. Biomarker and stable isotope studies have suggested that hot springs situated in less vegetated high elevations harbour autotrophic communities. By contrast, hot springs situated in more vegetated foothills harbour either heterotrophic or mixotrophic communities⁶. More recent studies have demonstrated that hot springs are predominantly inhabited by heterotrophs, with only a small fraction of autotrophs^{7,8}. The community structure of these heterotrophic biofilms varies from one site to another^{6,9} and is driven by physiochemical factors, including temperature, pH and dissolved organic carbon. Based on experimental studies, it has been hypothesized that these heterotrophic biofilms are energy-limited and largely depend upon the exogenous source of carbon (mostly light hydrocarbons)⁷. However, no study has been conducted as yet to demonstrate the *in situ* uptake rate of exogenous carbon by the heterotrophic thermophile community.

Although the sediment–water samples from hot springs in Ladakh area have been characterized for geochemical properties¹⁰ and microbial life^{11,12}, quantitative dataset on microbial activity is not available. Thus, the aim of this study is to measure the microbial exogenous carbon uptake in two distinct hot springs with temperature >70°C, by performing *in situ* slurry incubation with ¹³C-labelled glucose. The results of this study will provide some understanding of the microbial carbon metabolism at extreme temperatures, thereby generating data to update the model for elucidating carbon biogeochemical cycling during the advent of life on the Earth.

Ladakh is a part of Jammu and Kashmir, India, and borders Pakistan in the west and China in the north. The area has cold desert-type climate, where minimum temperature reaches -40°C during winter. Most of the Ladakh region lies 3500 m amsl (above mean sea level) and has scarce vegetation. The geology of Ladakh is comprised of basic to ultrabasic, plutonic and submarine volcanic rocks of middle to upper Cretaceous age. Three localities in the Ladakh region have thermal springs, namely Puga, Chumathang and Panamik, which have been studied for geothermal potential. Previous geological studies have demonstrated that these geothermal areas are concentrated near the Indus Tsangpo Suture Zone (ITSZ)^{10,13,14}. The Puga geothermal field is situated south of ITSZ. Panamik is situated in the Shyok valley, where the geothermal field is found in the rock sequence of the Shyok Suture Zone. The incubation experiments were conducted at Panamik (34°46'49.4"N lat. and 77°32'34.4"E long.) and Puga hot-spring sites (33°13'22.9"N lat. and 78°18'53.6"E long.; Figures 1 and 2).

First, the temperature and pH of waters in the Puga and Panamik hot-spring sites were recorded in the field using

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portable temperature indicator and digital pH LED meter (supplier: Basco Lucknow). Then biofilms along with hot-spring waters were collected to make five 100 ml slurries, which were subsequently transferred to five narrow-mouthed 125 ml glass bottles leaving 25 ml volume for headspace in each. These bottles were closed with a butyl rubber cap and then crimped to make them airtight. Then 37.4 mg mercuric chloride (in solution form) was added to the first bottle to stop any further bacterial activity. All the five bottles were subsequently injected with 2 ml of ^{13}C -labelled 1 ppm glucose solution with final concentration ~ 20 ppb of ^{13}C -labelled glucose. Incubation of the second, third, fourth and fifth bottles was terminated at 1, 2, 3 and 4 h intervals respectively, by adding mercuric chloride solution.

Fifty millilitres of each incubated samples were centrifuged at 4000 rpm for 15 min in the laboratory. Thereafter, the supernatants were removed and the residue was dried overnight at 60°C in an oven. The resulting particulate matter in each centrifuge tube was removed and crushed to fine powder in agate mortar. The powder was analysed using a spectrometer (MAT 253 IRMS; isotopic ratio mass spectrometer facility available in Birbal Sahni Institute of Palaeosciences, Lucknow) connected with an elemental analyser (Thermo Fisher) by combustion method.

Measurements of ^{13}C -labelled glucose uptake in particulate matter were derived from the conventional

equation using a standard calibrated for the natural abundance.

$$\delta^{13}\text{C}(\text{‰}) = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right] \times 1000, \quad (1)$$

$$(^{13}\text{C}/^{12}\text{C})_{\text{sample}} = (10^{-3} \delta^{13}\text{C} + 1)(^{13}\text{C}/^{12}\text{C})_{\text{standard}}. \quad (2)$$

Here $(^{13}\text{C}/^{12}\text{C})_{\text{standard}} = 0.011237$ (PDB). The $\delta^{13}\text{C}$ values are a linear transform of $^{13}\text{C}/^{12}\text{C}$ ratios and represent differences in sample and standard (see eq (1)). The precision of stable carbon isotope measurements by this method was 0.1‰, which is insignificant compared to ^{13}C enrichment in particulate matter through microbial uptake (i.e. $\delta^{13}\text{C} > 1000\text{‰}$).

