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EURO-CARES

A PLAN FOR EUROPEAN CURATION OF RETURNED EXTRATERRESTRIAL SAMPLES

PLANETARY PROTECTION

Report on protocols, methods and techniques for life and biohazard assessment

Work Package 2

Deliverable 2.1

Issue 1 Rev. 4

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1. Introduction

According to our current level of knowledge, Earth is the only planet to have biological processes that allow life to propagate on its surface. Nevertheless, the main ingredients for life (the full suite of stable elements in the periodic table and the complex organic molecules associated with the building blocks of life) can be found almost everywhere in space: on rocky planets, on moons, on asteroids and comets, and even in the depths of interstellar space. In our Solar System, the most promising objects that are most likely to contain life are thought to be:

- Mars
- Icy moons, in particular the Jupiter's ones Europa and Ganymede and Saturn's Enceladus
- Other satellites as Titan

There is a broad consensus in the scientific community that samples collected and returned to Earth must be contained and treated as potentially biologically hazardous until they are declared safe by applying recommended protocols, including rigorous physical and chemical characterization, life detection analyses, and biohazard testing (Race et al., 2009).
Before getting deep in the topics of this deliverable, we have to start from a couple of considerations:

1. There is well-documented history of successful biocontainment of pathogenic and infectious organisms in microbiological laboratories. These facilities typically use primary containment devices; negative-pressure gradients and inwards air flow to prevent harmful materials from being released. They are designed to leak in and as a result, are usually both chemically and biologically "dirty", although high containment facilities will filter the air to remove contaminating organisms.
2. Similarly, there is a record of successful containment for maintaining the integrity of extraterrestrial and planetary samples. These facilities typically use positive-pressure gradients to prevent contaminants from getting in. They are designed to leak out and as a result are ineffective for the containment of hazardous materials contained inside the samples.

Planetary protection must work in both directions: the samples have to be preserved to be as pristine as possible and, at the same time, the Earth environment must be protected from potential hazards. No single facility already exists that allows both containments, as required for a

Sample Receiving Facility (SRF) for materials returned from the previously mentioned objects, in particularly Mars.

Since it is impossible to foresee the actual risk factor of returned samples, the facilities need to have the most stringent containment level presently afforded to the most hazardous biological entities known on Earth. In conclusion, these facilities will require combining of maximum biosafety containment level (BSL-4) with cleanroom technologies.

Regardless of the fact that the assessment activities are directly performed inside the SRF or, preliminarily in a separate laboratory (e.g. in a dedicated transportable facility near the Earth re-entry capsule landing site), the primary objective for planetary protection is to asses Life Detection (LD) and Bio Hazard (BH):

- Life Detection analyses should be based on a broad definition for life and an approach for detecting life not limited by the specific features of life, as we know on Earth. This approach will rely on signatures of various types that encompass all known terrestrial life, and that might encompass non-terrestrial life (e.g., structures, biochemical compounds, isotopic patterns, and geochemical features).
- The biohazard testing process has the aim to determine if samples pose any threat to terrestrial organisms or ecosystems, regardless of whether the samples contain life forms or non-replicative hazards. Since potential hazards could take a multitude of forms, the spectrum of tests has to be diverse. In practical terms, biohazard testing should determine of whether the samples contain any biohazard and whether to distribute sub-samples while providing a reasonable assurance that the samples will not put humans or other terrestrial organisms at risk.

Given the extreme difficulty of inductively describing all possible living processes based on terrestrial biochemistry, no single approach is able to guarantee success with a given sample. Multiple approaches are key to the successful detection of possible life in a sample (Baross et al., 2000).

