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Abstract

The geologic histories of planetary surfaces reveal that Earth and Mars have been pummeled by cataclysmic impact events. The surface of Mars has been perused to have an impact origin for its hemispheric dichotomy. The spallation during impact events causes the interplanetary transfer of material from Mars to Earth or Mars to Phobos/Deimos. Assessing the survival of micro-organisms in impact conditions is critical for the development of planetary protection strategies for future missions. Shock waves are generated during such major impact events. The objective of the present investigation was to study the effect of shock waves on extremophilic bacteria isolated from Martian analogue site Laguna de Peña Hueca, Spain. Peña Hueca is a hypersaline sulphated lagoon rich in Mg-Na-SO4-Cl, epsomite and hexahydrate and it potentially serves as Planetary field analogue site for Martian chloride deposits. The microbial community structure of the lagoon was studied by 16S rRNA metagenomic sequencing. The phylogenetic studies indicated the presence of phyla Euryarchaeota, Proteobacteria, and Bacteroides in the hypersaline brines of the lagoon. The anoxic sediments of Peña Hueca showed the presence of Haloanaerobiaeta and Hadesarchaeota including the anoxic genus of Haloanaerobium, Desulfosalsimonas and Desulfovermiculum. The effect of impact shock on the halophilic bacterium Halomonas gomseomensis EP-3 isolated from Laguna de Peña Hueca was studied in a Reddy shock tube. The halophilic bacterium was exposed to shock waves at a peak shock pressure of 300 kPa and a temperature of 400 K. The results of shock recovery experiments of halophilic bacteria reveal 97 % killing at 300 kPa and Mach number of 1.47 in comparison with Bacillus sp. This study indicates that that gram positive spore-forming Bacillus sp. are better adapted to survival in impact shock waves in comparison to non-sporulating halophiles. In the current study, we present the first report on survival of halophiles in impact shock. Furthermore, we demonstrate a novel application of the simple hand held Reddy shock tube in astrobiology. The survival studies of halophiles isolated from Martian analogue sites in impact shock can provide valuable insights in astrobiology and microbial physiology in impact shock stress.

Keywords	Impact; Mars analogue; hypersaline; habitability; Astrobiology; Reddy shock tube

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Effect of impact shock on extremophilic bacterium *Halomonas gomseoemensis* EP-3 isolated from hypersaline Martian analogue site Laguna de Peña Hueca, Spain

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Highlights

- First report on effect of Impact on halophilic bacteria isolated from Martian analogue site
- Microbial Diversity of hypersaline Martian analogue site Laguna de Peña Hueca reported
- Novel application of Reddy shock tube in astrobiology to study impact events or fate of micro-organisms in impact driven interplanetary transport between planetary systems like Phobos/Deimos

Abstract

The geologic histories of planetary surfaces reveal that Earth and Mars have been pummeled by cataclysmic impact events. The surface of Mars has been perused to have an impact origin for its hemispheric dichotomy. The spallation during impact events causes the interplanetary transfer of material from Mars to Earth or Mars to Phobos/Deimos. Assessing the survival of micro-organisms in impact conditions is critical for the development of planetary protection strategies for future missions. Shock waves are generated during such major impact events. The objective of the present investigation was to study the effect of shock waves on extremophilic bacteria isolated from Martian analogue site Laguna de Peña Hueca, Spain. Peña Hueca is a hypersaline sulphated lagoon rich in Mg-Na-SO_4-Cl, epsomite and hexahydrate and it potentially serves as Planetary field analogue site for Martian chloride deposits. The microbial community structure of the lagoon was studied by 16S rRNA metagenomic sequencing. The phylogenetic studies indicated the presence of phyla *Euryarchaeota*, *Proteobacteri*a, and *Bacteroides* in the hypersaline brines of the lagoon. The anoxic sediments of Peña Hueca showed the presence of *Haloanaerobiaeta* and *Hadesarchaeota* including the anoxic genus of *Haloanaerobium*, *Desulfosalsimonas* and *Desulfovermiculum*. The effect of impact shock on the halophilic bacterium *Halomonas gomseomensis* EP-3 isolated from Laguna de Peña Hueca was studied in a Reddy shock tube. The halophilic bacterium was exposed to shock waves at a peak shock pressure of 300 kPa and a temperature of 400 K. The results of shock recovery experiments of halophilic bacteria reveal 97 % killing at 300 kPa and Mach number of 1.47 in comparison with *Bacillus* sp. This study indicates that that gram positive spore-forming *Bacillus sp.* are better adapted to survival in impact shock waves in comparison to non-sporulating halophiles. In the current study, we present the first report on survival of halophiles in impact

shock. Furthermore, we demonstrate a novel application of the simple hand held Reddy shock tube in astrobiology. The survival studies of halophiles isolated from Martian analogue sites in impact shock can provide valuable insights in astrobiology and microbial physiology in impact shock stress.

Keywords: Impact, shock waves, Mars analogue; hypersaline; microbial community; habitability; Astrobiology; Reddy shock tube

1. INTRODUCTION

The early Earth was subjected to heavy bombardment by comet and asteroid impact events during the Hadean era (Cockell, 2006; Kring and Cohen; 2002). Such impact events may have significantly affected origin of life on Earth (Cockell, 2006). The craters associated with impact events and deep-ocean hydrothermal systems are perused to have been unique sites for prebiotic chemistry. These impact altered lithological sites may have been favorable geomicrobiological environments for development of early life (Cockell et al. 2002). It is thought that the physical and chemical conditions around impact craters on Earth draw significant resemblance to Charles Darwin's "warm little pond" (Darwin, 1871; Cockell 2006). Impact events have been crucial in origin of life on Earth and development of planetary bodies in our solar system.

The surface of Mars has also been affected by impact processes and cratering (Chappaz et al., 2013). Impact events on Mars have been responsible for ejecting meteorites in space by spallation (Melosh, 1984,1988). Almost >37 Martian meteorites including Shergottites, Chassigny and Nakhlite have been ejected from Mars to Earth by hypervelocity impact (Chappaz et al., 2013). Impact spallation is a causal factor for the transfer of material from Mars to Phobos and Deimos (Chappaz et al., 2011).

Impact events are thus significant as they ubiquitously transport material between planetary bodies (Melosh,1985). Scientists often question, if such impact events can transfer life forms between planets? (Melosh, 1988). Addressing these questions are imperative to astrobiological studies of origin of life and planetary protection. Future exploration studies by JAXA like the MMX (Martian Moons Exploration) mission will focus on sample return from Phobos and Deimos. It is thus essential to study and classify the planetary protection strategies (restricted/unrestricted) for samples from Phobos and Deimos. If Mars would have life of any form (extinct/extant), it is possible that it may have been transferred to Martian moons by impact events (Chappaz et al., 2013). It is also possible that the pressure and hypervelocity can completely sterilize any form of life transported during impact. Thus the survival rate of bacteria in hypervelocity impact is crucial to classify the planetary protection guidelines for Phobos and Deimos (National Academies of Sciences, Engineering, and Medicine, 2019).

Most of the impact studies conducted previously focus on the effect of hypervelocity, pressure or temperature on the survival of bacteria (Burchell et al., 2001, 2003, 2004; Horneck et al., 2001, 2008; Fajardo-Cavazos et al., 2009; Hazael et al., 2016). However, there is a critical knowledge gap and research needed in the area of survival of extremophilic micro-organisms in impact shock. One of the key phenomenon observed during planetary impact events is the generation of shock waves (Shimamura et al., 2016). Shock waves are non linear high energy waves that propagate at supersonic speed and cause extreme change in pressure and temperature (Thombre et al., 2019).

Shock waves are observed during meteorite and asteroid impacts, volcanoes, explosions, and earthquakes. Shock waves are dissipative in nature and they increase static pressure and temperature in the medium during propagation (Gnanadhas et al., 2015). Shock waves can be produced in the laboratory by using shock tunnels, shock tubes, pulsed laser beam focusing,

electrohydraulic method, and controlled explosions (Divya et al., 2011).

More research is warranted to fill the gap in understanding the effects of impact shockwaves on survival of microorganisms. We have previously reported the effect of shockwaves on thermotolerant bacteria isolated from meteorite impact crater. (Thombre et al., 2019 Halophiles are considered as candidate 'exo-philes' in astrobiological studies (DasSarma et al., 2006). The candidate organisms chosen for this study were halophiles isolated from Laguna de Peña Hueca, Spain. Laguna de Peña Hueca is a hypersaline ion rich sulphated system in La Mancha, Central Spain. The salt rich deposits of endorreic origin makes this lagoon a terrestrial analogue for Martian chloride deposits and brines (Thombre et al., 2018). The microbial diversity of this Martian analogue site was studied and candidate extremophiles were chosen for impact shock studies.

In the current study, we present the first report on survival of halophiles in impact shock. Furthermore, we demonstrate a novel application of the simple hand held Reddy shock tube in astrobiology. The Reddy shock tube was used for generation of shock waves that impacts the target organism (Reddy and Sharath., 2013). The survival fraction of the organisms after impact shock at a peak shock pressure of 300 kPa, temperature of 400 K and Mach number of 1.47 was studied. The survival studies of halophiles isolated from Martian analogue sites in impact shock can provide valuable insights in astrobiology and microbial physiology in shock stress.

2. MATERIALS AND METHODS

Ethical Approval

This article does not contain any studies conducted with human participants or animals performed by any of the authors.

Sample collection and analysis

A visit to terrestrial analogue site Laguna de Peña Hueca, Tirez lagoon system was conducted between 9 to 13 April 2018 under Europlanet Transnational Access (TA) (17-EPN3-030) for collection of samples. The pink lagoon is located in the province of Toledo, Western La Mancha, Spain at 653 m altitude (39030' 56" N 30 20' 21"; UTM WGS84) with maximum depth of 40 cm (Figure 1). Different samples including water sample (PH-W), pink halite crust (PH-H), rocks submerged in the lagoon (PLR), green photosynthetic matt (PH-G) and black anoxic sediments (PH-AB) were collected in triplicate in pre-sterilized zip lock bags. The samples were processed in the mobile laboratory of INTA-CAB, Spain at site. The samples were analyzed for physicochemical parameters like pH, temperature, sodium, potassium, sulphate, chloride and magnesium content as per standard methods described earlier (Thombre et al., 2016; Greenberg et al.,1992). The chloride content was estimated by argentometric titration and the sodium, potassium and magnesium ion content was estimated using Flame photometric method as described by Greenberg et al. (1992).

