ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 4, Issue, 8, pp.1205- 1209, August, 2013

International Journal of Recent Scientific Research

RESEARCH ARTICLE

A COMPARATIVE STUDY OF TRENTEPOHLIA AND RED RAIN CELLS

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ARTICLE INFO

Article History:

Received 14th, July, 2013
Received in revised form 26th, July, 2013
Accepted 11th, August, 2013
Published online 30th August, 2013

Key words:
Red rain of Kerala; Extremophiles; Trentepohlia-aurea; Trentepohlia-umbrina

ABSTRACT

The red cells that caused the coloration of the red rain of Kerala gained scientific significance due to their possible extraterrestrial origin. A competing theory is that the red cells are spores of the *Trentepohlia*-genus that originated locally was lifted to the clouds and came down in rain. Hence a comparative study is required to decide whether the red rain cells are different from or indeed the same as *Trenetepohlia* spores. A comparative study was made of red rain cells and two species of *Trentepohlia* genus, namely *Trentepohlia aurea* and *Trentepohlia umbrina.* Red rain cells and *Trentepohlia* spores were differentiated on the basis of its morphology, absorption in the uv- vis regions, presence of DNA, ultrastructure, stability with regard to temperature, percentage of nitrogen and phosphorous in the cells and survivability in water. Our study showed that the red rain cells cannot be equated with *Trentepohlia* and still remain identified and distinct from known microbe on the earth.

INTRODUCTION

The red cells discovered in the red rain of the southern Indian State Kerala have gained much attention worldwide because of the possibility that they represent an extraterrestrial life form of alternate kind [Louis and Kumar, 2006]. At least 50 tons of red colored particles with a resemblance to biological cells fell in the rain during July to September 2001. Rain colored primarily red but also in a few cases yellow, green, or black, fell in scattered areas of the State. A detailed analysis of this phenomenon was carried out on the basis of geographical distribution patterns of incidence and we reported that these red colored biological cells might have originated from a cometary meteor. It was suggested that the atmospheric fragmentation of a fragile cometary meteor can be the reason for the geographical distribution of the red rain cases in a large area of size 450 km by 150 km. Such a bolide impact event was inferred from reports of a loud thunder that was heard by several people a few hours before the first colored rain fall. In a recent study McCafferty [2008] reported the connection between red rain occurred in Kerala and red particles may be consistent with historical accounts linking colored rain to meteor passing. Elemental analysis of the red rain cells by EDAX showed carbon and oxygen were the major constituents of the cells but substantial amount of silica and trace amount of Na, Al and Fe were also present [Louis and Kumar, 2006]. In addition to these elements 4.43 % of Hydrogen and 1.84 % of N_2 was also detected in the red cells using CHN analysis. The absence of Nitrogen determined in EDAX analysis and its low percentage in the CHN analysis were puzzling. Moreover the test for DNA in the red cells using ethidium bromide fluorescence enhancement technique showed negative results. Reports of the DNA detection using DAPI staining by Microscopic Probe technique after pigment removal by DMSO showed positive results. (Gangappa and Hogg, 2013)

Several episodes of red rain were also reported in the Central and Southern Provinces of Sri Lanka during late November and December 2012 (Nori et al., 2013). These events were preceded by fireball sightings and a meteor fall that happened approximately 10 days earlier. The cause of the red coloration to the Sri Lankan red rain was due to huge quantity of red cells and appears generally similar to Kerala red rain cells. The results of a TEM and EDAX studies of the Red rain cells showed that the outer cell wall unusually rich in uranium, and a nuclear region with a strong deficit or absence of phosphorus (Nori et al., 2013). The presence of DNA/RNA could not be established still, but continuing efforts are being made in other laboratories to test for. Despite all these facts several scientists (Sampath et al., 2002) argued that these red rain cells are of local origin in particular attributing them to spores of *Trentepohlia.* Sampath *et al* (2002) reported that the red color was due to the presence of spores of a lichen-forming alga belonging to the genus *Trentepohlia,* although they were unable to give the exact species name of the genus- *Trentepohlia.* The report also raises several questions regarding the origin of the huge quantities of red spore that can reach rain clouds, and the mechanism for their transport. In an experiment to culture red rain cells using medium suitable for algal growth, they found branched filamentous thallus of *Trentepohlia* in the cultures. Thus they concluded that the red color was due to algal spores in the rain which were of local origin. The local lichen that evidently contaminated the red rain water grew into algae, which had a vague similarity to the original red rain cells. This does not any way prove that the red spores are of local origin, although *Trentepohlia* may very well be included