2. **Life and Biosignatures**

To search for life must start from a definition of what actually constitutes life. Probably the simplest statement is that life is a chemical system able to transfer its molecular information via self-replication and evolve via mutations. For life as we know it there must be liquid water along with the necessary nutrients mainly based on C, N, H, O, S and P atoms. In any case, life evolved to survive in water that is very acidic, alkaline or is a strong brine solution. It also survived and flourished in water at temperatures above 100 °C (Wilson A. et al., 1999).
Evidences of microbial life on Earth starts about 3800 million years ago, within about 700 million years of its formation: it emerged very early and, once established, it evolved and adapted to a wide range of changing physical and chemical conditions (e.g. active biosphere found hundred meters below the Earth's surface and bacteria were found up to stratosphere). Thus, it is necessary to use an approach for life detection, not limited by the features of life on Earth. This approach relies on biosignatures that encompass terrestrial and non-terrestrial life (Rummel et al., 2002).
We can define biosignature as feature (such as a chemical compound, an isotope, a cellular component, a shape etc.) that only can be formed by a biological process and has no possibility of being formed abiotically, which indicates or suggests the presence of a biological process indicative of life.
Thus, biosignatures can be detected through different methods and in general, broadly-defined signatures offer the greatest opportunities for detecting life that is unfamiliar to us in its detail. However, broad signatures also carry the greatest chance for misleading or false-positive findings. In general, the greater the number of independently-defined signatures that are detected, and the greater the spatial co-localization of them, the stronger the evidence for life.
Biosignatures may have a variety of features that can be chosen to detect presence of life on returned samples. A short list of possible biosignatures categories is reported as follows (Rummel et al. 2002):

- **Morphological**. Patterns, complex and recognizable features: size, shape, structure, morphological indicators of replication or specialized features such as attachment and motility structures, septae, etc.
- **Structural chemistry**. Basic chemical features, such as organic, and higher-order features, such as polymers, membranes, and attachment and motility structures. There is a need for methods to characterize complex polymers and criteria for interpreting the patterns associated with complex carbon. Also possible structural complexity that can include silica and silica-carbon polymers, should also be considered.
- **Metabolism and bioenergetics**. The waste products that are released and the energy expended by all forms of life, as we know them, detected with physical and chemical

methods. Some products are created through specific enzyme catalysed reactions, such as the reduction of nitrogen that can occur from inorganic reactions. Other products are predicted to result from reactions in the absence of protein-enzymes, such as those involved in energy and CO_2 reduction. More work is needed to assess the range of metabolic mechanisms and products that occur on Earth, as well as theoretical studies of those that might occur in the absence of carbon.

- **Biosynthetic mechanisms**. Life has mechanisms to synthesize structural, metabolic and replicative macromolecules. Carbon-based life uses protein-enzymes and, to a limited extent, ribozymes (catalytic RNA). The synthesis of macromolecules involves a sequence of reactions that depends on the availability of basic organic components, such as amino acids for protein synthesis. Such synthetic mechanisms should provide detectable biosignatures, if they are present. In taking a broader view, there is the possibility of biosynthetic mechanisms and pathways catalysed by inorganic metals and minerals in non-protein matrices, or that are dependent on physical gradients (temperature, pH, Eh, magnetism), catalytic mineral surfaces, or various energy sources (UV and other forms of radiation and light). Such mechanisms may exist, but their detection may be as a consequence of first detecting other signatures of life.
- **Isotopic signatures**. Life with which we are familiar fractionate various elements; thus, isotopic fractionation can be indicative of life (e.g. Oxygen isotopic ratio associated with phosphate). Organisms that express different metabolic capabilities display distinctive patterns in the fractionation of carbon, nitrogen, oxygen and sulphur. This might be particularly important in assessing the possible origins of organic compounds and various volatiles such as methane, carbon dioxide, and carbon monoxide, if detected on Mars. While one cannot assume that extraterrestrial life will fractionate elements in the same manner as terrestrial life, it is reasonable to assume that local patterns of fractionation within or at sites of life-forms in the sample will vary from those measured in the surrounding sample environment. Some isotopes, such as those for oxygen (detected in carbon dioxide and phosphate), can be indicators of environmental temperature. There is promising new technology for measuring carbon isotope fractionation patterns in single organic molecules and fractionation patterns in transition metals. The latter may be very important in identifying a biological source for various minerals such as magnetite.
- **Geochemical signatures.** This includes findings such as magnetite, and other minerals out of equilibrium with their normal distribution in the environment. Redfield-like ratios of key elements (i.e. C, H, N, O, P, and S) are found in the pigments of terrestrial life, such as those known to be associated with photosynthesis, and other inorganic chemical anomalies (e.g., based on iron, sulphur, etc.). When specific biologically important elements are limited in the environment, there will be higher concentrations associated with life-forms or colonies