Enrichment, Isolation and identification of micro-organisms from Laguna de Peña Hueca

Micro-organisms from Laguna de Peña Hueca were isolated by enrichment culture method (Thombre et al., 2016). Different media and incubation conditions used for cultivation of organisms depending on their nutritional requirement. All bacteriological media were supplemented with 1.5 – 3 M NaCl and 0.1- 0.5 M $MgSO_4$. Briefly, 1 g or 10 ml water sample was enriched in 100 ml medium and incubated at 37 °C at 100 rev min-1 for 24 - 48 h or till growth appeared. The heterotrophic bacteria were enriched in Zobell's marine medium (HiMedia, India)

supplemented with 1.5 M NaCl and 0.5 M $MgSO_4$. A modification of Zobell's medium was devised in which the components of Zobell's marine medium (HiMedia, India) were dissolved in the lagoon water as a base instead of distilled water. Rock sample submerged in the lagoon (PLR) was surface sterilized in sterile brine, pulverized aseptically and enriched in Sehgal and Gibbons (SG) medium (Thombre et al., 2016) containing 1.5 -3 M NaCl and 0.1 - 0.5 M $MgSO_4$. 7 H_2O.

Sulphur reducing bacteria (SRB) from the black anoxic zone were enriched in anaerobic thioglycollate medium (HiMedia, India) containing 1.5 M NaCl and $MgSO_4$. 7 H_2O (Postgate, 1965). All media were incubated at 37 °C until visible growth was observed in the form of turbidity. After appropriate incubation, a loopful of the enrichment broth was isolated on respective agar and observed for growth of colonies. Colonies showing distinctive morphologies were subcultured and purified and used for microscopic, physiological and biochemical identification. Cell morphology was observed by gram staining and phase contrast microscopy at 1000X magnification (Olympus, Germany). The micro-organisms were identified by physiological and biochemical test as per Bergey's Manual of Determinative Bacteriology and 16 s rRNA gene sequencing using Sangers method as described earlier (Thombre et al., 2016; Thombre et al., 2019).

DNA Extraction, amplification of 16S rRNA genes for Metagenomic sequencing

PureLink®Genomic DNA extraction kit (Invitrogen ™, U.S.A) was used for the extraction of total DNA from four samples: water sample (PH-W), halite crust (PH-H), green photosynthetic matt (PH-G) and black anoxic zone (PH-AB). DNA was checked for purity using NanoDrop TM 2000 spectrophotometer (ThermoFisher Scientific, U.S.A) and quantified using Qubit 2.0 Fluorometer (Invitrogen™, U.S.A). The amplification of microbial 16S rRNA gene fragment (V3-V4 region)

was performed with specific primers 314F-5'CCTACGGGAGGCAGCAG3' and 518R-5'ATTACCGCGGCTGCTGG 3' and 10 ng of total DNA was used as a template for PCR reaction. The PCR reaction conditions followed were: 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 72°C for 30 s, extension at 72°C for 5s followed by a final hold at 4°C. The PCR product was separated on 2% agarose gel and visualized using SYBR® Safe DNA stain (10µl/100ml). The DNA amplicon was purified using PureLink® Quick Gel Extraction kit (Invitrogen™, U.S.A).

A second PCR was performed with primers that had Illumina indexed bar code sequences. The PCR master mix used had 2 µL each 10 pmol/µl forward and reverse primers, 1 µl of 40mM dNTP, 10 µL of 5 X Phusion® HF reaction buffers (New England BioLabs Inc., U.S.A), 0.4 µl of 2U/µl F-540 Special Phusion® Hot Start DNA Polymerase (Thermo Fischer Scientific™, U.S.A), 10 µl (minimum 5ng) of amplicon from the previous PCR cycle and water to make up the total volume to 50 µl. The amplicons were further purified using PureLink® PCR spin columns (Invitrogen™, U.S. A) and libraries were prepared. The libraries were validated using Tapestation® system (Agilent Technologies, U.S.A). After validation, the libraries were sequenced by paired- end sequencing (2 × 150 bp paired end run) on Illumina MiSeq platform utilizing a 300 cycle Illumina MiSeq reagent kit v.2. (Caporaso et al., 2010, Thombre et al., 2019). The raw metagenomic datasets from this study was deposited in NCBI's Sequence Read Archive under BioProject 'Metagenome of Laguna de Peña Hueca' and a DDBJ/EMBL/ GenBank Accession number for the SRA submissions was obtained.

Analysis of metagenomes, Annotation of taxonomic units and unculturable micro-organisms

The raw sequencing reads generated by sequencing of 16S rRNA amplicons were trimmed and the adapter sequences were removed using PERL. The sequences were trimmed by quality with Phred score ≥ 30 (>Q30; error-probability >= 0.001). Consensus V3 region sequence were constructed by FLASH program with default parameter settings (Magoc and Salzberg, 2011). The chimeras were removed using de-novo chimera removal method and the UCHIME algorithm was implemented in the USEARCH package (Edgar et al., 2011). Pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using UClust program (similarity cutoff = 0.97). The sequence data was analyzed using QIIME (Quantitative Insights Into Microbial Ecology software package) (Caporaso et al., 2010). A representative sequence was identified for each OTU and aligned against SILVA core set of sequences using PyNAST program (DeSantis et al., 2006; Quast et al., 2013). The taxonomic classification was performed using Ribosomal Database Project (RDP) classifier 2.2 (Wang et al., 2007) against SILVA 16S rRNA genes (Quast et al., 2013) and Greengenes database (DeSantis et al., 2006).

The taxonomic recruitment of phylum, class, order, family, genus and species was performed using OTU. The taxa other than the top ten were categorized as "others" and the ones that did not have any alignment against taxonomic database were categorized as "unknown". SILVA was used for procuring V3 sequences, taxonomic annotations and sample wise OTU. Heat maps and taxonomy abundance plots were created using QIIME. The genera-level taxonomic statistical analysis of metagenomic sequences was performed using, Statistical Analysis of Metagenomic Profiles (STAMP) v 2.0 software package. The corrected *p*-values were estimated on the basis of Fisher's exact test method using Storey's false discovery rate method of multiple test correction within STAMP and the *p*-values with <0.05 were considered significant for comparison (Storey and Tibshirani, 2003).

Statistical analysis

The microbial diversity within the samples was studied by calculating Shannon, Chao1 and observed species metrics. The chao1 (Schao1) metric estimates the species richness, the Shannon metric is the measure to estimate observed OTU abundances and accounts for both richness and evenness indices. The diversity indices in the sample were calculated using QIIME v1.7. The rarefaction curve for each metric was plotted. All the metrics were calculated using QIIME software.

Effect of impact shock on bacteria

The effect of impact shock on the survival of halophilic bacteria isolated from Laguna de Peña Hueca was studied in a Reddy shock tube (Figure 2). The target bacteria were impregnated on stainless steel coupons as described earlier (Thombre et al., 2019). Briefly, 24h old saline suspension of *Halomonas gomseoemensis* (10 µl) was impregnated on sterile stainless steel circular coupons of 5 mm diameter. The coupons were dried overnight in sterile conditions and then placed inside the driven section of the Reddy shock tube. A hand held piston was used to rupture the diaphragm (30 mm inner diameter) that separated the high-pressure (driver section) and low-pressure section of the shock tube. This rupture generated shock waves with peak pressures of 3 bar (300 kPa), temperature of 400 K, and Shock Mach number of ~1.47 for an exposure time of about 250–300 µs. The peak was measured using two piezoelectric sensors (PRESSURE SENSOR ABS AXIAL 6-SIP, MPX5700ASX-ND-NXP Semiconductors, USA, Inc.) of high response value. After impact, the coupons were collected and survival fraction was

estimated by total viable cell count (colony forming unit per ml) (Thombre et al., 2019). All experiments were performed in triplicates and appropriate controls were used.

3. RESULTS

Geochemistry of Terrestrial site

The mineralogical studies and physicochemical analysis indicates that Peña Hueca is a higly sulphated athalassohaline environment at near neutral pH. Laguna de Peña Hueca had a salinity of 12.5 % (water column, PH-W) to saturation (halite crust layer, PH-H). The elemental analysis of the lagoon water was compared to the elemental analysis of some of the representative hypersaline systems globally (Table 1) (Thombre et al., 2016). The water had a sulphate content of 18.75g/L, magnesium content of 9.04 g/L with a pH range of 7.5-7.9. The trend in concentration of elemental ions in the lagoon water was found to be SO_4^->Cl^->Na>Mg>K. The waters of the lagoon are known to be extremely rich in Mg-Na-SO_4-Cl, epsomite and hexahydrate (Montoya et al., 2013). The salt composition of this lagoon system depends on the inflow water arising from Tertiary age calcium sulphate marls and Triassic evaporites (Montoya et al., 2013).

Isolation and identification of organisms from *Laguna de Peña Hueca*

Halophilic organisms were isolated from the Spanish lagoon Peña Hueca. Only moderate and extreme halophiles were isolated and shortlisted for impact studies. The halophilic strains selected for the study were designated as EP-1, EP-2, EP-3, EP-4 (EP- Europlanet). The halophiles were identified as *Halomonas sp. , Halomonas lutea* and *Marinobacter persicus* and they were isolated from hypersaline lagoon water (Supplementary Table 1). The microscopic analysis of the water

samples of Peña Hueca revealed the presence of motile single celled red colored algae that was identified as *Dunaliella salina* strain EP-1 (NCBI GenBank accession no: MH553054) using morphological characteristics and 18 S rRNA gene sequencing (Supplementary Fig.S1).

Halomonas gomseomensis was isolated from saline lagoon water (strain EP-3) and a rock submerged in the saline lagoon (strain PLR). The halophilic organism was identified on the basis of biochemical, physiological and morphological and phylogenetic analysis and used for impact shock studies. *H. gomseomensis* EP-3 is an aerobic, chemoorganotrophic, gram negative, moderately halophilic bacterium that belongs to the family *Halomonadaceae* of the class Gammaproteobacteria. The isolate was Voges–Proskauer test negative, indole negative, nitrate reductase negative and could produce acid from D-arabinose, glucose, galactose, fructose, maltose and sucrose.The biochemical and physiological characteristics of isolate EP3 were comparable to characteristics reported for the type strain *Halomonas gomseomensis* M12T isolated from saline water of Gomseom solar saltern in Anmyeondo, Korea. EP-3 produces creamish colonies on SG agar with a temperature optimum from 37-40°C. This isolate grows in medium containing 10- 20 % NaCl. The optimum pH of the isolate was from 6 to 9.

Anoxic SRB were also cultured in the laboratory using anaerobic medium containing thioglycollate. The anaerobic medium showed presence of typical blackening in liquid enrichment cultures, however the growth could not be further isolated on solid medium. This may be owing to the complex requirement of salts and ions by Peña Hueca isolates. Hence, metagenomic studies were further undertaken for studying the microbial community structure of this extreme lagoon especially in the anoxic zones.

Metagenomic insights in Microbial community structure of Laguna de Peña Hueca

The taxonomic complexity of microbial communities in Laguna de Peña Hueca was observed by subjecting the 16 S rRNA gene sequences to taxonomic classification using RDP classifier against SILVA 16S RNA genes database. The 16S rRNA gene sequences of Peña Hueca metagenome that could not be affiliated to any known bacterial class by the RDP classifier and were classified as 'unknown'. The analysis was performed using the pre-processed consensus V3-V4 sequences. The pre-processed reads from all samples were pooled and clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity using Uclust program (similarity cutoff = 0.97). A total of 1559135 OTUs were identified from 2933230 reads. From 1559135 total OTUs, 1522067 Filtred OTUs (OTUs with reads with >or =5) were removed and 37068 OTUs were selected for further analysis. The results of phylogenetic analysis of the16S rRNA gene sequences from water sample (PH-W), pink salt halite crust (PH-H), green photosynthetic matt (PH-G) and black anoxic zone (PH-AB) of Peña Hueca are depicted using the abundance heatmaps from phylum and genus level (Fig. 2 a, b).

