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Responsible WP Leader: INTA, Felipe Gómez

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Dissemination level		
PU	Public	x
PP	Restricted to other programme participants (including the Commission Service)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (excluding the Commission Services)	

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Abstract: Under Horizon 2020, the Europlanet 2020 Research Infrastructure (EPN2020-RI) is providing external users access to a series of Earth Analogues of planetary surfaces through their Trans National Activity 1 (TA1). In order to add to the number of facilities accessible through TA1programme, Joint Research Activity 1 (JRA1) has prepared two new field sites providing realistic terrestrial analogues of the surface and near surface geological-geomorphological environments of Mars, Europa and Titan.

These sites are Lake Tirez (Spain) (D 7.1) and the Danakil depression (Ethiopia) (D 7.2).

1. Explanation of the work carried out by the beneficiaries and Overview of the progress

1. Objectives:

The main objective of JRA1 activity is to provide two more field sites for TA 1 external user visits during the last two years of EPN2020-RI infrastructure. The objective is to validate two extreme environments as planetary field analogues (PFAs). Those sites are Tirez Lake (Spain) and the Danakil depression (Ethiopia).

The preparation of these sites as Facilities for TA1 required extensive groundwork to develop theories and models of their interior, surface and atmospheric conditions. Extensive field and laboratory work was required to fully characterize the PFAs for geology, hydrology and geomicrobiological environments, determine the feed-back mechanisms between the regional geology, soils, climate and biology and establish how these mechanisms vary throughout the year. As part of TA1 in years 3 and 4, these two extreme and diverse environments will represent a tremendous new resource for the planetary science community, and will undoubtedly become sites on ongoing research for external users. In order to prepare the sites for these future activities we needed to develop ground work as well as laboratory work in order to validate those sites as Earth Analogues this was the objective of JRA1 with INTA-CAB responsible for Lake Tirez and IRSPS for Danakil depression.

Task 7.2. Characterisation of PFA 1 – Lake Tirez

Three visits were organised to the Lake Tirez l field site during the first year of the project and one during the second year. During these visits groundwork was undertaken for regional geological studies as well as sampling for laboratory work.

Tirez lagoon, together with Peña Hueca and Laguna Larga, are members of the lacustrine Villacañas region, a group of small endorheic hypersaline lagoons of western La Mancha (known as Campo de Calatrava). It is included in one of the three areas of endorheic regions of the Iberian Peninsula: La Mancha, Aragón and Andalucía, rich in hypersaline lakes (Guerrero and de Wit, 1992). Tirez has a maximum extension of 0.8 km^2 and a depth of 40 cm. It is located at 653 m altitude, N 39º32´695´´ and W 03º21´073´´ (UTM 50469821-4377309). With a continental climate, there are wide daily and seasonal thermal oscillations.

From a limnological point of view, Tirez is a peculiar lagoon, with differential precipitation of various types of salts and crust formations during the summer. Due to the high sulfate and magnesium content in the water it can be classified as a Na-Mg-Cl-SO_4 type of lagoon. The inner part of the lagoon is covered during the dry season by a saline crust made up of, from top down: halite, epsomite, thenardite, bloedite and gypsum. From a lithological point of view, the dominant material is sand, which alternates with gypsum and limestone. The bottom is made of clay with a sopropel layer produced by decomposition of algae with epsomite crystals. The decomposition occurs in an anaerobic environments isolate from the oxygen from the atmosphere. Anaerobic metabolism is developed in the subsurface.

The main idea of the study was to characterize the geology but the conditions of surface and subsurface as well. Samples were taken to be analysed at the laboratory for microbial diversity characterization.

Several techniques and protocols were used in the groundwork as well as in the laboratory work.

Sampling and sample conservation. Sampling was done with a RingKit core-sampler for soft soils. An homogeneous mixture of the first centimetres of sediments with the water retained in the core-sampler, corresponding to a 1-2 cm of the water column, was taken. The samples were kept refrigerated at 4ºC during their transport to the lab and until further use for chemical and microbiological analysis. The sampling was done in January, the first days of April, which climatologically corresponds to the end of the wet season, with average temperature and rain values of 13ºC and 45 mm, respectively, and in June 2016 (summer season). More visits are expected in near future for winter sampling in order to follow the evolution of the physic-chemical parameters of the water of the associated lagoons.