of life-forms. Usually, the limiting element in the environment will limit the extent of growth and productivity of organisms (known as Liebig's Law of the Minimum). Some of those key elements that are limited in terrestrial environments and that life could concentrate to higher-level respect to the surrounding environment include iron and molybdenum (essential for nitrogen cycle reactions), and tungsten (essential for specific enzymes in hyperthermophilic archaea).

3. **Biohazard**

A biohazard is defined as a hazard that either can replicate by itself or be amplified by a biological system (e.g. viruses). The main point is the concept of replication, which is a key distinction between a biohazard and a simple toxin hazard. Potential hazards could take one or more of a multitude of forms: toxic, mutagenic, life cycle altering, hazardous through genetic recombination, disruptive to ecosystems, capable of biasing phenotypes or even behaviour, etc.
Incorporating a biohazard assessment as part of any sample return mission is essential when developing a sample-return facility here on Earth.
The spectrum of tests has to be diverse. In case of the detection of a living, self-replicating organisms within a sample returned from e.g. Mars, biohazard analysis should follow clearly defined chemical and biological risk assessment components that include:

- **Hazard assessment**, which involves an evaluation of the intrinsic hazard characteristics of biochemicals and macromolecules.
- **Dose-response evaluation**, which in the case of a biological agent involves parameters such as minimal dose for infectivity, pathogenicity, environmental transmission, and distribution in the ecosystem populations.
- **Exposure assessment,** such as those involved in occupational, clinical, and general environment-related activities using a set of realistic exposure scenarios.
- **Risk characterization**, a formalized approach to combine the characteristics of hazard, toxicity, and exposure to derive a measure of risk associated with the biological agent.

Based on this data-driven approach, similar to the previously described approach by Kminek et al., (2014), samples should be classified following an amended Weight-of-Evidence (WoE) procedure as suggested by the World Health Organization (WHO) for the ranking of potential biohazards. The risk-based designations for the agents ought to be as follows:

- Risk Group 1: Agents do not indicate danger to humans or the environment.
- Risk Group 2: Agents show a small effect on model systems (e.g. Human cell lines, mice); however diseases are rarely serious. There is minimal risk that the biomolecule cause an effect on the environment.
- Risk Group 3: Agents show serious or lethal disease/effect in model systems. High individual risk but low community risk.
- Risk Group 4: Agents that cause serious or lethal diseases in model systems for which preventive or therapeutic interventions are not usually available — high individual and high community risk.

4. **Procedures for Life detection and Biohazard**

The testing of bodily fluids for biohazardous biological materials is a common procedure in biocontainment laboratories worldwide. However these laboratories do not routinely test soils, rocks, and other materials unless they are implicated in some form in a disease.
As previously mentioned the samples collected and returned to Earth must be contained and treated as potentially biologically hazardous until they are declared safe. This is the reason why dedicated facilities require the combination of BSL-4 facilities with cleanroom technologies.
A set of dedicated procedures is needed to assess the biohazard of samples. Once sufficient information is available for characterizing and understanding the biological materials in question, informed decisions can be made to downgrade or even eliminate containment requirements, if deemed appropriate.

The procedural assessment for LD and BH can be summarized as shown in Figure 1, where only the main LD/BH activities are reported. Sterilization activities are optional but they also require a validated procedural approach. For each of the displayed phases (the boxes in Figure 1) a whole fully comprehensive procedural approach is required:

- Re-entry capsule containment integrity verification
- Sample container containment integrity verification
- Sample container sterilization
- Gas phase analysis (at sample container opening)
- LD/BH analysis
- Samples selection
- Sample container sterilization
- Sample sterilization