To determine microbial uptake of ^{13}C -labelled glucose using the mass balance approach, all the $\delta^{13}\text{C}$ values were converted to absolute abundance ratios (A) using eq. (5)¹⁵.

$$A = \left(\frac{^{13}\text{C}}{^{12}\text{C} + ^{13}\text{C}} \right). \quad (3)$$

Equation (3) can also be written as

$$A = \left(\frac{^{13}\text{C}/^{12}\text{C}}{1 + ^{13}\text{C}/^{12}\text{C}} \right). \quad (4)$$

Replacing $(^{13}\text{C}/^{12}\text{C})$ of eq. (4) with eq. (2) gives eq. (5).

$$A = \left[\frac{(10^{-3} \delta^{13}\text{C} + 1)(^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{1 + (10^{-3} \delta^{13}\text{C} + 1)(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \right]. \quad (5)$$

Mass-specific microbial uptake rate of ^{13}C -labelled glucose for a short-term experiment can be measured if the following values are known: A_{PC_0} , the natural ^{13}C -particulate abundance (i.e. $\delta^{13}\text{C}$ value) at the initiation of the incubation experiment (i.e. 0 h) and A_{PC_t} , the ^{13}C -particulate abundance after the incubation experiment (calculated from post-incubation $\delta^{13}\text{C}$ -particulate using eq. (5)). After the incubation experiment and stable isotope measurements, the ^{13}C -labelled glucose uptake rate was calculated using a simple mixing model.

$$\text{PC}_f = \text{PC}_0 + \text{PC}_\Delta. \quad (6)$$

Therefore, the isotopic composition of the final particulate organic pool, PC_f , is represented by a mass-weighted average of ^{13}C enrichment of the original particulate carbon pool in the slurry, PC_0 , and the particulate carbon added in the slurry through microbial uptake of ^{13}C -labelled glucose, PC_Δ (see eq. (7)).



Figure 1. Location of the field site in Ladakh, India. Red dots represent the Panamik and Puga hot springs where incubation experiments were conducted.

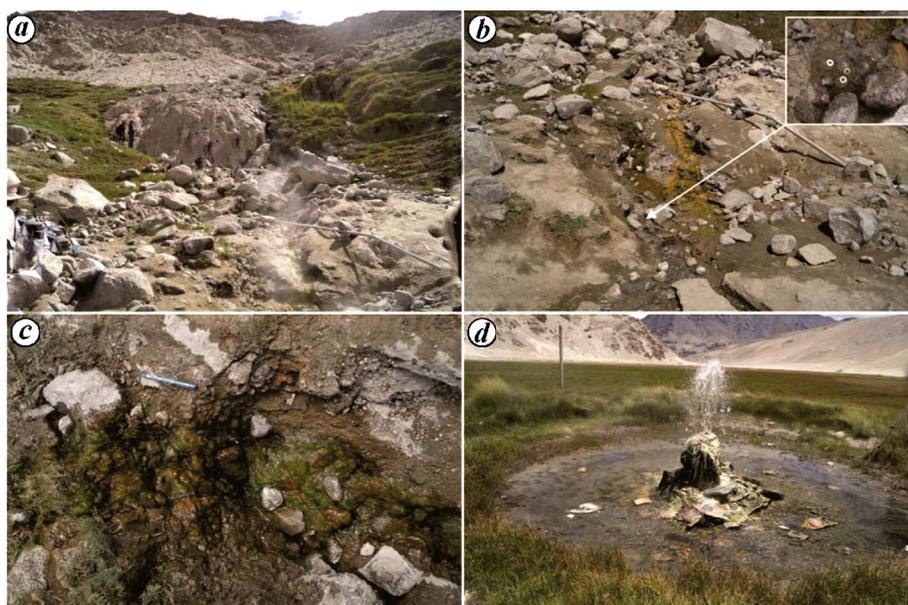


Figure 2. Field photographs of hot springs in the Ladakh region. (a) Panamik hot spring site. (b) Incubation bottles. (c) Algal biofilm in flowpath of hot-spring water. (d) Puga hot-spring site.

$$A_{PC_f} = \frac{[(PC_0)(A_{PC_0})] + [(PC_\Delta)(A_{PC_\Delta})]}{PC_f} \quad (7)$$

A_{PC_0} is the natural ^{13}C -particulate abundance at the initiation of the incubation experiment (i.e. 0 h), while A_{PC_f} is the final ^{13}C -particulate abundance at the end of the incubation experiment (calculated from natural $\delta^{13}\text{C}$ -particulate using eq. (5)).