The heatmap depicts the most abundant taxon in Peña Hueca differs in each sample. The black anoxic zone has abundance of *Proteobacteria*, *Euryarchaeota, Haloanaerobiaeota, Hadesarchaeaota, Firmicutes and Bacteroides*. The phylum *Nanoarchaeota* and *Euryarchaeota* were the most abundant in water sample (PH-W). The pink halite crust had an abundance of *Firmicutes, Bacteroides*, *Actinobacteria* and *Proteobacteria* while *Cyanobacteria*, *Bacteroidetes* and *Haloanaerobiaeota* were recruited in green matt. (Fig. 2a). The lagoon demonstrated an abundant presence of different extreme, halophilic, anaerobic and facultative organisms belonging to the genera *Halothiobacillus*, *Natronomonas*, *Halorientalis*, *Marivirga*, *Haloanaerobium, Halorubrum* and *Halomonas* sp (Fig. 2b). Halophilic archaea are known to thrive in Cl^- rich environment however it is still unclear how the SO_4^{-2} ions may contribute in the physiology and

adaptation of these haloarchaea in the presence of Cl^- ions. Many anaerobic SRB, like *Haloanaerobium*, *Desulfosalsimonas* and *Desulfovermiculum* were also observed in the PH-AB zone (Fig. 3).

The species diversity of Peña Hueca were further studied using Shannon's diversity index and the Chao1 index was used to estimate total species richness in the microbial community**.** The alpha-diversity rarefaction plot was constructed based on Shannon index calculation depicted that the microbial community of pink halite crust had more species diversity as compared to other samples. The Principal coordinate analyses (PCA) was performed and Bray-Curtis distances were calculated using OTU counts. The weighted UniFrac distances considered the presence/absence of OTUs, the relative abundance of OTUs and was used to identify the significant taxon features among all four sample groups. Hierarchical clustering based on Ward method was performed to further study the correlation of microbial species within samples (Fig.4a).

The dendrogram shows the correlation of samples obtained by hierarchical cluster analysis based on Ward method. The plot shows Pena Hueca water (PH-W) and green matt (PH-G) taxon reads are closely related to each other and in turn with taxon from Anoxic zone (PH-AB). The taxon reads of pink crust halite (PH-H) sample is distantly clustered as compared with the other three samples (Fig. 4a).

The Principal Component Analysis (PCA) of reads from the four samples of Laguna de Peña Hueca was performed using MetageneAssist server with default parameters. The result shows that PH-G shares common taxon with PH-W; next to PH-AB and PH-H sample taxons are uniquely distributed among other samples (Figure 4c).

An explicit comparison of microbial communities between the Laguna de Peña Hueca samples was performed. At first, the distance matrix using both weighted and unweighted UniFrac

approach was generated. Sequence abundances were taken in to account in Weighted UniFrac for comparing microbial diversity. A jackknife test was performed to construct a consensus UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree for all samples in this set. The resulted consensus was taken for UPGMA trees built using weighted and unweighted UniFrac distance matrix as shown in Fig 4.

Overall, the metagenomic studies of Laguna de Peña Hueca revealed that the microbial community structure was dominated by phyla *Euryarchaeota* (90 % in water – 50 % in Anoxic zone) followed by *Proteobacteria* (50 % in pink salt crust – 10 % in green matt), while *Bacteroidetes* dominated the pink salt zone (38%). *Cyanobacteria* were prevalent in green photosynthetic matt while *Hadesarchaeota, Haloanaerobium*, *Desulfosalsimonas* and *Desulfovermiculum* was found only in the black anoxic zone.

Effect of impact shock on bacteria

The effect of impact shock waves on *Halomonas gomseomensis* EP-3 was studied in a Reddy shock tube (Figure 6). The Reddy shock tube is a hand driven shock tube that contains a driver and driven section separated by a diaphragm. The rupture of diaphragm at higher pressure produces shock waves that impacts the target placed in the driven section. The peak pressure and shock speed was measured using piezoelectric sensors and peak pressure was calculated using the voltage output of an oscilloscope. A peak pressure of 300 kPa (3 bar) was impacted on *Halomonas gomseomensis* that lasted for ~250 microseconds. The Mach number was calculated as 1.47. Shock wave velocity was calculated using Time of Flight method (v =x/t) using the trigger of 2 sensors placed 15 cm apart as 500 m/s. The Rankine-Hugoniot jump equations for Normal Shocks were used for the calculation of peak temperature using experimentally calculated Mach number value

(Equations 1 and 2). The Temperature jump in terms of Mach number due to primary shock wave was calculated using equation 1:

$$\frac{T_2}{T_1} = \frac{\left(2\gamma_1 M_s^2 - \left(\frac{\gamma_1 - 1}{2}\right)\right) \times \left(M_s^2\left(\frac{\gamma_1 - 1}{2}\right) + 1\right)}{M_s^2\left(\frac{\gamma_1 - 1}{2}\right)^2} \quad (1)$$

The Temperature jump in terms of Mach number due to reflected shock wave was calculated using equation 2:

$$\frac{T_5}{T_1} = \frac{[2\gamma_1 M_s^2(\gamma_1 - 1) + (3 - \gamma_1)] \times [M_s^2(3\gamma_1 - 1) - 2(\gamma_1 - 1)]}{[M_s^2(\gamma_1 + 1)^2]} \quad (2)$$

Where $T_5 \rightarrow$ *Temperature jump of the system due to reflected shock wave.*

$T_3 \rightarrow$ *Temperature jump of the system due to primary incident shockwave.*

$\gamma \rightarrow$ *The ratio of specific heats* (C_p *and* C_v) *for a particular gas.* $\gamma = 1.4$ *for air.*

$M \rightarrow$ *Mach number of the incident shockwave.* $M = \frac{Velocity of the Shockwave}{Velocity of the sound in the medium}$

The results in the current experiment indicate that 97 % killing of *H. gomseomensis* EP-3 was obtained by shock waves that were 1.47 times faster than the local speed of sound in air (Mach number 1.47) with a velocity of 500 m/s. We compared the survival of *H. gomseomensis* EP-3 after impact shock at 300 kPa to the survival of *Bacillus thermocopriae* IR-1 and other control organisms (Table 2) that had been impacted at 400 Kelvin for 250 microseconds previously (Thombre et al. 2019*). A summary and comparison of parameters used in shock tube and the

results are depicted in Table 2.

4. DISCUSSION

The Earth has been affected by a cataclysmic proportion of impactors during its evolution. Life on Earth originated around 3.5 to 3.8 Gya. This period coincides with the bombardment period known as the late heavy bombardment (LHB) on Earth (Gomes et al., 2005). Such impact events are known to be the only extraterrestrial mechanisms of generation of shock waves. Many studies also report that these impact events may have contributed to organic molecules and prebiotic inventories involved in origin of life on Earth (Chyba and Sagan, 1992). Thus it is imperative to study the role of shockwaves produced during impact on cellular life. The surface of Mars has also been subjected to impact events. Hence there has been considerable speculation on what is the probable effect of impact events on life (extinct/extant) on Mars. Similar studies need to be conducted on what would be the effect of impact events on transfer of material to Phobos/Deimos. The recent discovery of liquid water on Mars in the Planum Australe region using MARSIS (Mars Advanced Radar for Subsurface and Ionosphere Sounding), a low-frequency radar on the Mars Express spacecraft has significant implications in future human explorations and Martian Astrobiology (Orosei et al., 2018). The radar profiles indicate the presence of subglacial stable liquid salt water body that resists freezing due to high concentration of salts (Orosei et al., 2018; Diez, 2018). The presence of liquid water, organics and complex Noachian fluviolacustrine environments near the Knobel crater rich in chloride, basalt, iron and salt deposits makes these sites as ideal potential plausible habitable zones for microbial life (Orosei et al., 2018; Diez, 2018; Huang et al., 2018; Witze at al., 2018; McKay, 2010). The presence of evaporite deposits, phycosilicates and hypersaline environments reported to have been formed around 3.7 Ga are

potentially similar to the terrestrial analogue salt deposits and hypersaline environments that harbor extreme microbial life on earth (Huang et al., 2018; Witze at al., 2018). As liquid water on Mars seems to be in the form of concentrated salt brines, it is perused that if any potential Martian microbial life may ever exist, it may be similar to terrestrial halophilic extremophiles (McKay, 2010; Landis, 2001).

Hypersaline environments on earth rich in Cl- and SO4 –ions are considered terrestrial analogue sites or Planetary Field analogues (PFA) of Martian deposits (Pontefract et al., 2017). Previous studies and observations of Mars indicate that the red planet may have had immense water flows and saline fluids in the past (Tosca et al., 2008). The Hesperian (3.7 Ga) times of Ancient Mars were perused to have ion rich ephemeral lakes that resulted in the presence of chloride and sulphate deposits in present day Martian surfaces and sediments (Tosca et al., 2008; Goudge et al., 2016; Gendrin et al., 2005). In fact, certain reports state that the high amount of magnesium sulphate in Columbia crater was due to the plausible presence of a hypersaline paleolake on Ancient Mars (Gendrin et al., 2005; Wray et al., 2011). Overall, magnesium, sulphate and chloride deposits are dominant (10-30 % in some sediments) on majority of the Martian surfaces. Most hypersaline environments on earth like saltpans, salt lakes, crystallizer ponds are rich in chloride ions (Thombre et al., 2016) but have less abundance of magnesium and sulphate salts. Hence, the potential terrestrial analogue hypersaline sites for investigation of survival of life in extremes should contain high amount of sulphates and ions in addition to chlorides (Pontefract et al., 2017). In the present investigation, we attempted to study a Laguna de Peña Hueca as a Planetary field analogue (PFA) of Mars. We attempted to isolate representative halophiles for impact experiments and investigate the microbial community structure in the extreme environment.

Laguna de Peña Hueca, is an interesting PFA site for Mars and Europa under Europlanet TA activities. Peña Hueca is a part of the Tirez lagoon system and is a small hypersaline sulphated lagoon in Villacañas**,** Toledo, Spain (Fig. 1). It is one of the several endorrheic lagoons that constitutes the lacustrine Tirez lagoon system. Infact, Tirez lake was is proposed as a terrestrial analogue of Europa's ocean owing to its similarities and hydro geochemical properties as based on the analysis data generated by Galileo's Near Infrared Mapping spectrophotometer (Montoya et al., 2013, Prieto-Ballesteros et al., 2003). The inflow water from Triassic evaporates, dolomites and calcium-sulfate deposits from Tertiary age contribute to the saline and sulphate composition of these lagoons (Montoya et al., 2013, Prieto-Ballesteros et al., 2003). The physicochemical properties of the lagoon water with circumnetral pH suggest that sulphate and chloride ions have a major role in shaping the microbial community structure of Peña Hueca. The lagoon is characterized by its surreal bright pinkish colored water with pink colored salt crust. The pink color of the lagoon is attributed to the presence of halophilic photosynthetic microalgae *D. salina* EP-1. The shallow stagnant environment of the lagoon with high salinity and sulphur content metabolically favors the combined biogeochemical activities of Sulphur reducing bacteria, purple or green Sulphur bacteria and halophilic bacteria. A distinct gradation of microbial communities was observed and a black anoxic layer indicates the biological production of hydrogen sulphide that causes precipitation in sediment.