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as a minor contaminant. Besides Sampath et al. (2002) had chosen a culture medium that was suitable for growth of algae and fungi so that traces of algae or fungi in the rain water would have grown easily. A comparative study is necessary to elucidate the difference between red rain cells and *Trentepohlia* spores. In this paper we report the results of a comparative study on both red rain cells and *Trentepohlia.*

Trentepohlia is a common genus of sub-aerial green algae occurring on tree bark, leaves, rock and several types of artificial substrata (Printz, 1939; Chapman, 1984; Lopez et al., 2002). The main characteristics that distinguish the order *Trentepohliales* from other *Chlorophycean* green algae are the presence of ß-carotene, haematochrome and its unique flagellar apparatus (Lopez et al,, 2002; Christiaan et al,, 1995). The genus includes about 40 species (Christiaan et al,, 1995), mainly distributed in tropical and subtropical areas (Printz, 1939; Cribb, 1958; Krishnamurthy, 2000) Additionally it was reported that, *Trentepohlia* species grow very slowly, and in culture they will rarely produce structures such as zoosporangia, useful for species–level determination (Uyenco,1965)

MATERIALS AND METHODS

For the present study two species of *Trentepohliales* namely *Trentepohlia- aurea* and *Trentpohlia- umbrina* were collected. These samples were collected from the trees located at the Mahatma Gandhi University Campus, Kottayam, India where red patches (fig. 1a & fig. 2a) attributable to these algae could be recognized with the unaided eye. The samples are found in very few trees and are not very common. Samples of red rain cells were collected from various locations where the red rain occurred during July to September 2001. The characteristics including morphology of the red cells contained in the red rain samples collected at different places were the same showing a common origin. The red rainwater is basically a pure suspension of red cells and is practically devoid of any dust content, although a very few samples contain contaminants like filamentous algae, protozoa, etc. The detection of DNA can be performed using a microscopic probe technique on both red rain cells and *Trentepohlia* genus spores. For this purpose each sample is placed on the surface of a microscopic slide and DNA staining was done using ethidium bromide stock solution. The samples were then examined microscopically using an epifluorescence microscope (Olympus fluorescence microscope BX-51). The absorption spectrum of the red rainwater in the uv and visible region was recorded using Spectrophotometer (Shimadzu Model No. UV-2401 PC).

RESULTS AND DISCUSSION

Fig 1b (40×), fig1c (100×) shows a microphotograph of *T. aurea* whereas fig. 2b (40×) and fig. 2c (100×) shows that of *T. umbrina*. The erect axes were poorly branched and distinctly longer in *T. aurea* than in *T. umbrina.* In *T. aurea* the cell were regularly cylindrical in erect parts (fig.1b & 1c) and swollen in the prostate parts. *T. umbrina* form small compact masses of short irreguilary branched filaments consisting of either globular or swollen cells (fig. 2b & 2c). Both species contains gametes inside the cell. Unreleased motile cells are clearly visualized (fig.1c & 2c) under higher magnification (100×). It has been reported that reproduction mechanism of both *T. aurea* and *T. umbrina* was the production of presumptive gamatangia that release biflagellate swarmers (or gametes) and this swarmers never observed to fuse sexually. The resulting swarmers were initially oval in shape and later become swollen or globular (Rindi and Guiry, 2002). The red rain cells and *Trentepohlia* –genus spores are differentiated on the basis of their morphology, stability with respect to temperature, presence of DNA/RNA, ultrastructure, and survivability in water.

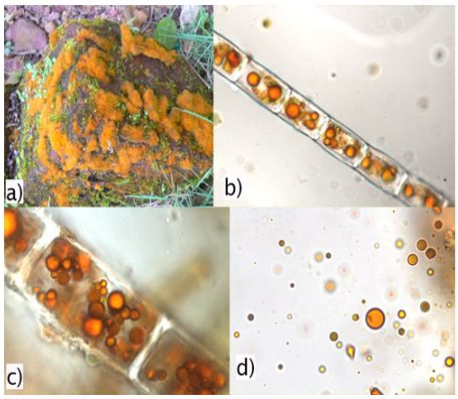

Fig.1 Images of *Trentepohlia aurea* a) as grown in a brick b) Bright field microscopic images at 40x, c) Bright field microscopic images a 100x, d) Released motile cells at 100x

Morphological difference

Red rain cells were differentiated from those of the *Trentepohlia* –genus spores by their morphology. Figure 1d & fig. 2d shows the microphotograph of the released motile cells of *T. aurea* and *T. umbrina* respectively whereas fig.3a shows the microphotograph of red rain cells. The red rain cells are about 4 to 8μm in size, almost transparent red in color and are well dispersed in the rainwater. The majority of the red rain particles have a reddish brown color under transmitted light but a small percentage of particles are white or have colors with light yellow, bluish gray and green tints. The microscopic images of each motile cell of both *T. aurea* (Fig. 1d) and *T. umbrina* (fig. 2d) shows the cells were spherical in shape with a color of deep orange.