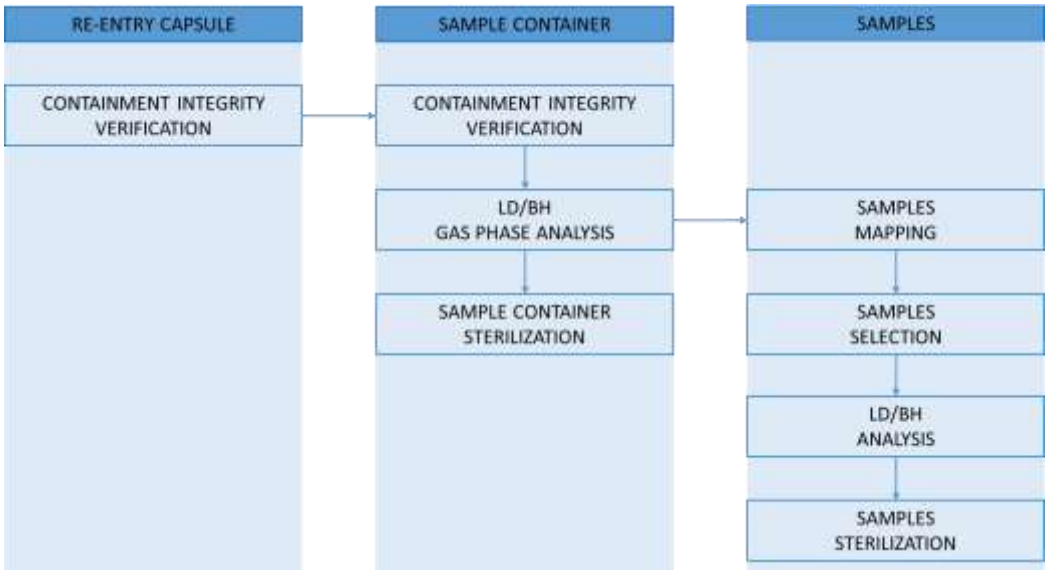

Figure 1 – LD/BH phases and procedures

4.1. Sample selection

Although it is not possible a priori to foresee the actual amount of samples returned from each sample return mission, it is presumed that life detection and biohazard testing will require a selection of aliquots taken from larger samples. It is also important to consider that the sample size of material used for destructive life detection and biohazard tests should be appropriate to give significance to the results and assurance that life is/isn't present. But it would also be desirable to preserve an amount of material for further scientific investigations, so biological assays that require small quantities are highly preferable if the results will be meaningful.
The question is how to obtain an aliquot that will yield reliable results during testing. This problem is emphasized by the fact that the biosignatures of rocks, soils, and ices may show heterogeneous distributions within samples at microscopic scales of observation: spatial variations in mineral (and elemental) compositions, sizes, shapes, and sorting of grains, distributions of void spaces (e.g., intergranular porosity, dissolution voids, vesicles, and interconnected networks of microfractures), etc.
Thus, it is very important that subsamples subjected to life detection and biohazard testing be acquired in samples that have the highest potential for containing life or its biosignatures.
The following steps define a general approach to creating a proper context for subsample selection for life detection and biohazards testing:

1. Samples - exteriors tested for organic compounds and any released gases

2. Samples - non-destructive methods of analysis to map the microscale spatial distributions of minerals and biological elements in samples;
3. Acquisition of sample aliquots from most promising areas, targeted by compositional and microtextural mapping, performed at step 2.

Step 1 refers to LD/BH gas phase analysis, step 2 samples mapping phase and 3 is the sample selection.
If, for some reason, this approach is impossible, pre-sorting of the samples in terms of relevant biologically interesting sub-samples or an approach simply based on a statistical method should be applied.
The model system for life detection and biohazard testing should be relevant to a probable hazard scenario and to systems of ecological interest, sensitive, meaningful and clear to interpret, robust, well documented, able to provide an answer in a reasonably short time, compatible with the curation in terms of handling operations and available analysis technologies.