Metageneomic studies revealed the presence of anoxic bacteria belonging to the the genus *Haloanaerobium*, *Desulfosalsimonas* and *Desulfovermiculum* in the black anoxic (PH-AB) zone (Figure 3). The sulphides produced by these SRB are oxidized by cyanobacteria that form a typical green biofilm like mat and representing photosynthetic communities. Peña Hueca lagoon forms a typical habitat called "Sulfuretum" (Plural: sulfureta) that is sulphur rich due to biological

activities and geological deposits. The extremely high concentration of sulphates in this lagoon makes it an interesting site to understand how sulphate can affect the microbial community structure (Montoya et al., 2013). Survival in high concentration of sulphate, ions and chlorides impose severe osmotic stress on microbial life. The hypersaline brines with magnesium and sulphate salts have low water activity and are highly chaotropic and membrane destabilizing (Pontefract et al., 2017).

It was interesting that despite the harsh ionic stresses, high osmotic pressure, potentially low water activity with high amount of kosmotropic sulphates, microbial life thrived in the pink salt crust and saline waters of Pena Hueca. As expected archaeabacteria, especially haloarchaea belonging to the genus *Halonotius, Halorubrum, Halobacterium, Halothiobacillus, Haloorientalis, Haloanaerobium* and *Natronomonas* were found in abundance in Peña Hueca lagoon (Figure 2). Though most were halophilic archaea, they demonstrate different modes of metabolism and nutritional diversity. *Halothiobacillus* is an aerobic chemolithoautotrophic, sulfur-oxidizing bacterium, *Haloanaerobium* is an anaerobic chemoorganotrophic, *Natronomonas* is haloalkaliphilic while other haloarchaea were chemoheterotrophs. The chemolithotroph, *Halothiobacillus* is Sulphur oxidizing bacteria (SOB) classified under gamma Proteobacteria. It has the 'tetrathionate pathway' of thiosulfate oxidation to sulfate. Amongst all the chemolithotrophic bacteria, it is perused that SOB can adapt better extreme ionic stresses due to a very high energy yield available during complete oxidation of sulfide/thiosulfate to sulfate. Sulphate reducing bacteria and archaea (SRB, SRA) both survive by utilization of SO_4^{-2} as terminal electron acceptor. Their respiration is anaerobic and they can also reduce other sulphate rich compound like sulfite, thiosulfate, tetrathionate, trithionate, polysulfide and elemental sulfur by a process termed dissimilatory sulfate reduction using sulfate adenylyltransferase and ATP

sulfurylase (Postgate, 1965). The occurrence of these SRB's especially archaeal phylotypes in sulphate rich environment was an interesting finding. The presence of SOB and SRB in close vicinity indicates the interdependence of the communities on each other for the metabolism and inter conversions of 'S' in a sulfureta.

Despite the strong ion rich environment, halophiles were dominant in Peña Hueca microbial community structure. These halophiles seem to have adapted to the extreme sulphate and chloride environments by evolving various mechanisms to combat the ionic environmental perturbations. Halophiles adopt two major strategies for survival in these stresses, the 'organic osmolytes strategy' and the 'salt-in strategy'. Besides, they also produce protective carotenoid pigments, molecular chaperones and utilize proton electrochemical gradient in juxtaposition with Na^+/H^+ antiporters to maintain ionic balance (Thombre et al., 2016). However, future studies are warranted to understand the role of SO_4^- ions in the physiology of these halophiles.

The halophilic bacterium *Halomonas gomseomensis* EP-3 was isolated from hypersaline lagoon water. *H. gomseomensis* EP-3 could grow over a wide range pf pH (6-10), temperature (25-40) and NaCl concentration (10-20 % NaCl). Due to its unique ability of physiological adaptation over wide range of pH, temperature and NaCl concentration, it was selected for further impact studies. The bacterium was exposed to impact shock waves using a Reddy shock tube (Fig. 5). The shock recovery experiments revealed that the halophiles demonstrated only 3 % survival (97 % killing) at a peak shock pressure of 300 kPa and a peak shock temperature of about 400 K. In our previous study, we had exposed spores of *Bacillus thermocopriae* IR-1 to similar conditions and they demonstrated ~ 28 % survival while vegetative cells of psychrophile *Kocuria rosea* PRL-1 and *Staphylococcus aureus* could not survive impact shock (Figure 5 c). The results are interesting as our experiments have exposed impact shock only upto 300 kPa which result in substantial killing

of bacterial cells. The results shed new light on the need to study the survival mechanism of the surviving fraction of *H. gomseomensis* EP-3 after impact shock. Currently, the mechanism of survival or inhibition of bacteria in impact shock has not yet been examined in detail. Future studies are warranted to investigate the exact mechanism of survival of organisms in impact shock.

5. Conclusion

The present investigation reports the effect of impact shock on halophilic bacteria using Reddy shock tube. Previous experiments have been reported the effect of hypervelocity impact on bacteria at higher pressures (2- 32 GPa) using different impact systems (Horneck et al., 2001). Impact experiments at such high pressures (upto 32 GPa) have been conducted using Ames Vertical Gun Range (AVGR) (Fajardo-Cavazos et al., 2009), 2 stage light gas gun (Burchell et al., 2001, 2003, 2004) and high explosive and air gun plane wave impact techniques Horneck *et al.,* 2008). All these methods have reported the effect of impact on bacteria like *Bacillus* sp. (Burchell et al.,2004; Horneck et al., 2008; Fajardo-Cavazos et al., 2009) and other organisms like yeast and *Rhodococcus erythropolis* DSM 13002 (Burchell et al., 2001). However, there are no reports on effect of impact shock on halophilic bacteria. The major strength of our novel methodology to study impact using Reddy shock tube is that it is simple, cost effective and does not involve the use of hazardous and costly explosives, pistols or guns. This study also emphasizes the application of Reddy shock tube in astrobiology to study impact events or fate of micro-organisms in impact driven interplanetary transport between planetary systems like Phobos/Deimos.

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CONFLICT OF INTEREST STATEMENT

The authors declare absence of any commercial or financial relationships that was conducted during the research which could be construed as a potential conflict of interest.

References

1. Burchell, M.J. and Whitehorn, L. (2003) Oblique incidence hypervelocity impacts on rock. *Mon. Not. R. Astron. Soc.* 341:192–198 doi: 10.1046/j.1365-8711.2003.06385.
2. Burchell, M.J., Mann, J., Bunch, A.W., and Brandao, P.F.B. (2001) Survivability of bacteria in hypervelocity impact. *Icarus* 154: 545–547 doi: 10.1006/icar.2001.6738.
3. Burchell, M.J., Mann, J.R., and Bunch, A.W. (2004) Survival of bacteria and spores under extreme shock pressures. *Mon. Not. R. Astron. Soc.* 352:1273–1278 doi: 10.1111/j.1365-2966.2004.08015.
4. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; Huttley, G.A.; Kelley, S.T.; Knights, D.; Koenig, J.E.; Lev, R.E.; Lozupone, C.A.; McDonald, D.; Muegge, B.D.; Pirrung, M.; Reeder, J.; Sevinsky, J.R.; Turnbaugh, P.J.; Walters, W.A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* , *7*, 335–336. doi:10.1038/nmeth.f.303.
5. Chappaz L. Melosh H.J. Vaquero M. Howell K.C. Supplement 2. NASA Headquarters; Washington, DC: 2011. Transfer of material from the surface of Mars to Phobos and Deimos. Final Report: NNX10AU88G.

6. Chappaz, L., Melosh, H. J., Vaquero, M., & Howell, K. C. (2013). Transfer of impact ejecta material from the surface of Mars to Phobos and Deimos. *Astrobiology*, 13(10), 963–980. doi:10.1089/ast.2012.0942
7. Cockell C. S. (2006). The origin and emergence of life under impact bombardment. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 361(1474), 1845–1856. doi:10.1098/rstb.2006.1908
8. Darwin, C. 1871 Letter to Hooker, J. D.
9. Dassarma, Shiladitya. (2006). Extreme Halophiles Are Models for Astrobiology. *Microbe.* doi: 10.1128/microbe.1.120.1
10. DeSantis, T. Z.; Hugenholtz, P.; Keller, K.; Brodie, E.L.; Larsen, N.; Piceno, Y.M.; Phan, R.; Andersen, G.L. (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res.*,*34*, 394-9 doi: 10.1093/nar/gkl244
11. Diez, A. (2018) Liquid water on Mars. *Science*, 361, 448-449 doi: 10.1126/science.aau1829
12. Divya P, G., Anish, R. V., Jagadeesh, G., and Chakravortty, D. (2011) Bacterial transformation using micro-shock waves. *Anal Biochem* 419:292–301 doi: 10.1016/j.ab.2011.08.038
13. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, *27 (16)*, 2194-2200 doi: 10.1093/bioinformatics/btr381
14. Fajardo-Cavazos, P., Langenhorst, F., Melosh, H.J., and Nicholson, W.L. (2009) Bacterial Spores in Granite Survive Hypervelocity Launch by Spallation: Implications for Lithopanspermia. *Astrobiology* 9: 647–657 doi: 10.1089/ast.2008.0326.