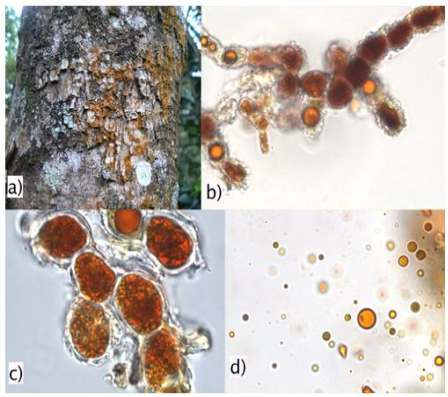

Fig. 2 Images of *Trentepohlia umbrina* a) as grown in a tree bark b) Bright field microscopic images at 40x , c) Bright field microscopic images a 100x, d) Released motile cells at 100x

Some cells have yellowish red color. The cells have sizes ranging from 1micron to 10 microns. All the gametes were morphologically circular in shape. But the morphology of the red rain cells can vary from spherical to ellipsoid, slightly elongated types and few even have triangular in shape (Fig. 3). The inner constituent of the red rain cells can be seen under light microscope whereas it cannot be seen in the *Trentepohlia* spores. The light microscopic images and scanning electron microscopic images of the biflagellar motile cell of *Trentepohlia- aurea* were given by Graham & McBride (1975). Report on the light microscope image

of *Trentepohlia –aurea* (using Nomarski optics, 1700×) showed the motile cells were oval in shape with biflagellate apparatus and the SEM image also showed biflagellar structure (Roberts, 1984). They also reported that motile cells of all *Trentepohliales* were initially oval in shape - 8-10 micron long and 5-8 micron wide and later becoming swollen or globular. The SEM image of *Trentepohlia – aurea* shown in figure 4b was oval in shape and with size about 12 microns. Some of the cells were damaged while impinging the electron beams. The SEM image of the red rain cells showed its outer surfaces to be smooth and round with inward depression of the spherical surface to form cup-like structures (Fig. 4a). The amount of such surface deformation varies from cell to cell and some of the cells do not have these surface depressions. There are no flagella or filamentous structures attached to the outer surface, which mainly differentiated the red cells from all *Trentepohliales.*

uv-vis absorption studies

The absorption spectra of both red rain and *T. aurea* motile cells are given in figure 5. Spores inside the filamentous *T. aurea* can be released by grinding the cells gently in a agate mortar. Most of the spores will be released in and thus a few cells got fully crushed. Absorption spectra were recorded using a spectrophotometer (Shimadzu UVPC) in the wavelength region 200 -900 nm. Both samples showed an absorption in the uv region but the intensity of absorption for the red rain sample was comparatively high. The absorption spectrum of a red rain sample showed an absorption peak at 211 nm while no characteristic peak was found for the *Trentepohlia* cells. The absorption spectra of *Trentepohlia* cells in the visible region showed few peaks (Fig 6) respectively at 359, 442, 463, 497, 573, 662, 787, 808 nm. In some *Chlorophytes* such as in *Trentepholiales* the cholorophyll is masked by haematochrome which is red in color and consist of a mixture of carotenoid pigments (Lopez et al., 2002). The absorption peaks observed at 442, 463, 497 nm are due to carotenoid pigments and the peaks found at 662, 787 nm are due to the absorption of chlorophyll. The peak due to carotenoid pigments and chlorophyll are absent in the red rain which has peaks at 503 and 600 nm (Fig 6).