In this short-term experiment, where PC_Δ constitutes only a small fraction of PC_f , $PC_0 \sim PC_f$. Equations (6) and (7) can be combined to provide an expression for the contribution of ^{13}C -glucose fixed into particulate carbon to the total pool of particulate carbon at the completion of the incubation experiment.

$$(PC_\Delta)(A_{PC_\Delta}) = (A_{PC_f} - A_{PC_0})(PC_f) \quad (8)$$

The microbial incorporation of ^{13}C -labelled glucose in particulate matter over a time span of Δt can be expressed as specific glucose uptake rate ($\text{mg g}^{-1} \text{h}^{-1}$)⁷.

$$\left(\frac{1}{\Delta t}\right)(PC_\Delta)(A_{PC_\Delta}) = (A_{PC_f} - A_{PC_0})(PC_f) \left(\frac{1}{\Delta t}\right) \quad (9)$$

The pH and temperature of the waters were 7.4 and 74°C in the Panamik hot spring, and 7.8 and 86°C in the Puga hot spring incubation sites respectively. The 0–4 h *in situ* incubation experiment with ^{13}C -labelled glucose demonstrates that glucose uptake rate in the Puga hot spring ranges from 168 to 1196 $\text{ng C g}^{-1} \text{h}^{-1}$, and in the Panamik hot spring from 28 to 147 $\text{ng C g}^{-1} \text{h}^{-1}$ (Table 1). This dif-

ference in the glucose uptake rate could be driven by the nitrogen availability and/or temperature change. For example, a study on *Escherichia coli* has demonstrated that rapid increase in bioavailable nitrogen can result in increased glucose uptake rate. However, experimental studies with glucose treatment on lagoonal water at different temperatures and oxygen conditions suggest that glucose uptake rate is dependent more on temperature than other parameters¹⁶. Antonio and Bianchini Jr¹⁶ calculated the temperature response as $Q_{10} = 1.12$, suggesting that glucose uptake was favoured in low-oxygen and high-temperature conditions. Incubation experiments have also been performed with cultured heterotrophic plant cells at normal temperature, which suggest two parallel mechanisms for glucose uptake by these cells: (1) plasmalemma-bound carrier framework delivering glucose to the cytoplasm, and (2) an endocytic framework that directly transports glucose into vacuoles^{17,18}. These mechanisms are apparently controlled by a gradient of hydrophilic ions between the intracellular and extracellular milieu¹⁸. Nevertheless, how temperature, nitrogen and ionic gradient control glucose uptake mechanism remains unknown.

Specifically, for glucose, the uptake rate at Panamik hot-spring site was up to ~70 times and at Puga site up to ~1400 times higher compared to that demonstrated by laboratory based incubation experiments with *Thermus*, *Firmicutes* and some unspecific bacteria from alkaline hot springs having temperature ~75–88°C and pH ~7.64–8.06 (ref. 7). However, in comparison to the in-lab acetate uptake by the above-mentioned bacteria at ~75–88°C temperature and ~7.64–8.06 pH, and glucose uptake by heterotrophic plant cells at normal temperature, the

Table 1. The $\delta^{13}\text{C}$, TOC (total organic carbon), $A^{13}\text{C}$ (absolute abundance of ^{13}C) and glucose uptake for each sample

Sample	$\delta^{13}\text{C}$ (‰)	TOC (mg g ⁻¹)	$A^{13}\text{C}$ (atom %)	Uptake rate (ng C g ⁻¹ h ⁻¹)
PG-C	-11.7	120.8	1.082	0
PG2	2527.0	60.6	3.739	1196
PG3	1893.0	100.7	3.062	789
PG4	4365.0	50.3	5.773	491
PG5	1253.0	60.7	2.459	168
PN-C	-9.1	90.9	1.081	0
PN2	135.7	80.4	1.230	28
PN3	2472.0	30.4	3.892	147
PN4	565.0	60.9	1.675	82
PN5	218.0	100.1	1.307	55