15. Gendrin, A.; Mangold, N.; Bibring, J.P.; Langevin, Y.; Gondet, B.; Poulet, F.; Bonello, G.; Quantin, C.; Mustard, J.; Arvidson, R.; LeMouélic, S. (2005) Sulfates in Martian layered terrains: the OMEGA/Mars Express view. *Science,* Mar;307(5715) 1587-1591. doi:10.1126/science.1109087
16. Gnanadhas, D. P., Elango, M., Thomas, M. B., Jagadish, G., and Chakravortty, D. (2015) Remotely triggered micro-shock wave responsive drug delivery system for resolving diabetic wound infection and controlling blood sugar levels. *RSC Advances* 5(17):13234–13238 doi: 10.1039/C4RA15270K
17. Gomes, R., Levison, H. F., Tsiganis, K. & Morbidelli, A. (2005) *Origin of the cataclysmic Late Heavy Bombardment period of the terrestrial planets*. *Nature* 435, 466–469 doi: 10.1038/nature03676.
18. Goudge, T. A.; Fassett, C. I.; Head,J. W.; Mustard,J. F.; Aureli, K. L. (2016) Insights into surface runoff on early Mars from paleolake basin morphology and stratigraphy. *Geol. Soc. Am.* , *44*, 419−422 doi: 10.1130/G37734.1
19. Greenberg, A.; Clesceri, L.; Eaton, A. (1992) Standard Methods for the Examination of Water and Wastewater (18th ed.) American Public Health Association, Washington, 4–72.
20. Hazael, R., Meersman, F., Ono, F., & McMillan, P. F. (2016). Pressure as a Limiting Factor for Life. *Life (Basel, Switzerland)*, *6*(3), 34. doi:10.3390/life6030034
21. Horneck, G., Stöffler, D., Eschweiler, U., and Hornemann, U. (2001) Bacterial spores survive simulated meteorite impact. *Icarus* 149: 285–293 doi: 10.1006/icar.2000.6543
22. Horneck, G., Stoffler, D., Ott, S., Hornermann, U., Cockell, C.S., Moeller, R., Meyer, C., De Vera, J-P., Fritz, J., Schade, S., and Artemieva, N.A. (2008) Microbial rock inhabitants

survive hypervelocity impacts on Mars-like host planets: first phase of lithopanspermia experimentally tested. *Astrobiology* 8(1):17-44 doi: 10.1089/ast.2007.0134

23. Huang, J (黄俊).; Salvatore,M.; Edwards,C.; Harris, R.; Christensen,P. (2018) A Complex Fluviolacustrine Environment on Early Mars and Its Astrobiological Potentials. *Astrobiology*, 8. doi:10.1089/ast.2017.1757
24. Implications for exploring habitability of Mars and Europa. European Planetary Science Congress, EPSC2018 Berlin, Germany. Vol. 12. 2018.1180.
25. Kring, D. A., and Cohen, B. A., (2002) Cataclysmic bombardment throughout the inner solar system 3.9–4.0 Ga, *J. Geophys. Res.*, 107(E2), doi:10.1029/2001JE001529
26. Landis, G. A. (2001) Martian water: are there extant halobacteria on Mars? *Astrobiology*, *1(2)*, 161-164 doi: 10.1089/153110701753198927
27. Magoc, T.; and Salzberg, S. (2011) FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, *27(21)*, 2957-63 doi: 10.1093/bioinformatics/btr507
28. McKay, C.P. (2010) An Origin of Life on Mars. *Cold Spring Harbor Perspectives in Biology*, 2(4): a003509. doi:10.1101/cshperspect.a003509.
29. Melosh H.J. (1985) Ejection of rock fragments from planetary bodies. *Geology*. 1985;13:144–148 doi: 10.1130/0091-7613(1985)13<144:EORFFP>2.0.CO;2
30. Melosh HJ. (1988) The rocky road to panspermia. *Nature*. Apr; 332(6166) 687-688. doi:10.1038/332687a0.

31. Montoya, L.; Vizioli, C.; Rodríguez, N.; Rastoll, M.J.; Amils, R; Marin, I. (2013) Microbial community composition of Tirez lagoon (Spain), a highly sulfated athalassohaline environment. *Aquat Biosyst.* 2013; 9(1) 19. doi:10.1186/2046-9063-9-19

32. National Academies of Sciences, Engineering, and Medicine. 2019. PlanetaryProtection Classification of Sample Return Missions from the Martian Moons.Washington, DC: The National Academies Press. https://doi.org/10.17226/25357.

33. Orosei, R.; Lauro , S.E.; Pettinelli,E., Cicchetti, A.; Coradini,M.; Cosciotti,B.; Di Paolo F.; Flamini,E.; Mattei, E,; Pajola,M.; Soldovieri,F.; Cartacci,M.; Cassenti, F.; Frigeri, A.; Giuppi, S.;Martufi,R., Masdea,A.; Mitri,G.; Nenna,C.; Noschese,R.; Restano, M.; (2018) Seu. Radar evidence of subglacial liquid water on Mars. *Science*, doi: 10.1126/science.aar7268.

34. Pontefract, A.; Zhu, T.F.; Walker, V.K.; et al. (2017) Microbial Diversity in a Hypersaline Sulfate Lake: A Terrestrial Analog of Ancient Mars. *Frontiers in Microbiology*, *8*, 1819. doi:10.3389/fmicb.2017.01819.

35. Pontefract, A.; Zhu, T.F.; Walker, V.K.; et al. (2017) Microbial Diversity in a Hypersaline Sulfate Lake: A Terrestrial Analog of Ancient Mars. *Frontiers in Microbiology*, *8*, 1819. doi:10.3389/fmicb.2017.01819.

36. Postgate, J. R. (1965) Recent Advances in the Study of the Sulfate-Reducing Bacteria. *Bacteriological Reviews* , *29.4*, 425–441.

37. Prieto-Ballesteros, O.; Rodríguez, N.; Kargel, J.S.; González-Kessler, C.; Amils, R.; Fernández-Remolar, D. (2003) Tirez Lake as a Terrestrial Analog of Europa. *Astrobiology*, *3*, 863–877. doi:10.1089/153110703322736141

38. Quast, C.; Pruesse, E.; Yimaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Opens external link in new window. *Nucleic acids research*, 41(Database issue), D590–D596. doi:10.1093/nar/gks1219

39. Reddy, K.P.J., and Sharath, N. (2013) Manually operated piston-driven shock tube. *Current Science* 104(2):172-176. doi: 10.1007/978-3-642-25688-2_86

40. Shimamura, K., Shimojo, F., Nakano, A., and Tanaka, S (2016) Meteorite Impact-Induced Rapid NH_3 Production on Early Earth: *Ab Initio* Molecular Dynamics Simulation. *Sci. Rep* 6:38953. doi: 10.1038/srep38953

41. Storey, J.D.; Tibshirani, R. (2003) Statistical significance for Genome wide studies. *Proceedings of the National Academy of Sciences of the United States of America*, 100(16), 9440–9445. doi:10.1073/pnas.1530509100

42. Thombre R, Kulkarni P, Gomez F, Sivaram B. Extremophiles from Tirez and Peña Hueca: Implications for exploring habitability of Mars and Europa. European Planetary Science Congress,EPSC2018 Berlin, Germany. Vol. 12. 2018.1180

43. Thombre R, Shivakarthik E, Sivaraman B, Vaishampayan P, Seuylemezian A, Meka JK, Vijayan,S, Kulkarni P, Pataskar T, Patil B (2019) Survival of extremotolerant bacteria from the Mukundpurameteorite impact crater. *Astrobiology*. doi: 10.1089/ast.2018.1928.

44. Thombre, R.; Shinde, V.; Oke, R.; Dhar, S.; Shouche, Y. (2016) Biology and survival of extremely halophilic archaeon *Haloarcula marismortui* RR12 isolated from Mumbai salterns, India in response to salinity stress. *Sci Rep*, *6*, 25642. doi: 10.1038/srep25642, 2016.

45. Tosca, N. J.; Knoll, A.H.; McLennan, S.M. (2008) Water activity and the challenge for life on early Mars. *Science*, 320 (5880), 1204-7. doi: 10.1126/science.1155432

46. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*, 73(16), 5261-67. doi: 10.1128/AEM.00062-07

47. Witze, A. (2018) There's water on Mars! Signs of buried lake tantalize scientists. *Nature*, *560* (7716), 13-14.doi: 10.1038/d41586-018-05795-6.

FIGURE LEGENDS

FIGURE 1 Pink colored hypersaline Laguna de Peña Hueca. b. Location of site in Spain.

FIGURE 2 Heatmap showing the Microbial diversity and relative abundance of bacterial taxonomies distributed among Laguna de Peña Hueca based on PCR amplifications and high-throughput Illumina sequencing of 16 S rRNA gene sequences. a) Comparison of reads between four samples PH-AB, G, H and W at genus level. The heatmap illustrates top 20 abundance hits, b) The plot shows the genus differences between four samples. Only well annotated known taxonomy hits from Laguna de Peña Hueca were considered for abundance comparison. The colors depict the extent of abundance in the sample as indicated in the legend. In gradient scale, the less abundant reads for particular taxon in a given metagenome are represented in green, the most abundant taxa are shown in red and the null read counts are represented in black color.

FIGURE 3 Phylogenetic tree based on 16 S rRNA gene sequences from Anoxic black zone (PH-AB) of Laguna de Peña Hueca. The scale bar represents expected changes per site.

FIGURE 4 a. Dendrogram tree showing the sample clustering generated based on Ward Method; b: Pearson correlation coefficient profile at phylum level; c: Principal component analysis of all the four samples of Laguna de Peña Hueca

FIGURE 5 a. Schematic representation of Reddy shock tube, b Reddy shock tube used for study of impact shock, c: Graphical representation of Percentage killing of bacteria due to Effect of shock waves (300 kPa, 400 K, Mach Number: 1.47), d. Calculation of Peak pressure using fast-acting piezoelectric sensors.

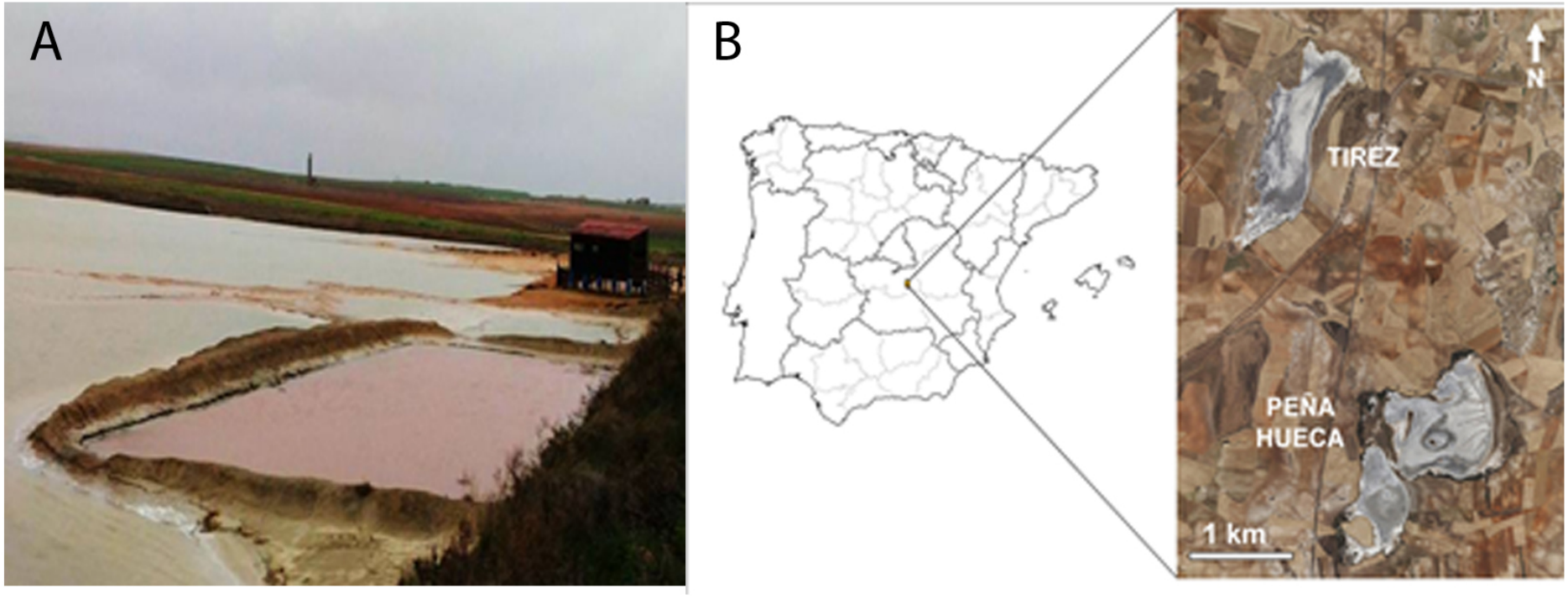
TABLES

Table 1. Elemental analysis of Laguna de Peña Hueca with representative hypersaline sites (Thombre et al., 2016). Values are expressed as g L^{-1}, (nd, not determined).