Physico-chemical characterization. Physico-chemical parameters were measured in situ: temperature and conductivity (Orion 120), pH and redox potential (Orion 420), and dissolved oxygen (Symplair Sylaud Ins.). Sulfate and carbonate were also determined in situ with Hanna Instruments kits (Hanna Sulfate LR-HR H1-38001A for sulfate and Hardness HR H1-3812 for carbonate). Other anions and cations (Cl-, Na+, K+, Mg_2+, Ca_2+, etc.) were determined in the laboratory by elemental TXRF analysis and NO_3- by ionic chromatography.

Metabolic assays and isolation of pure cultures. Some particular cultures and isolates were of special interest. Sulfate-reducing and methanogenic activities were analyzed using the mixture of sediment in water as inocule. For sulfate-reducing bacteria (SRB) enrichment cultures the following media was used: 0.2% $MgSO_4$. $7H_2O$, 0.35% Na-lactate, 0.1% $Fe(NH_4)_2$-$6H_2O$, 1 ml/L of trace elements, in Tirez lagoon water. The sulfide production was qualitatively detected with the lead acetate-paper method. Acetate and formate were used as substrates. The methane produced was analyzed with a Shimadzu GC-8A gas chromatograph.

In order to isolate pure cultures, SRB enrichment cultures and methanogenic cultures were used as inoculum. Agar plates with specific media were inoculated and incubated in an anaerobic chamber with N2:H2 atmosphere at room temperature. Colonies were picked, diluted in 100 µl of water and 1µl was directly used, without cell disruption, for PCR amplification according with the protocol described below.

DNA extraction and PCR. Cells from the homogeneous sediment-water samples were disrupted and DNA was extracted using FastDNA kit for soils BIO101 according to the manufacturer's protocol. The 16S rRNA genes from mixed microbial DNA were amplified by PCR. To obtain 16S rRNA genes two oligonucleotide primer pairs were used: 27F and 1492R (annealing T: 56ºC) for the Bacteria domain and 25F and 1492R (annealing T: 52ºC) for the Archaea domain. The thermal profile for amplification included 30 cycles of denaturation at 94ºC for one min, primer annealing for one min, and primer extension at 72ºC for three min. The DNA concentration was 25-50 ng for each reaction.

Clone libraries and sequencing. The amplified 16S rRNA genes (length 1465-1467 bp for bacteria and archaea respectively) were cloned using TOPO Cloning Kit (Invitrogen Corporation, San Diego, California) and then transformed into competent *E. coli* cells. Plasmid DNA inserts were extracted by alkaline lysis method (Miniprep). The archaeal clones were grouped according to their restriction pattern obtained after digestion with Sau3AI. Plasmid inserts were amplified by PCR using the M13 primer set (Invitrogen). Automated DNA sequencing was performed with an ABI model 377 sequencer (Applied Biosystems).

Sequence analysis. Sequences were compared with the NCBI database by using the basic local alignment search tool (BLAST, http://www.ncbi.nim.gov) to identify the closest sequence. After that, sequence data were aligned and analyzed with the ARB program package (available at http://www.mikro.biologie.tu-muenchen.de/). Parsimony was used to construct phylogenetic trees. Sequence alignments of the clones to determine their identities were made using the SeqLab program (http://www.accelrys.com/support/bio/fags_wis_html).

Nucleotide sequence accession numbers. The sequences obtained in this study have been deposited in the GenBank database.

Atmospheric measurements. Weather stations were settled on the site in order to follow atmospheric parameters during the duration of the field campaigns.

