1 Introduction

Halophiles, including halophilic archaea, bacteria, and algae, are highly evolved groups of organisms that thrive in hypersaline environments. For the past several decades, halophiles preserved from ancient evaporites have been intriguing microbiologists and astrobiologists who seek to decipher the origin and evolution of life in extreme environments. Most hypersaline environments are restricted seas, lagoons or lakes, promoted by a high rate of evaporation, and possibly high temperature and low precipitation. Some evaporite successions are several hundred meters thick, indicating prolonged hypersaline environments, which provoked the evolution of microorganisms (Dombrowski, 1963, 1966). Moreover, evaporites have also been found on some extraterrestrial planets, such as Mars (McLennan et al., 2005). Evaporites hold great potential for preserving signals of potential life and organic matter in extraterrestrial surface habitats (e.g., Squyres et al., 2004; Osterloo et al., 2008).

Many researchers have reported isolating halophiles from ancient rock salt or brine in salt mines, but doubts remain about their ages because of potential contamination from surface water or other introduced fluids (Dombrowski, 1963, 1966; Vreeland et al., 2000, 2007; Stan-Lotter et al., 2002). Primary fluid inclusions in halite preserve the first-hand evidence of original depositional fluid and therefore can serve as a potential window to the halophilic ecology. Moreover, the presence of haloarchaea bacteria possibly affects the mineral crystallization and leads to increases in size and amount of fluid inclusions (Schubert et al., 2010). Adamski et al. (2004) experimentally proved that microorganisms could be trapped by and preserved in fluid inclusions in synthetic halite. In fact, Dombrowski (1963) reported the isolation of

**Ancient Microorganisms and Carotenoids Preserved in Fluid Inclusions in Halite from Chaka Salt Lake, Western China:**
Evidence from Micro-observation and in situ Raman Spectroscopy

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Abstract: Trapped ancient microorganisms in halite fluid inclusions are of special interest to the understanding of biology and ecology in salt lake systems. With the integration of petrologic, microthermometric, and Raman spectroscopic analyses, this study utilizes fluid inclusions from Chaka Salt Lake, eastern Qaidam Basin, NW China, to assess the possibility of microorganism-trapping by fluid inclusions. Here, we report that the solid phase of some primary fluid inclusions contains carotenoids, which is interpreted as evidence of *Dunaliella* algae, and that the coexisting liquid phase comprises SO$_4$$^{2-}$. The homogenization temperatures of single-phase primary fluid inclusions indicate that the precipitation temperature of the Holocene halite in Chaka Salt Lake ranges from 13.5°C to 36.4°C. This suggests that fluid inclusions in halite are a good medium for trapping and preserving ancient microorganisms and organic matter in salt lakes, and that Raman spectroscopy has good potential to identify halophilic archaea.

Key words: invertebrate paleontology, halophile microorganisms, halite, fluid inclusions, carotenoids, Qinghai Province

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Recently, a series of studies have employed a combination of petrographic observations with traditional molecular methods to document microorganisms in halite up to \( \sim 100,000 \) years old from Death Valley and up to \( \sim 150,000 \) years old from Saline Valley, California, USA (Schubert et al., 2009a, 2009b, 2010; Lowenstein et al., 1998; Winters et al., 2013). Prokaryotes, eukaryotes, and associated organic compounds have been described from these fluid inclusions in modern halite (Schubert et al., 2010). In this case, \textit{in situ} discrimination of microorganisms, as one of the applications of Raman spectroscopy used in microbiology, is of particular value.

In this study, we performed petrographic and Raman spectroscopic analyses on fluid inclusions in the natural halite deposit of Chaka Salt Lake, Qinghai province, western China. The objectives of this study are to: 1) document the entrapment and preservation of microorganisms in halite fluid inclusions; 2) apply Raman spectroscopy to \textit{in situ} discrimination of microorganisms in fluid inclusions; and 3) discuss the concerns and limitations of the preservation of microorganisms in fluid inclusions in halite.