No	**Hypersaline environment**	**Na^+**	**K^+**	**SO_4^-**	**Cl^-**	**Ca^{+2}**	**Mg^{+2}**
1	Laguna de Peña Hueca, Spain	43.0	0.195	18.75	116.89	nd	9.04
2	Bhandup, Mumbai saltern, India	94.05	nd	nd	211.28	nd	8.26
3	Mumbai saltern, India	46.55	0.75	nd	262.3	76.0	24.3
4	Great Rann of Kutch, India	46.0	2.0	nd	157.0	Trace	16.1
5	Great Salt Lake, USA	105.5	6.7	nd	181.0	0.3	11.1
6	Dead Sea, Israel	40.1	7.6	nd	225.0	nd	nd
7	Wadi Natrun, Egypt	142.0	2.3	nd	155.0	nd	nd

Table 2: The various parameters used to study effect of impact shock on *H. gomseomensis* in Reddy shock tube

No.	Parameters	Reddy shock tube
1.	Diameter of the Tube	30 mm
2.	Cross-sectional Area	706.5 mm^2
3.	Energy of the Shock Wave	212.5 J
4.	Energy per unit area at exit	300 mJ/mm^2
5.	Peak-over pressures	3 bar (kPa)
6.	Peak Temperature	400 kelvin
7.	Mach number	1.47
8.	Duration of peak pressure & Temperature	250 µs
9.	Percentage Killing : *Halomonas gomseomensis* EP-3	97%
10.	Percentage Killing : *Bacillus thermocopriae* IR-1*	71 %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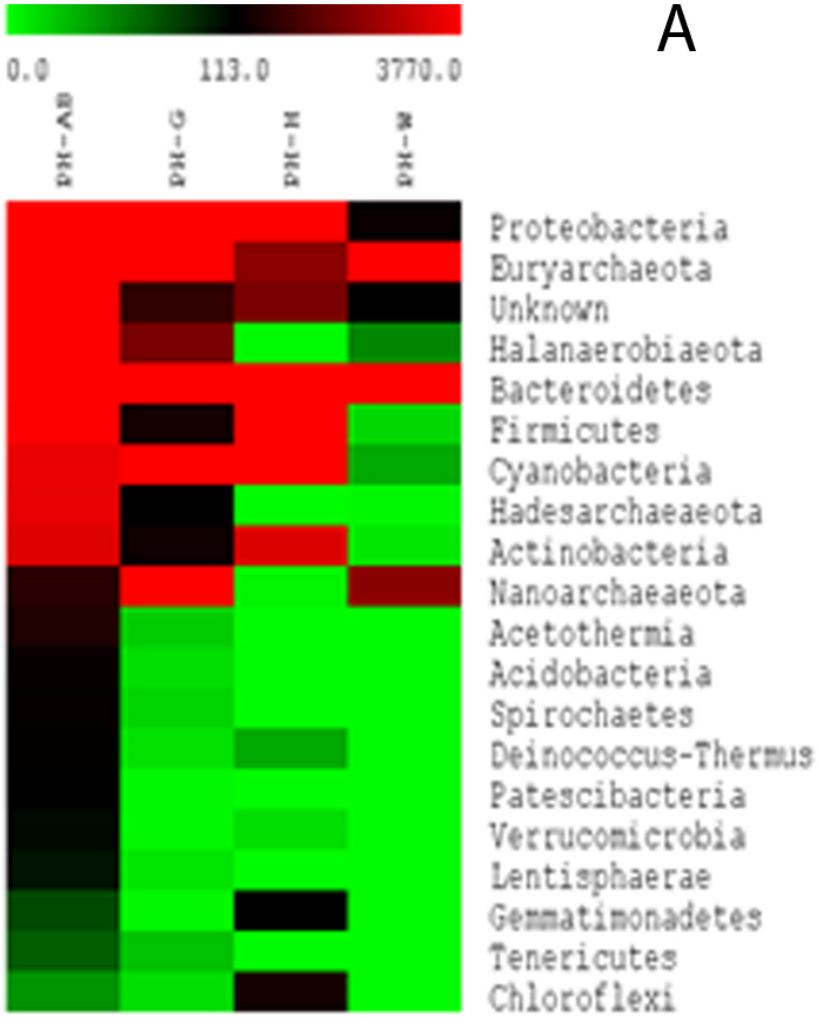
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Proteobacteria
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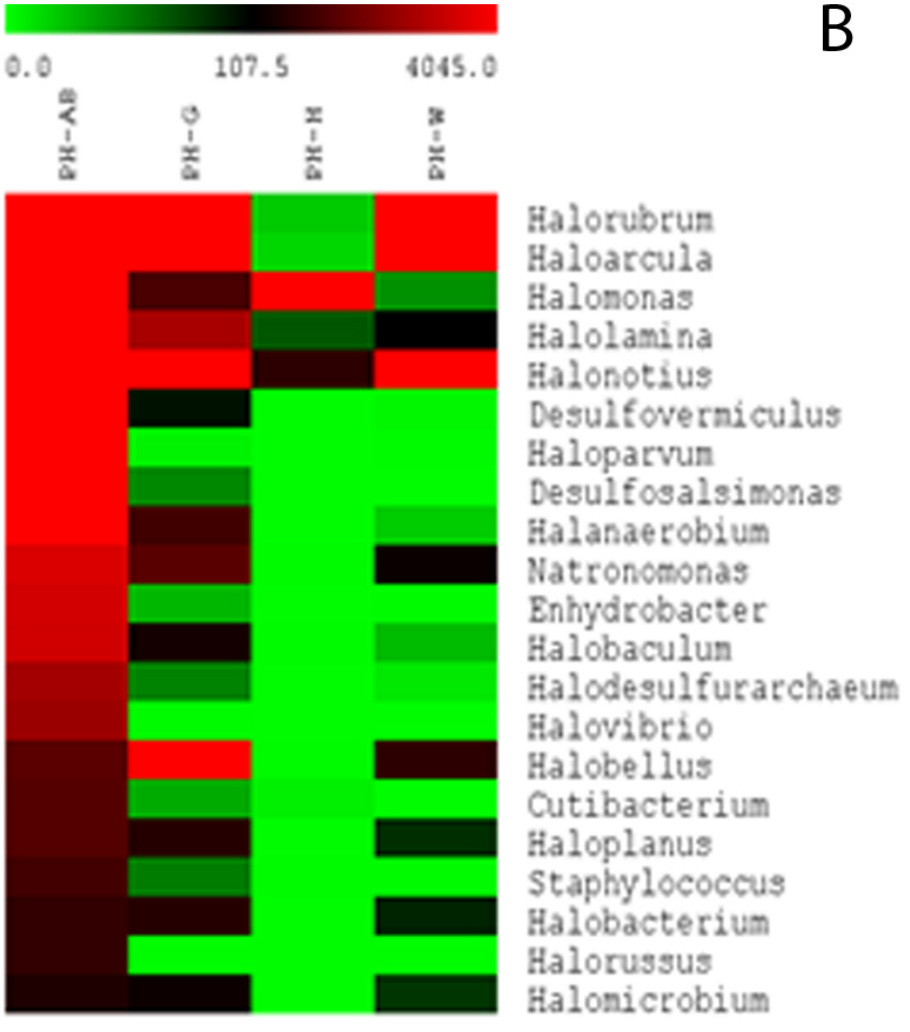
Unknown
Halanaerobiaeota
Bacteroidetes
Firmicutes
Cyanobacteria
Hadesarchaeaeota
Actinobacteria
Nanoarchaeaeota
Acetothermia
Acidobacteria
Spirochaetes
Deinococcus-Thermus
Patescibacteria
Verrucomicrobia
Lentisphaerae
Gemmatimonadetes
Tenericutes
Chloroflexi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Halorubrum
Haloarcula
Halomonas
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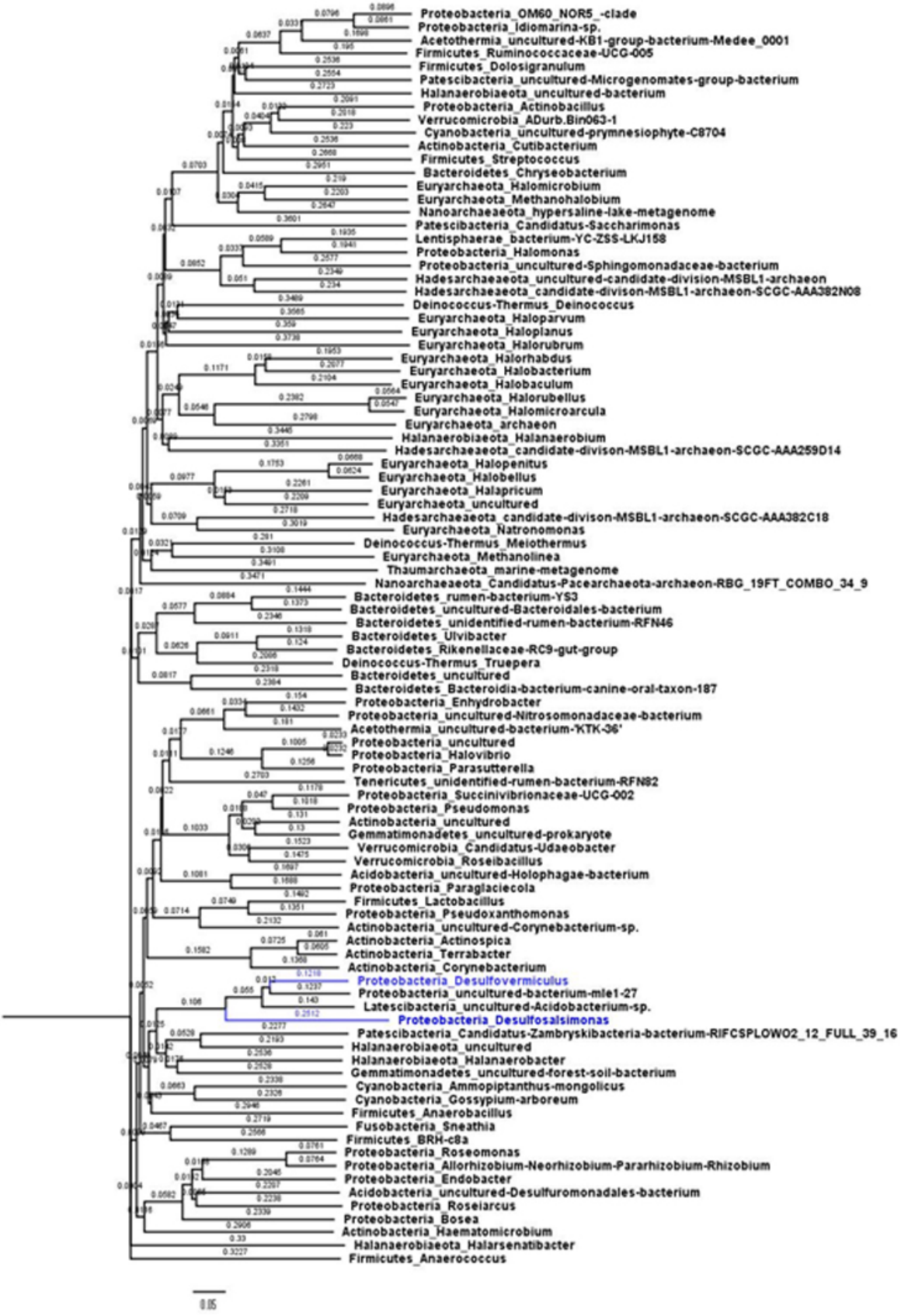
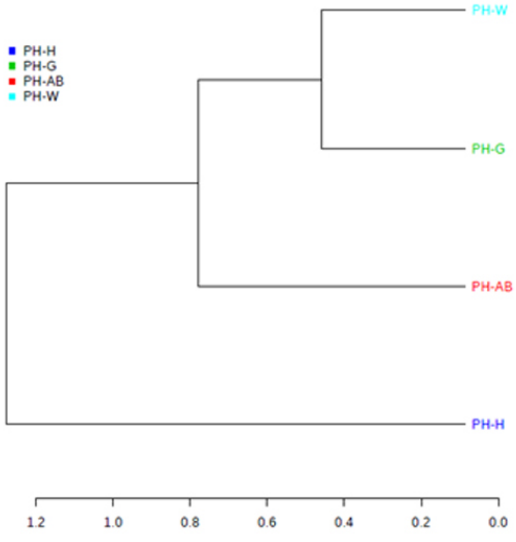
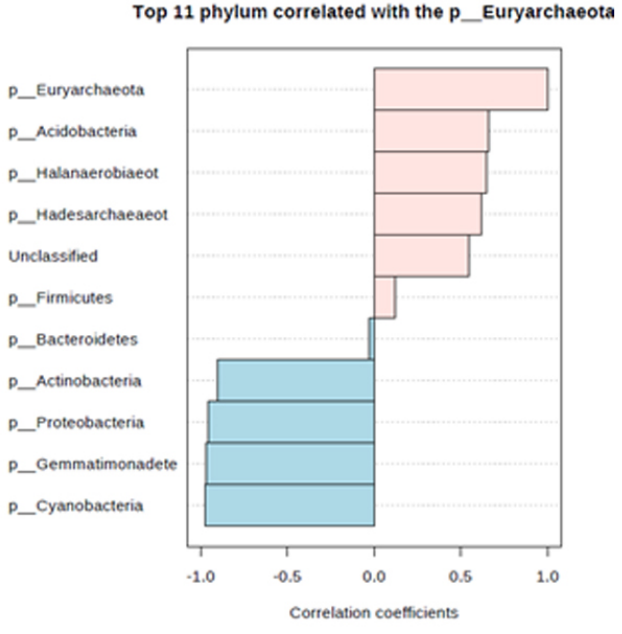
Desulfovermiculus
Haloparvum
Desulfosalsimonas
Halanaerobium
Natronomonas
Enhydrobacter
Halobaculum
Halodesulfurarchaeum
Halovibrio
Halobellus
Cutibacterium
Haloplanus
Staphylococcus
Halobacterium
Halorussus
Halomicrobium