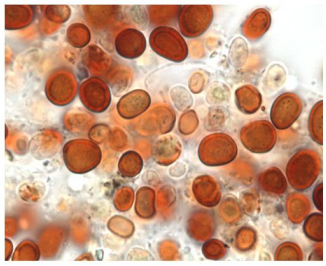

Fig.3 Bright field microscopic image of red rain cells at 100x

Presence of DNA in the cells

DNA detection was done on red rain cells, *T. aurea* and *T. umbrina* by microscopic probe technique using ethidium bromide dye. Fig. 7a, b and c shows the microphotograph of the ethidium bromide stained *T. aurea, T. umbrina* and released motile cells of *T. aurea* respectively whereas fig. 7d and fig. 7e shows ethidium bromide stained red rain cells. Results showed that the red rain cells are not fluorescing after stained with ethidium bromide (fig. 7d and 7e). However intense fluorescence was observed for a rod shaped bacteria (contaminant) that was present in the rain water along with red cells when stained with ethidium bromide (fig. 7e). Results of the DNA detection using microscopic probe technique in *T. aurea* and *T. umbrina* showed intense fluorescence for both unreleased (fig. 7a & 7b) and released gametes (fig. 7c). Gametes inside the filamentous cell can be clearly visualized after stained with ethidium bromide (fig. 7a & 7b).

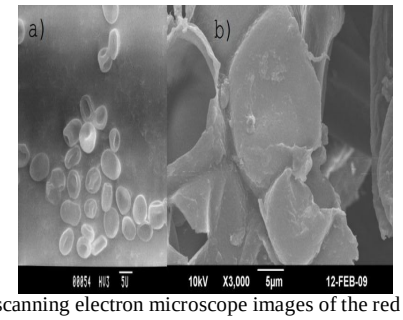

Fig 4 a) scanning electron microscope images of the red rain cells indicating no flagella attached. b) SEM image of the *Trentepohlia aurea* spore

Results of DNA detection in the red rain cells shows negative results indicating the non-detection of DNA and that on *Trentepohlia* cells showed positive result. It can be argued that the red rain cells did not absorb ethidiuim bromide dye due to its thick cell wall. But the light microscopic bright field image (Fig. 7f) shows that the red rain cells are intact absorbing ethidium bromide dye. We have already reported that the red rain cells appear to be free from DNA/RNA by use of fluorescence enhancement - spectrofluorimetric techniques using ethidium bromide dye (Louis and Kumar, 2006). But, later study on the detection of DNA in the red cell (Gangappa and Hog, 2013) showed positive result by extraction of red pigment from the outer layer with DMSO which enhances the permeability of red rain cells to chemical stains, allowing positive DAPI staining, indicating the presence of DNA. This result is not conclusive because of the following reason. When the red pigment was removed from the outer layer, then the cell will auto -fluoresce in wide wavelength. The wide auto fluorescence behavior of the pigment removed red cells has been described in their paper. The red cells are to be assumed as spore and contain inner vegetative cells, which can auto-fluoresce very intensively. Hence the inner blue fluorescence is not due to DNA rather due to autofluorescence of the red cells. Moreover the red cells lack phosphorous (Nori et al., 2013), which is an essential constituent of the DNA bearing life forms.

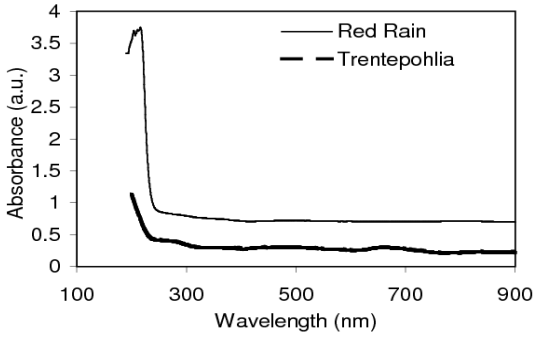

Fig 5 UV absorption spectrum of red rain water and *Trentepohlia* spores in water

Temperature stability of Trentepohlia and red rain cells

To compare the temperature stability of both *Trentepohlia* and red rain cells, all the samples were placed in a heating stage and the temperature was increased. The samples were then observed under a microscope after heating at intervals of 10 °C. It was observed that the gametes inside the filamentous cell of both *T. aurea* and *T. umbrina* retain their shape and color up to 70^{o} C. After 70^{o} C the pigments start to smear off inside the filamentous

cell. This may be due to the instability of the carotenoid pigments at elevated temperatures. By further heating *Trentepholiales* to 100° C almost all gametes inside the filamentous cells vanished (Fig. 8a & 8b) although the filamentous cells of both *T. aurea* and *T. umbrina* retained its shape. The size and shape change of the filamentous cell of both *T. aurea* and *T. umbrina* starts after 140° C (Fig. 8c & 8d). The red cells however retained their color, size and shape even after dry heating at 140° C (Fig. 8e). On further dry heating the color and size reduction started occurring only above 200° C. No rupture or breaking of the cell wall took place, but a certain amount of shrinking in the size of the cells could be noticed at 370° C (Fig. 8f). The cell wall could be seen clearly even after heat treatment. However the color of the cells changed more and more brownish and finally blacker if the temperature increased further. It can be argued that the pigment which causes the red coloration in the red rain cell is not a carotenoid.