### 2 Sampling

Samples were collected from Chaka Salt Lake (36°38'–36°45'N, 99°02'–99°12'E; 3200 m a.s.l.), which is located on the eastern edge of the Qaidam Basin, about 150 km west of Qinghai Lake (Fig. 1). The Qaidam Basin, covering an area of 120,000 km\(^2\), is a large intermontane depression on the northern margin of the Tibet Plateau, bounded by the Kunlun, Aerjin, and Qilian mountains. The Chaka Salt Lake was formed in a regional rifting system during the depression of the Qaidam Basin (Liu et al., 2008). The present-day Chaka Salt Lake is characterized by a continental climate with a mean annual temperature of 3.5°C and an annual mean precipitation of 197.6 mm, and is fed by fresh water rivers (Mo R. and Hei R.) and springs without outlet. Sedimentary successions in Chaka Salt Lake consist of halite, gypsum, thenardite, epsomite, bloedite and hydrohalite. The halite layers outcrop on the lake surface, and are generally 1.2–9.68 m thick, with a maximum of 15 m and an average of 4.9 m (Liu et al., 2008). Originated from a clastic-dominated freshwater lake, Chaka gradually evolved into a hypersaline lake as the result of oscillating warm-cold climatic periods during the late Pleistocene (Liu et al., 2007; 2008). With minor amounts of gypsum and anhydrite, the salt deposit comprises mainly halite. The water in Chaka Salt Lake has a salinity of 322.4 g/L, a density of 1.2178 g/cm\(^3\), pH of 7.8, and the chemical type of brine is the sulfate type, a subdivision of magnesium sulfate (Zheng, 2001; Yuan et al., 1995).

The samples in this study are Holocene primary halite from near the surface of the lake. We collected halite crystals with good crystallinity and transparency for analysis. In order to facilitate observation, we separated the halite into smaller transparent thin sections by cutting along directly the cleavage surface, rather than by grinding the sample, in order to avoid dissolution of halite. The samples are stored in the lab of fluid inclusions in China University of Petroleum (East China).

### 3 Methods

Samples were subject to systematic microscopic observations under the microscope, to record the morphology, quantity and distribution of fluid inclusions and identify which inclusions are likely to contain organisms. Particular attention was paid to distinguish primary and secondary inclusions. Microscopes used are equipped with digital imaging systems. The compositions
of the fluid inclusions were analyzed using a laser Raman spectrometer, including the gaseous, liquid and solid phases. Raman spectrometry is a spectroscopic technique typically used to determine vibrational modes of molecules. Laser Raman spectroscopy was used to identify the matters in halite and fluid inclusions. The spectrometer used is a LabRam-010 Microscopic confocal Raman spectrometer made by HORIBA Jobin Yvon. The wavelength of exciting laser is 514.53 nm and the spectral resolution is 1.7 cm$^{-1}$.

Homogenization temperatures of the chosen primary monophase liquid inclusions were measured according to the methods described in previous studies (Petrichenko, 1979; Shela and Spencer, 1995; Lowenstein et al., 1998; Benison and Goldstein, 1999). Selected halite samples were kept in a laboratory freezer at $\text{–18°C}$ for two weeks for monophase liquid inclusions to nucleate a vapor bubble before the measurement of homogenization temperatures. The homogenization temperatures were measured with a Linkam THMSG 600 Cooling-Heating Stage, with an error ±0.1°C after correction. The homogenization temperatures are thought to represent the capture temperatures of the fluid inclusions and they indicate the paloetemperatures during the deposition of halite, because the trapping (surficial) pressure of the primary fluid inclusions is essentially the same as the pressure when measurement was performed, both being under atmospheric pressure (Shela and Spencer, 1995).

4 Results

4.1 Primary fluid inclusions and their homogenization temperatures

Primary halite usually shows chevron bands (see Fig. 2a). During the precipitation of halite from saline lake brine, fluid inclusions are trapped along the growth bands, possibly preserving any micro-components of the system, including the lake water, tiny atmospheric bubbles, salt crystals, and microorganisms (Benison and Goldstein, 1999; Lowenstein, 2001, 2011; Blamey et al., 2016). Primary fluid inclusions in cumulates and chevrons from the Chaka Salt Lake are oriented parallel to the crystal growth faces, and have negative crystal shapes, as shown in Fig. 2b. The main morphology of the primary fluid inclusions is negative-crystal cube and cuboid. Primary fluid inclusions range in size from 1 μm to 200 μm, mainly between 5 μm and 30 μm. Most fluid inclusions are filled with liquid, but some contain a vapor phase and gypsum crystals that were trapped during halite growth. Gaseous phase boundaries appear black under transmission light, whereas the liquid phases of all the fluid inclusions are clear and colorless.

The homogenization temperatures were measured after samples had been frozen as noted above to nucleate vapor bubbles. The range of temperatures is 13.5–36.4°C, mainly distributed in the 19–30°C range, with an average of 23.9°C and a maximum of 36.4°C (Fig. 3).

4.2 Raman Spectra of greenish solid phase

Detailed petrological observations revealed that some solid phases exhibit rounded or subcircular shape with green or brown color and are not salt crystals (Fig. 4). These solid materials like *Dunaliella* algae reported by Abbas et al. (2011). In order to confirm the type of these materials in the fluid inclusions, laser Raman spectroscopy was performed on targeted solid phases. Spectra show consistent patterns (Fig. 5a–c) with large Raman peaks appearing near 1010 cm$^{-1}$, 1160 cm$^{-1}$ and 1525 cm$^{-1}$, as well as the H$_2$O band at 3400–3500 cm$^{-1}$. A Raman band also exists at 983 cm$^{-1}$ (Fig. 5d).