Proteobacteria_OM60_NOR5_-clade
Proteobacteria_Idiomarina-sp.
Acetothermia_uncultured-KB1-group-bacterium-Medee_0001
Firmicutes_Ruminococcaceae-UCG-005
Firmicutes_Dolosigranulum
Patescibacteria_uncultured-Microgenomates-group-bacterium
Halanaerobiaeota_uncultured-bacterium
Proteobacteria_Actinobacillus
Verrucomicrobia_ADurb.Bin063-1
Cyanobacteria_uncultured-prymnesiophyte-C8704
Actinobacteria_Cutibacterium
Firmicutes_Streptococcus
Bacteroidetes_Chryseobacterium
Euryarchaeota_Halomicrobium
Euryarchaeota_Methanohalobium
Nanoarchaeaeota_hypersaline-lake-metagenome
Patescibacteria_Candidatus-Saccharimonas
Lentisphaerae_bacterium-YC-ZSS-LKJ158
Proteobacteria_Halomonas
Proteobacteria_uncultured-Sphingomonadaceae-bacterium
Hadesarchaeaeota_uncultured-candidate-division-MSBL1-archaeon
Hadesarchaeaeota_candidate-divison-MSBL1-archaeon-SCGC-AAA382N08
Deinococcus-Thermus_Deinococcus
Euryarchaeota_Haloparvum
Euryarchaeota_Haloplanus
Euryarchaeota_Halorubrum
Euryarchaeota_Halorhabdus
Euryarchaeota_Halobacterium
Euryarchaeota_Halobaculum
Euryarchaeota_Halorubellus
Euryarchaeota_Halomicroarcula
Euryarchaeota_archaeon
Halanaerobiaeota_Halanaerobium
Hadesarchaeaeota_candidate-divison-MSBL1-archaeon-SCGC-AAA259D14
Euryarchaeota_Halopenitus
Euryarchaeota_Halobellus
Euryarchaeota_Halapricum
Euryarchaeota_uncultured
Hadesarchaeaeota_candidate-divison-MSBL1-archaeon-SCGC-AAA382C18
Euryarchaeota_Natronomonas
Deinococcus-Thermus_Meiothermus
Euryarchaeota_Methanolinea
Thaumarchaeota_marine-metagenome
Nanoarchaeaeota_Candidatus-Pacearchaeota-archaeon-RBG_19FT_COMBO_34_9
Bacteroidetes_rumen-bacterium-YS3
Bacteroidetes_uncultured-Bacteroidales-bacterium
Bacteroidetes_unidentified-rumen-bacterium-RFN46
Bacteroidetes_Ulvibacter
Bacteroidetes_Rikenellaceae-RC9-gut-group
Deinococcus-Thermus_Truepera
Bacteroidetes_uncultured
Bacteroidetes_Bacteroidia-bacterium-canine-oral-taxon-187
Proteobacteria_Enhydrobacter
Proteobacteria_uncultured-Nitrosomonadaceae-bacterium
Acetothermia_uncultured-bacterium-'KTK-36'
Proteobacteria_uncultured
Proteobacteria_Halovibrio
Proteobacteria_Parasutterella
Tenericutes_unidentified-rumen-bacterium-RFN82
Proteobacteria_Succinivibrionaceae-UCG-002
Proteobacteria_Pseudomonas
Actinobacteria_uncultured
Gemmatimonadetes_uncultured-prokaryote
Verrucomicrobia_Candidatus-Udaeobacter
Verrucomicrobia_Roseibacillus
Acidobacteria_uncultured-Holophagae-bacterium
Proteobacteria_Paraglaciecola
Firmicutes_Lactobacillus
Proteobacteria_Pseudoxanthomonas
Actinobacteria_uncultured-Corynebacterium-sp.
Actinobacteria_Actinospica
Actinobacteria_Terrabacter
Actinobacteria_Corynebacterium
Proteobacteria_Desulfovermiculus
Proteobacteria_uncultured-bacterium-mle1-27
Latescibacteria_uncultured-Acidobacterium-sp.
Proteobacteria_Desulfosalsimonas
Patescibacteria_Candidatus-Zambryskibacteria-bacterium-RIFCSPLOW02_12_FULL_39_16
Halanaerobiaeota_uncultured
Halanaerobiaeota_Halanaerobacter
Gemmatimonadetes_uncultured-forest-soil-bacterium
Cyanobacteria_Ammopiptanthus-mongolicus
Cyanobacteria_Gossypium-arboreum
Firmicutes_Anaerobacillus
Fusobacteria_Sneathia
Firmicutes_BRH-c8a
Proteobacteria_Roseomonas
Proteobacteria_Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Proteobacteria_Endobacter
Acidobacteria_uncultured-Desulfuromonadales-bacterium
Proteobacteria_Roseiarcus
Proteobacteria_Bosea
Actinobacteria_Haematomicrobium
Halanaerobiaeota_Halarsenatibacter
Firmicutes_Anaerococcus
0.05