4.2. Life Detection and Biohazard Procedures

Given the extreme difficulty of describing all possible living processes based on terrestrial biochemistry, no single approach will be able to guarantee a success with a given sample. A multiple approach, both chemical and microscopic, seems to be more appropriate for life detection in a sample and, consequently, multiple analytical techniques should be capable of distinguishing between abiogenic and biogenic signatures.
At this stage of our study is difficult to define the fine procedures for life detection and biohazard assessment. Nevertheless, the general principles can be described as follows:

- Start with a broad optical survey of different sample types for more general features suggestive of life, then turn to a higher resolution microscopy with suggestive features for a more detailed characterization;
- Emphasize structural signatures of life and other inhomogeneity that can be easily detected as a first order task;
- Apply non-destructive analytical methods in the early stages and use their results as a guide for more definitive but destructive methods;
- Start with samples least likely to contain life (e.g. surface fines) and, if negative[1], use these as blanks and controls;

[1] Resolution of analytical instruments currently available for laboratory analyses is high enough to get as far as molecular and atomic scale. Thus, if negative life detection is obtained, this would not be due to instrument detection levels.

- Multiple independent signatures are necessary for the recognition of life;
- If inactive or extinct life is found, treat it as potentially active life;
- Generalize a carbon-centred methodology to other chemical species;
- Use an iterative approach.

Analytical methods for life detection can be divided into those that facilitate a wide survey of a representative portion of different sample types, and those that facilitate a more focussed, but high-resolution, examination:

- **Survey methods** are less destructive of samples, and include microscopy, broadband fluorescence, surface scanning and chemistry, tomography, and isotopic measurements. These methods seek structural and basic chemical signatures, and local inhomogeneity.
- **Higher resolution methods** are generally more destructive, and include mass spectroscopic methods, combustion, isotope analysis, and electron microprobe procedures for elemental mapping. These methods seek to characterize inhomogeneity and more complex structures, and are discussed below in further detail.

One factor to be considered is the difficulty in detecting or interpreting signatures if life forms are inactive or have become fossilized. One of the large challenges is an understanding of the stability of various biosignatures over time and their dependence on continued metabolic activity.
Some indicators, either structural or chemical, which may indicate "past" or inactive life, should be treated as potential indicators of active life.
A useful strategy for life detection is to replicate analytical measurements over time. Repeated analyses for the biosignatures may reveal changes in the sample or surrounding atmosphere due to metabolic activity. This do not account for viruses and infectious agents such as prions. Viruses are intracellular pathogens and are incapable of replication outside the host organism. However, the time required for an organism to replicate could be so long to be considered ineffective for our purposes – e.g. sub surfaces below the oceanic floor, prokaryotes have been postulated to have a doubling time of the order of 10^3 years (Whitman et al. 1998). The probability of life based on a chemical species other than carbon is very low, but cannot be eliminated: carbon centred methodologies and approaches need to be generalized to other chemical species, whenever possible. An iterative general approach is recommended, with results obtained by one method or analysis being used to specify and direct any subsequent use of such methods or analyses.
Changes to biological material may occur during growth (e.g. mutations) or due to stress situations (formation of DNA damages or protein folding errors) if self-replicating life is detected and amplified in Earth environmental conditions. This could increase the biohazard potential of the obtained extra-terrestrial system. A continuous testing of changes in pathogenicity with human

cell lines is essential to monitor for possible changes in the toxicity of the obtained sample (for more details see Deliverable 2.2).

According to the general suggestions, a possible approach should be implemented as follows, with an increasing degree of difficulties:

1. Verification of containment materials integrity: coupons of containment materials would be incubated with sample material at different conditions and monitored for degradation using optical methods, mechanical tests and chemical analyses. This should be done in order to verify that the samples could be kept in storage container without risk of inadvertent release.
2. Pathogenicity testing: to determine adverse effects on cell lines. First tests will involve exposing human primary cell cultures (with an emphasis on epithelial cells such as skin, lung or gut cells) to sample material for at least three months, preferably using robotic technologies. Cell cultures should be examined twice a week for microorganisms that may have replicated.
3. Subsequent pathogenicity testing and possible reduction in the necessary containment level: to accommodate a variety of test systems and representative organisms from different biological domains and ecologically and economically important phyla. (This reduction in containment would not happen for a long time if life was found to ensure there wasn't long term issues with the lifeform such as mutagenesis e.g. human papilloma virus)
4. Broader-spectrum BH tests: direct culture, exposure of "small" models (e.g. mice), molecular and biological tests, genetic tests, whole organism tests, ecosystem tests.