5 Discussion

5.1 Paleotemperature of Chaka Salt Lake

Homogenization temperatures of halite fluid inclusions can be used as a proxy for surface environment temperatures. Published records show a good correlation between homogenization temperatures of halite (liquid) fluid inclusions and brine temperature. The maximum homogenization temperature matches the maximum brine temperature during halite precipitation (Shela and Spencer, 1995; Benison and Goldstein, 1999; Satterfield et al., 2005a; Meng et al., 2011a, 2011b; Zambito and Benison, 2013). Meng et al. (2011b) suggested that different morphologies of halite crystals might reflect their depositional environments: primary fluid inclusions in funneled halite crystal formed at the air-water interface and those in chevron-shape crystals deposited at the
bottom of water, and, thus, their homogenization temperatures can reflect the paleotemperatures of different levels of the water body. For samples from Chaka Salt Lake, measured homogenization temperatures range from 13.5–36.4°C with an average of 23.9°C and a maximum of 36.4°C. Given that the halite is formed in the air-water interface under atmospheric pressure, no pressure correction is required. This suggests that the brine temperatures at the time when halite was precipitated were 13.5–36.4°C, which is consistent with previous studies (e.g., Liu et al., 2008). Modern observations show that the difference between surface water temperatures and local air temperature is ~ ±5°C (Benison and Goldstein, 1999). Thus, the homogenization temperature of halite fluid inclusions can not only be used to indicate the precipitating hypersaline water but also provide regional climatic information. Our microthermometric records suggest that the paleoclimate was comparatively mild when the halite was precipitated at a temperature that is suitable for the growth of microorganisms.

5.2 Microorganisms trapped in halite fluid inclusions?

The green solid phase observed in our halite fluid inclusions is petrologically distinguishable from salt crystals. Previous studies have reported organic matter or microorganisms trapped in fluid inclusions from the Permian or older halites (Dombrowski, 1963; Vreeland et al., 2000; Satterfield et al., 2005b; Conner and Benison, 2013). However, considerable skepticism was raised due to contamination issues during the extraction procedures. Therefore, a non-destructive method is very important to detect microorganisms in fluid inclusions. Fendrihan et al. (2009) applied the Raman scattering technique to identify microorganisms captured in synthetic halite. The present...

Fig. 3. Histogram of homogenization temperatures of single liquid phase fluid inclusions in halite from Chaka.

Fig. 4. Micrographs of Dunaliella algae (solid phase) in selected halite fluid inclusions.
study has applied the same technique to natural halite from Chaka Salt Lake. As shown in Fig. 5, the Raman bands near 1010 cm\(^{-1}\), 1160 cm\(^{-1}\) and 1525 cm\(^{-1}\) match the Raman spectrum of the carotenoid pigments of *Dunaliella* as shown by Marshall et al. (2007) and Winters et al. (2013). In fact, *Dunaliella Salina* is one of the most common algae living in the Chaka Salt Lake (Liu et al., 2007; 2008). The bands at 1525 cm\(^{-1}\) and 1160 cm\(^{-1}\) belong to the stretching vibration of conjugate alkene C=C and C-C bonds in carotene, whereas the in-plane vibrational mode of the -CH\(_3\) connected with the conjugate alkene C-C bond shows the Raman band at ~ 1000 cm\(^{-1}\) (Abbas et al., 2011; Jehlicka et al., 2012, 2013). The Raman spectra collected from all green solid phase examples show similar patterns, indicating the same microorganism was preserved widely in fluid inclusions. *Dunaliella* algae are unicellular microalgae without cell walls exhibiting a green color due to chlorophyll pigments in their chloroplasts. *Dunaliella* algae usually thrive in hypersaline ponds and salt lakes with a strong ability to adapt to the environment so that it can survive in brine with low salinity to saturated brine, with pH from 1 to 11.5 and a temperature range from 0°C to 38°C (Schubert et al., 2010). The halotolerance of *Dunaliella* algae is mediated by glycerol as an osmoticum (Morgan et al., 1987). The middle Raman band at 983 cm\(^{-1}\) belongs to the vibration of the sulfate radical (Fig. 5d) indicating that the contained water dissolved the sulfate component when the halite precipitated. This supports the existence of *Dunaliella* algae given that they inhabit hypersaline waters and utilize sulfate for assimilation, since *Dunaliella* algae are one of the most active microorganism communities in Chaka Lake (Jiang et al., 2006, 2007). Importantly, this finding also confirms that fluid inclusions do have the capability to capture and trap living microorganisms in the environment of salt precipitation.