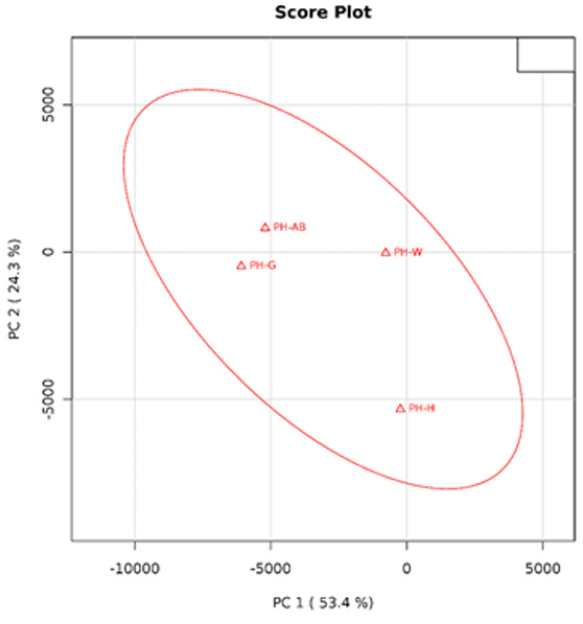
A


PH-H
PH-G
PH-AB
PH-W
PH-W
PH-G
PH-AB
PH-H
1.2
1.0
0.8
0.6
0.4
0.2
0.0

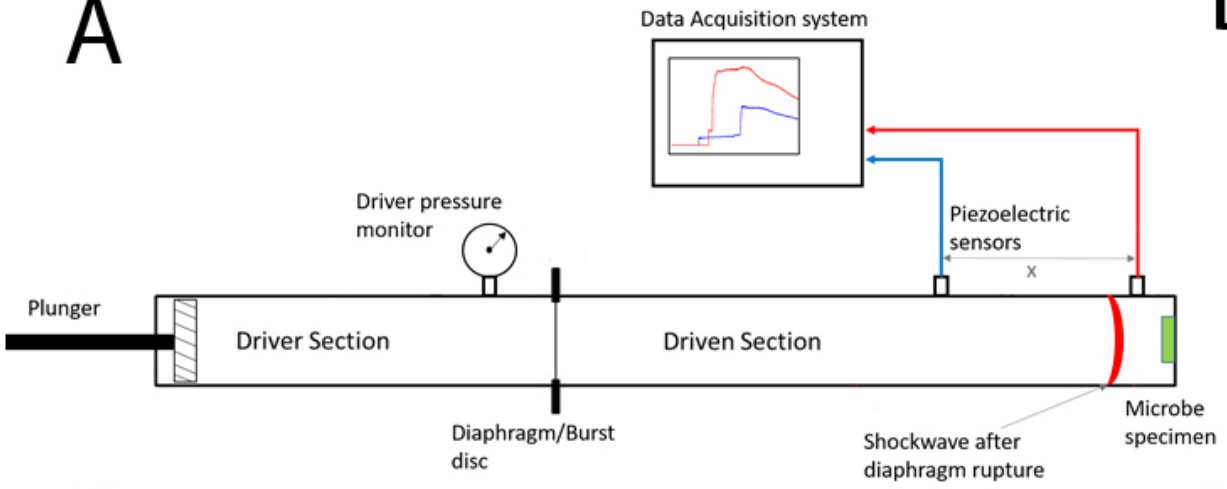
B


Top 11 phylum correlated with the p__Euryarchaeota
p__Euryarchaeota
p__Acidobacteria
p__Halanaerobiaeot
p__Hadesarchaeaeot
Unclassified
p__Firmicutes
p__Bacteroidetes
p__Actinobacteria
p__Proteobacteria
p__Gemmatimonadete
p__Cyanobacteria
-1.0
-0.5
0.0
0.5
1.0
Correlation coefficients

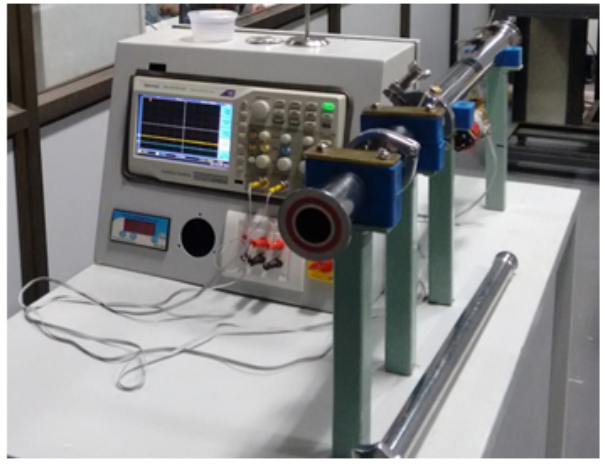
C


Score Plot
PH-AB
PH-W
PH-G
PH-H
5000
0
-5000
PC 2 (24.3 %)
-10000
-5000
0
5000
PC 1 (53.4 %)

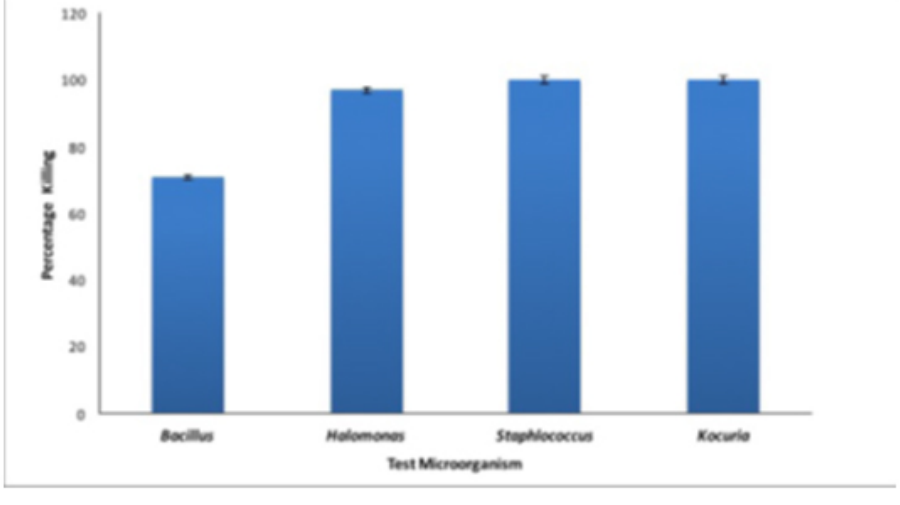
A


Data Acquisition system
Driver pressure monitor
Piezoelectric sensors
x
Plunger
Driver Section
Driven Section
Diaphragm/Burst disc
Shockwave after diaphragm rupture
Microbe specimen

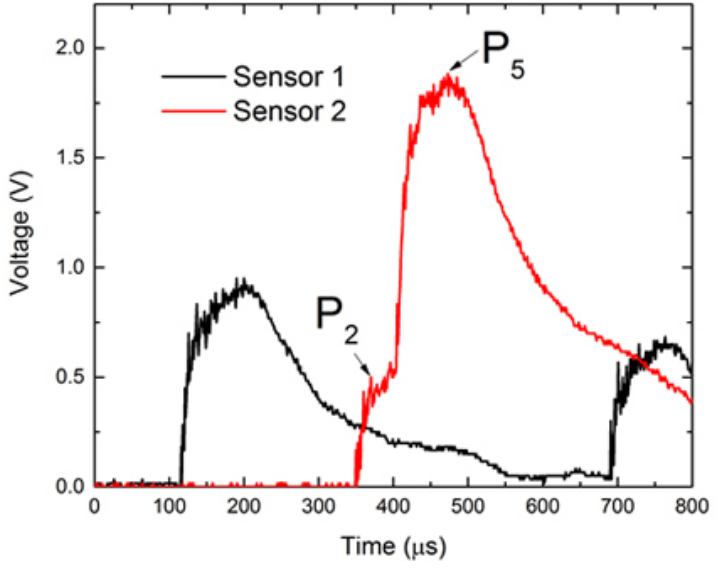
B



C


120
100
80
60
40
20
0
Percentage Killing
Bacillus
Halomonas
Staphlococcus
Kocuria
Test Microorganism

D


Sensor 1
Sensor 2
P5
P2
Voltage (V)
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
700
800
Time (μs)

Effect of impact shock on extremophilic bacterium *Halomonas gomseoemensis* EP-3 isolated from hypersaline Martian analogue site Laguna de Peña Hueca, Spain

Thombre R[1,2]*, Gomez F[3], Parkhe R[4], Kaur K[1], Vaishampayan P[5], Kartick S [6], Sivaraman B[6], R. Perumal[7], Mason N[2]

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[5]Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 911009.

[6]Atomic, Molecular and Optical Physics Division, Physical Research Laboratory, Ahmedabad, India.

[7]Agrigenome Laboratory, Kochi, Kerala, India

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

'Declarations of interest: none'

Supplementary Data

Effect of impact shock on extremophilic bacterium *Halomonas gomseoemensis* EP-3 isolated from hypersaline Martian analogue site Laguna de Peña Hueca, Spain

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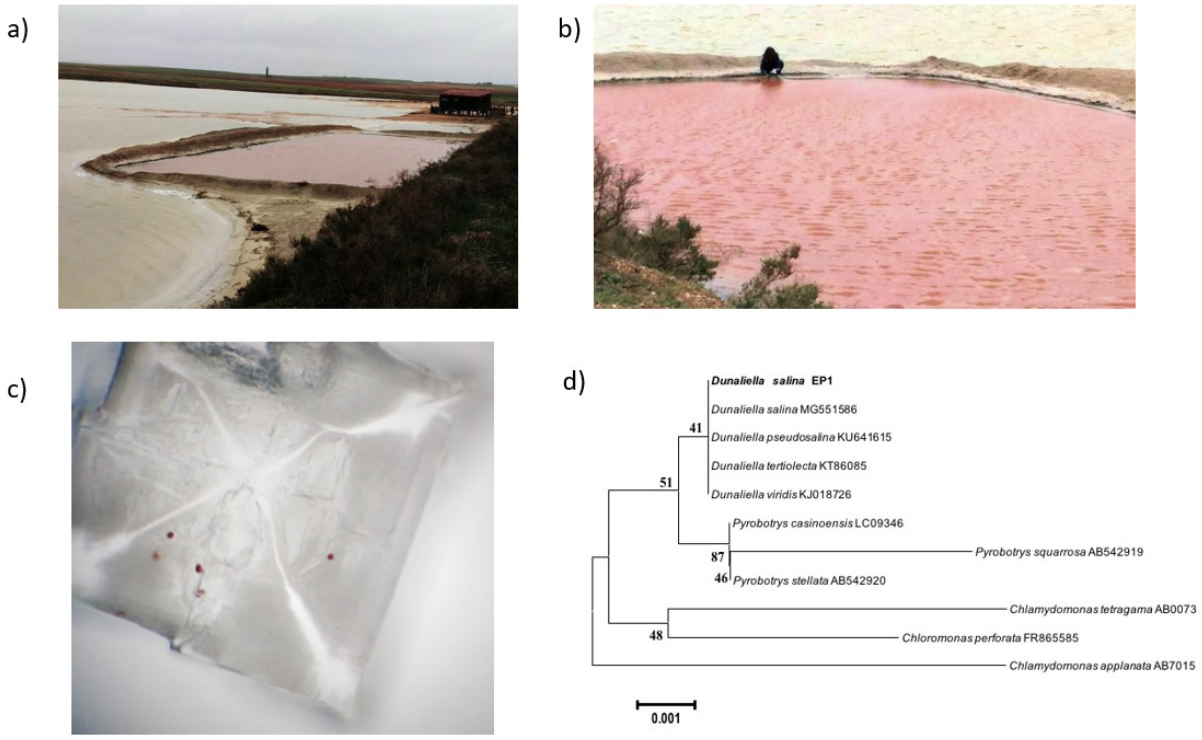
Supplementary Table

Supplementary Table 1. Identification of micro-organisms using 16 S/ 18 S* rRNA gene sequencing

Isolate Name	Sample	Nearest phylogenetic match	Medium used	Identity (%)
PLR	Rock	*Halomonas gomseomensis* strain M12	SG	99
EP-1	Water	*Dunaliella salina** isolate VAT2	Johnsons	100
EP-2	Water	*Marinobacter persicus* strain M9B	Zobell	98
EP-3	Pink crust	*Halomonas gomseomensis* strain M12	SG	99
EP-4	Yellow halite	*Halomonas lutea* strain YIM 91125	SG	99

Supplementary Figures



Supplementary Figure S1. a) Laguna de Peña Hueca, Spain b) Bright pink colored water of Laguna de Peña Hueca bordered by green waters of rest of the lagoon system, c) Red cells of algae entrapped in halite crystals collected from Peña Hueca (1000X), d) Construction of phylogenetic tree based on 18S rRNA gene sequencing. The evolutionary history was inferred using the Neighbour-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of transitional substitutions per site. Evolutionary analyses were conducted in MEGA 6.