Ultrastructure of red rain cells and Trentepohlia-spores

The ultrastructural details of reproductive structures, quadriflagellate zoospores, and biflagellate gametes in the *Trentepohliales* have been reported by several phycologists (Christiaan van den 1995; Graham and McBride,1975; Roberts, 1984). Studies of *Trentepohlia*, flagellar apparatus in the *Trentepohliales* show distinct and unique features. Detailed ultrastructural observations were made by Graham and McBride (1975) on both released and unreleased cells of *Trentepohlia aurea*.

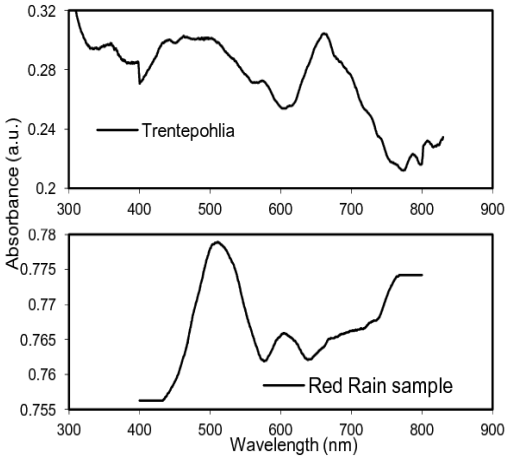

Fig 6 Visible absorption spectrum of red rain water and *Trentepohlia* spores

They reported that the motile cells possess 2 equal flagella which show the typical 9+2 arrangement of axonemal microtubules. They further showed the motile cell development in the green alga *Trentepohlia aurea* to reveal the presence of multilayered structures (MLS) associated with flagellar bases. Again the ultrastructure of ***T. aurea*** showed the relationships between the flagella, basal bodies, multi layered-structure (MLS), mitochondria, nucleus, and other cell components. The TEM images of red rain cells showed fine-structured membranes. Their Cell wall is enormously thick (fig. 9a) and encased inside the thick outer wall there appears to be a detached inner capsule.

When the ultrastructure of red rain cells is compared to the *Trentepholiales*, several striking differences are apparent. No multilayered structure was observed in the red rain cells as in *Trentepohliales*.Flagellar apparatus which is a unique characteristic of all *Trentepohliales* was absent in the red rain cells.

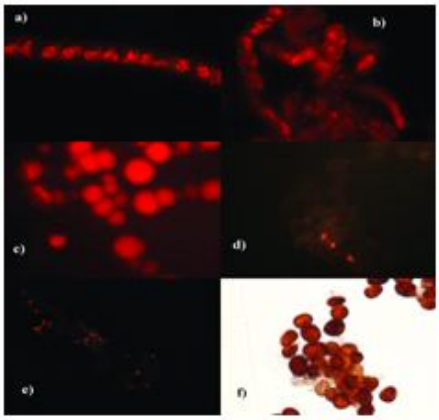

Fig. 7 DNA detection of the cells by fluorescence microscope probe technique after dyed with ethidium bromide a) fluorescence image of the *T. aurea* cells 40x b) fluorescence image of the *T. umbrina* 40x c) fluorescence image of the released motile cells of the *T.aurea* 100x d) fluorescence image of the red rain cells at 100x shows the cells are not fluorescing e) fluorescence image of the bacteria contained along with red rain cells showing bright fluorescence and act as a control experiment, f) bright field image of the red rain cells after dyed with ethidium bromide shows it is absorbing by the cells.

Similarly the other constituents, namely nucleus, mitochondria and chloroplast, seen in the *Trentepohliales* are absent in the red rain cells. Further the TEM images of some red rain cells shows daughter cells inside (Fig. 9b). If the red rain cells are considered to be the spores of a *Trentepohlia*-genus type then the presence of daughter cells inside a motile cell is extraordinary. It is incorrect to say that these *Trentepohlia* motile cells contain daughter cell inside it.