Fluid inclusions trapped in minerals act like sealed micro-chambers (Roedder, 1984), so that they can preserve paleo-fluids and provide clues about the temperature, pressure, density, and composition of the fluid that precipitated the mineral or rock. Unlike most minerals, halite can form in environments where microorganisms inhabit. As we demonstrated above, halite fluid inclusions can give insights into both paleoclimatic and paleoecologic conditions. Raman spectroscopy is sensitive to organic and inorganic compounds and is able to distinguish specific spectra within biological and chemical components (Schmid et al., 2009; Abbas et al., 2011; Osterrothová and Jehlička, 2011). Taking this advantage of the non-intrusive in situ analysis of Raman spectroscopy, our work demonstrates an effective detection method for paleoecological investigations in hypersaline systems. This work may provide an alternative method for such investigations given that evaporites are developed widely throughout the entire geological history (Warren, 2010). Moreover, evaporitic deposits are not exclusive to Earth. Astrophysicists and astrobiologists have found that many planets, including Mars, contain paleolake craters and evaporates. Saturated salt solutions may be envisaged on Mars due to the Martian

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**Fig. 5.** Raman spectra of (a, b, c) solid phase in halite fluid inclusions indicating ancient halophilic bacteria, where red circles illustrate test points of the Raman analyses, and (d) the magnified version of the field in red box in C.
temperatures and atmospheric pressure, and Na, Mg, Cl and Br are found extremely concentrated in soil and rocks of Mars (Rieder et al., 2004). Evidence for halite was also found on Mars in such as shergottite, nakhlite, and chassigny (SNC) meteorites (Treiman et al., 2000; Rieder et al., 2004). Determining whether life ever arose on Mars was among the top science objectives for NASA’s 2020 Mars Mission (Williford et al., 2018). If life had ever existed on Mars, freezing hypersaline brines, paleolakes, and acid saline lakes would be the most promising sites for investigation and halophilic microbial forms would be the best prospect (e.g. Mancinelli et al., 2004; Benison and Bowen, 2006; Knoll and Grotzinger, 2006). Among different detection techniques, non-destructive Raman spectroscopy holds its special value for this mission. Our work illustrates that fluid inclusions can capture microbial organisms in hypersaline environments during the precipitation of evaporites, which can be regarded as a potential analog for investigating Martian life.

5.3 Importance and concerns of trapped microorganisms in fluid inclusions

Although it is of great significance to explore for information of life in fluid inclusions hosted by halite, caution should be exercised when investigating and interpreting such information. Halite is easily affected by its external environment owing to its characteristics of low hardness, easy deliquescence and ease of melting in heat. Especially for those that experienced a long geological history, halite can be reformed by any geological stress and active fluid. The fluid inclusions trapped in halite are, therefore, apt to experience some secondary change, including recrystallization, extension, leakage, contraction, and so on (Roedder, 1984). Therefore, detailed observation in determining primary and secondary fluid inclusions is critical. On the other hand, high temperatures and pressures are the main reasons for changes in microorganisms trapped in fluid inclusions, particularly those caused by deformation and metamorphism of protein, nucleic acid and bio-macromolecule. For the microorganisms in fluid inclusions that have gone through high temperature and pressure, it is questionable whether the physical and biological information could be completely preserved. Therefore, seeking out unaltered primary fluid inclusions is vitally important in the research on ancient depositional environments and microorganisms of halite.

6 Conclusions

Based on this study, the following conclusions can be reached.

1) The paleotemperatures of salt lake water varied between 13.5°C and 36.4°C when the Holocene halite was deposited in Chaka Salt Lake, Qinghai. The alga Dunaliella salina lived in the Holocene saline lake and was preserved in halite fluid inclusions.

2) Raman spectroscopy is a contributive means to analyze microorganism characteristics non-intrusively. The Raman spectra show carotene information of Dunaliella salina in the halite inclusions from Chaka Salt Lake, providing evidence for identifying the microorganism type in inclusions.

3) For studies of halite fluid inclusions, the primordial quality of inclusions must be ensured. The integrity of organic matter preserved in fluid inclusions also deserves more attention and further investigation. There are still restrictions and limitations regarding the methods used that need to be examined.

4) This study demonstrates a working example for studies on ancient microorganisms and their preservation in halite fluid inclusions. Evaporites of various ages have been found in terrestrial strata as well as on extraterrestrial planets. This makes fluid inclusions that trap microorganisms a prospect for paleoecological and astrobiological investigations.

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