PG represents samples from Puga hot spring. PG-C is sampling at 0 h incubation (control), while PG2, PG3, PG4 and PG5 represent incubation for 1, 2, 3 and 4 h respectively, PN represents samples from Panamik hot spring. PN-C is sampling at 0 h incubation (control), while PN2, PN3, PN4 and PN5 represent incubation for 1, 2, 3 and 4 h.

glucose uptake rate in this study at the Panamik hot-spring sites was equivalent and at the Puga site was ~20 times higher⁷. The consumed glucose undergoes the glycolysis process before its conversion to acetyl-CoA enzyme, which is then utilized for lipid biosynthesis⁷. The $\delta^{13}\text{C}$ (between -9.1 and -11.7‰) of Panamik and Puga hot spring samples having no ^{13}C -enriched glucose uptake suggests the presence of heterotrophic microbiota⁶.

Thus, the present study suggests that preferential exogenous carbon compound for alkaline hot-spring microbes varies from one place to another depending upon physico-chemical conditions of the habitat and principal metabolic pathways of the inhabiting microbes. The glucose uptake rate measured under laboratory conditions does not represent the real value in the natural environment. Furthermore, this study also demonstrates that heterotrophic glucose uptake rate measured in high-temperature alkaline hot springs is comparable or even higher than that under normal temperature conditions. The property of glucose integration in lipids by heterotrophic microalga has currently been exploited in the field of biotechnology to develop commercial methods for the production of high-value metabolites, including biodiesel¹⁹. Therefore, results presented in this study tentatively suggest high commercial value of microalga present in the Puga hot spring.

This study indicates that hot springs having temperature above 70°C not only host heterotrophic microorganisms in their immediate ambience, but also maintain a significant biomass by utilizing the exogenous carbon which is released into the pool by weathering or aeolian deposition. The study demonstrates that *in situ* microbial glucose uptake rate in alkaline hot springs is several times higher than that demonstrated in lab incubation studies. Also, the hot-spring heterotrophic microbial glucose uptake rate is higher than the heterotrophic glucose up-

take rate at normal temperature. Interestingly, the rate of heterotrophy also shows a positive correlation with temperature and pH. However, the mechanism that controls glucose uptake remains unknown and requires more detailed studies involving major controls like temperature, nutrients and hydrophilic ions.

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Some aspects of seed dispersal by Asian elephants (*Elephas maximus*) in Kaudulla National Park, Sri Lanka

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Our understanding of the qualitative and quantitative aspects of seed dispersal by Asian elephants is at its infancy. We explored some of these aspects at Kaudulla National Park, Sri Lanka, focusing on the germination potential of dispersed seeds, and the influence of gut passage on germination and latency in the seeds of *Bauhinia racemosa*. Seeds of ten species were dispersed and their germination potential was generally poor. However, gut passage significantly reduced the latency period of *B. racemosa*. Long-term research on frugivory, passage times of seeds and ranging behaviour will help develop wildlife management plans.

Keywords: Asian elephants, *Bauhinia racemosa*, latency, seed dispersal.

SEED dispersal has a profound impact on plant spatial structure, dynamics and community composition. The spatial distribution of seeds relating to their source plant sets the initial template for future processes that lead to plant recruitment^{1,2}. As a result, seed dispersal has received significant attention of ecologists³. It has become the key to understanding several ecological phenomena such as community structure^{4,5}, population dynamics⁶, evolutionary trade-offs⁷, ecological restoration⁸, interaction networks⁹ and biological invasions¹⁰. Animal-mediated seed dispersal is the most prevalent dispersal mechanism among plant species, as many plants possess highly adapted fruits that depend on vertebrates for recruitment^{11,12}.

Endozoochoric seed dispersal, in which dispersal is effected by animals after passage through the gut, has been shown to reduce seed or seedling mortality due to higher predation rates, pathogen infection¹³ and sibling competition near the parent plant¹⁴, and aid colonization of disturbances or locating microhabitats suitable for establishment and growth¹⁵. For instance, seeds dispersed 45 m away from the mother plant of baboon wood (*Virola surinamensis*) recorded a 44-fold advantage in seed survivorship when compared to seeds deposited beneath the crown¹⁶. Over evolutionary time, animal seed dispersal

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