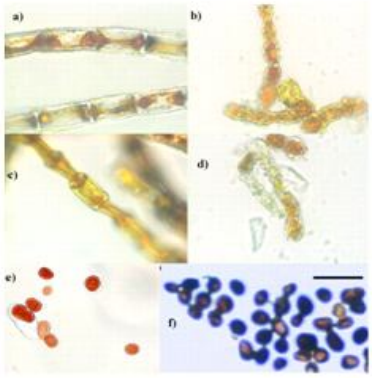

Fig.8 Microscopic images of the cells after dry heatinga) cells of *T.aurea* heated at 100 Deg C, b) cells of *T. umbrina* heated at 100 Deg C, c) filamentous cells of *T.aurea* heated at 140 Deg C, d) filamentous cells of *T. umbrina* heated at 140 Deg C, e) red rain cells heated at 140 Deg C and, f) red rain cells heated at 370 Deg C.

Survivability in water

The red cells contained in the rain water were capable of surviving for long periods without any preservative. The cells are very stable against decay with time. No decay or discoloration of the cells could be found when stored in the original rainwater at room temperature without any preservative for about 11 years. But *Trentepohliales* do not survive in the rain water for more than 1 year. It was reported that (Gupta and Agrawal, 2004) *T. aurea* vegetative cells do not survive submerged conditions for more than 5 months, but can survive air-exposed conditions for more than 1 year. Disintegration and rapid death of algal cells was

observed more markedly when submerged than exposed to air. Under submerged conditions, *T. aurea* did not form any sporangium while prolific formation occurred under air-exposed conditions. Under submerged conditions, algal cells formed few-celled, filamentous, cytoplasmic type setae (Chris and Edward, 1991).

Presence of elements like nitrogen and phosphorous

The reported elemental composition of the red rain cells using EDAX showed absence of nitrogen and the CHN analysis showed a low percentage (1.84%) of nitrogen (Louis and Kumar, 2006). Sampath et al., (2002) reported the percentage of phosphorous in the red rain cells to be 0.08 % using an Ion Coupled Plasma Mass Spectrometer (ICPMS) technique. It was reported (Chris and Edward, 1991) that the minimum percentage of nitrogen in human beings, green plants and bacteria were 5.14, 0.83 and 3.04 respectively. The percentages of phosphorous in these three species were 0.63, 0.71 and 0.60 respectively (Chris and Edward, 1991). The percentage of nitrogen and phosphorous for all living things in the earth may be significantly more than that observed in the red rain cells. This difference is intriguing and extraordinary.

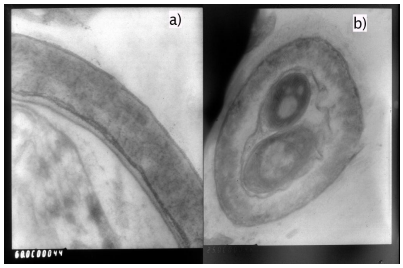

Fig. 9 TEM images of red rain cells a) shows outer wall is very thick b) shows inner daughter cells

CONCLUSION

Our studies of the comparison of *Trentepohlia* with red rain cells showed marked differences in their morphologies, temperature stability, ultra-structure, detection of DNA, survivability in water, and the content of nitrogen & phosphorous. The red rain cells and spores of *Trentepohlia* are morphologically dissimilar. The red rain cells are stable at elevated temperatures at which no *Trentepohliales* can survive. Flagellar apparatus which was a unique characteristic of all *Trentepohliales* was absent in the red cells. Hence from the above results it is erroneous to equate the red rain cells with the *Trentepohlia*-genus spores, and it can be concluded that both red rain cells and *Trentepohlia* shows different lineaments. We have shown that the red cells can grow after incubation for periods of up to two hours at 121^{o}C (Gangappa et al., 2010). These novel microbes can also be grown at temperatures below 100^{o}C which shows their unique biological nature (Louis and Kumar, 2010). Their ability to multiply at extreme high temperature of 300^{o}C, at which no known microorganism on earth could survive, and the unusual autofluorescence of their biomolecules (Kumar and Louis, 2008) are some of their extraordinary defining properties. Conventional biomolecules or organisms are not known to have this type of autofluorescence and hence the presence of novel biomolecules can be inferred in the red rain microbes. Organisms replicating at very high temperature and showing unusual autofluorescence properties are currently unknown to exist on Earth, yet several thousand kilograms of these cells came down through the red rain. The red rain cells cannot therefore be equated with *Trentepohlia* and thus remains unidentified. The answer regarding the origin of the red cells is still puzzling, but the possibility of red cells representing an extraterrestrial life form that originated from a cometary source remains more compelling.

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