Some results:

Physico-chemical characterization

Sediment cores of the Tirez Lake sampled at different depths were subject of physico-chemical analysis. The sulfide showed higher concentrations at 0-10 cm depth. The occurrence and distribution of sulfide along the profile reflects the presence of sulfate-reducing bacteria (SRB) in the hypersaline sediment. The SRP activity, which was inferred by the concentration of H_2S, coincided with the presence of a black deposit of iron sulfide below the surface of the sediment. The sulfate increased with the depth and the concentration ranged from 10^2 mM, up to a ~300 mM, the latter detected at 10-15 cm . These values are located below the highest concentration zone of sulfide. The entire profile was anoxic and was in accordance with the redox potential. The redox potential and oxygen increased slightly in the deeper zones (15-20 cm in depth). The redox conditions of the majority of the sediments were in the range of -300 and -200 mV, which was enough to allow the presence of SR and MT processes. The lowest Eh values were reached at 0-10 cm in depth. The ammonium concentration fluctuated between 1-6 µM. Likewise, the highest NH_4^+ concentration (4-6 µM) was observed at 10-15 cm in depth. The $Cl:SO_4$ proportion fluctuated between 0.1 and 0.3, which reflected the athalassic nature of the system. The sulfate concentration in Tirez Lake was lower than in Chaka Lake sediment (10^{-1} mM), which is also an athalassic system. Even though Chloride was undetermined in Chaka Lake sediment, the $Cl:SO_4$ proportion of the water phase in Chaka Lake is two times higher than the highest values registered for the water phase in Tirez Lake.

A dual aerobic vs. anaerobic ecosystems were identified in the surface and subsurface of Lake Tírez.

Anaerobic diversity in the hypersaline sediment

Some kinds of microbes inhabit an ecosystem whenever their biotope requirements are realized. However, not all the types of metabolism occur in all the biotopes, including the halophilic ones. In addition, few studies have attempted to quantify the microbial and metabolic composition in highly sulfated and saline environments. Thereby, a functional gene approach was applied here to identify genetically and unambiguously the sulfate reducing and sulfur oxidizing bacteria as well as the methanogenic archaea, who could be probably the main responsible for the biogeochemical processes in the anoxic, sulfated and highly saline sediment of the Tirez's lake. Even though we were able to characterize several phylotypes involved in these distinctive metabolically lineages on this ecosystem, the Denaturing Gradient Gel Electrophoresis (DGGE) and phylogenetic analyses revealed a low sulfate-reducing prokaryotes (SRP), sulfur-oxidizing prokaryotes (SOP) and Methanogenic Archaea (MA) diversity, which agrees with the fact that the diversity of the three domains of life decreases in extreme hypersaline communities. Nevertheless, Tirez, as in other hypersaline environments, supports the presence of low energetic anaerobic metabolisms such as the extreme halophiles fermenters from the *Halanerobiales* group, who employ the Wood–Ljungdahl pathway to ferment organic compounds producing volatile fatty acids (VFA), such as acetate and H_2. Wood-Ljungdahl pathway is also utilized during terminal carbon oxidation by some SRP organisms such as *Desulfonema* spp.; however no extreme halophilic anaerobes from the genera have been reported from Tirez.

Microbial diversity in Tirez Lake

The genera phylotypes reported in this work have not previously been reported from athalassic environments. These genera have, however, been reported from athalassic natronophilic or thalassic sediments, which have been more extensively studied

The Gram Positive *Desulfotomaculum* spp. perform the oxidation of a broad spectrum of electron donors including acetate (*D. acetoxidans*), lactate and H_2. Unfortunately the assignment of *aprA* gene fragment was not conclusive at species-level, thus their function in Tirez system remain uncertain. The presence of *Desulfohalobium* and *Desulfonatronovibrio* at Tirez can be explained based on their metabolism but not in the case of *Desulfonema* sp.

Interestingly, in summer samples, *Desulfonema magnum* (apsam25) is present, which are functionally significant in the system because they are complete carbon mineralizers, thereby, organisms encoding corresponding *aprA* gene face the summer increase in salinity in their natural habitat. It has been argued that any hypersaline environment is inappropriate for the survival of anaerobic acetate oxidation as a consequence of the low negative balance of the standard ΔG yield this metabolism supplies and the high maintenance energy needed for compatible solute synthesis/accumulation under high osmotic conditions. Despite their ecological significance very little is known about the mechanisms of energy conservation in acetatoclastic SRP that allows them to thrive in extreme saline conditions. Though the

relationship between band intensity and relative abundance of the corresponding phylotype is questionable, other studies have shown that *Desulfobacteriaceae* are present in thalassic environments and soda lakes. Probably *Desulfonema* exerts the energy conservation strategy of methanogens, as proposed for *Desulfobacterium autrotrophicum*, to result in an extra transference of electrons from membrane complexes and H^+ pumping that enhances the known SRP chemiosmotic way of energy conservation.

The proteobacterial families *Ectothiorhodospiraceae* and *Hydrogenophilaceae* phylotypes from non-axenic cultures and environmental samples are expected to thrive in the extreme saline soil as strict anaerobes or at least facultative anaerobes given the low Eh and partial O_2 pressure observed in the sampling site. In contrast to the SRP and MA, their anaerobic chemotrophic lifestyle probably generally relies on high energetic reactions.

None of the genera detected in either season belongs to acetoclastic MA, this absence has been described in earlier studies (of what? Saline environments) and was argued to be a consequence of the low Gibbs energy liberated during metabolism. In the Tirez sulfated sediment the SR is probably responsible for H_2S production at 0-10 cm depth where *Methanosarcinales* were also detected. The presence of *Methanohalobium* and *Methanolobus* genera was unsurprising because their metabolism is based on methylated substrates, which are non- competitive with SRP. On the other hand, the increase of ammonium (4-6 µM) at 10-15 cm depth and decrease in Eh through the sediment column suggests that increase in NH_4^+ might be attributed to the strict anaerobic and methylotrophic MA, precluding aerobic/anaerobic ammonium oxidation because of energy constraints for these metabolic pathways under hypersaline conditions.

The hydrogenotrophic *Methanoculleus* was detected in the surface DGGE profile from winter sediment, when water column salinity averaged 6% w/v. It is interesting to note that *Methanocalculus halotolerans* also hydrogenotrophic (H_2-MA), was not detected even though *M. halotolerans* has been described as the most halophilic H_2-MA with an optimal growth at 5% NaCl. So far, halophilic species within the *Methanoculleus* genera have not been reported, although clones of the genera have been obtained from thalassic (proportional to marine) sediment communities, whose salinities fluctuate between 3.5 and 5.3% w/v. The failure to detect *Methanoculleus* in summer samples correlates with the low energy yield of CO_2/H_2 and formate pathways in comparison with the methylotrophic. A paper that describes the above results is in preparation to be submitted to Microbial Ecology journal.

This site will be open for external users visits in the period 2018-2019 and at the moment of this report finalization several external users are interested in visiting the site.

2. Deviations from Annex 1 (if applicable)

2.1 Tasks (if applicable)

Not applicable: No task for this wp at this moment.

2.2.1 Unforeseen subcontracting (if applicable)

Not applicable

2.2.2 Unforeseen use of in kind contribution from third party against payment or free of charges (if applicable)

Not applicable

Annex. PFA participants

PFA participants are listed below.

Participant	**Permanent personnel**	**Personnel hired by the project**
5. INTA	Dr. Felipe Gómez Gómez Dr. José Antonio Rodriguez Manfredi Prof. Ricardo Amils Dr. Olga Prieto-Ballesteros Dr. Juan Angel Vaquerizo Nuria Rodríguez Fernando Camps	Beatriz Flores
9. IRSPS	Prof. Gian Gabriele Ori Dr Goro Komatsu Dr Monica Pondrelli	

PFA Structure – wp7 – JRA 1: Earth Analogues Validation Coord.: INTA with partners INTA and IRSPS

PFA activities consist of one work package with two different sites managed by two partners. These activities consist of the following tasks:

- *Task 7.2. Tírez Lake validation (Spain). Applicable as TA1 report after second year of the project. Managed by INTA. (D7.1)*
- *Task 7.3. Danakil Depression (Ethiopia). Applicable as TA1 report after second year of the project. Managed by IRSPS/INTA. (D7.2)*