4.3. Outcomes

Once a life detection and biohazard evaluation is complete, there are three possible outcomes (Rummel 2002):

1. **No detection of biosignatures**. In this case the containment level may be downgraded to allow controlled distribution of samples.
2. **Clear evidence of living organisms of non-terrestrial origin** In this case only sterilised samples would be distributed outside the facility and work would take place within the containment facility to better understand the living organisms. The highest priority must then be given to biological experimentation and biohazard assessment. It must be emphasized that the most likely source of life detected returned samples is expected to be

terrestrial contamination (introduced just prior to, or following the spaceflight portion of the mission, especially with a non-nominal landing).

3. **Not clear evidence of life or its absence**. For example complex carbon bearing molecules may be detected but without any other biosignatures.

5.4 Environmental Monitoring and Personnel Security Issues

The procedures for monitoring the health and safety of the personnel of the curation and the environment in and around the curation must be developed, whenever possible using existing regulations and standards (Casey Chosewood, 2009). The procedures will require a continued monitoring plan, beginning before the arrival of the samples and continuing during/after the curation work on samples.

Considering that the risk associated to samples is actually unknown, the strictest standards should apply. Because of the unique nature of potential hazards, additional controls beyond those routinely used for hazard monitoring may be required.

The monitoring plan should be designed to maintain a balance between the estimated risks to individuals, the environment, the general population and the personal and practical imposition of the monitoring plan.

The procedures should be designed in order that the data arising from life detection and biohazard testing are correlated with those from the monitoring of the curation personnel and the environment, in order to allow quick subsequent modifications of either set of tests.

Some categories of potential hazard have to be considered, as described below.

- **Physical hazard -** Standard radiation hazards already have protocols for protection and monitoring. A radioactivity measurement should be assessed and be appropriate to verify a biohazard risk. Standard radiation safety protocols should be in place prior to the arrival of the samples, but if the radioactivity does not represent a biohazard, monitoring for radioactivity can be discontinued.
- **Chemical hazard -** The presence of non-biological toxins for living organisms has to be assessed. If an unusual substance or chemical is identified, specific monitoring methods for that substance must be designed.
- **Containment -** Standards for monitoring the containment will have to be designed for use in a high BSL curation facility. Most containment measures have a degree of redundancy and duplication. For example if one HEPA filter fails on the exhaust of a BSL4 facility and secondary filter is in place. Therefore loss of protection is designed to be a very rare occurrence. However, if a breach occurs inside the curation facility it will be detected by alarm systems and quickly corrected and personnel exposures can be assessed. If a breach occurs to the environment outside the curation, a standard procedure should be

developed to assess possible consequences to the environment and to humans. Procedures for handling a breach of the SRF due to different causes (e.g. leak, disaster, security breach, etc.) should be considered in the design of the building and be reviewed using standard risk assessment procedures (HAZOP, LOPA, SWIFT). These are discussed within WP 3 and 6 of Euro-Cares.

- **Environment -** An assessment of the pre-existing conditions (environment and humans in the nearby populations) should be performed before the arrival of the samples, to define a baseline for monitoring of environmental changes. In case changes are noted after arrival of the samples, the biohazard testing results could assist in determining if the changes are related to the samples themselves. In this case, procedures could be undertaken to assess whether an undetected breach has occurred.
- **Personnel -** Before the sample's arrival a process of training and health surveillance certification for people who will work in the facility should be developed. It will include training in the use of all containment equipment and PPE, security clearances, medical examinations and tests, and a thorough program of training about procedures to be employed in health monitoring as well as on the risks and requirements for employees. During the sample handling at the facility, a schedule for regular evaluations of personnel should be established, using the same evaluation methods adopted for the baseline data collection. In particular:
    - If an exposure occurs the individual is quarantined for a defined period of time.
    - If an exposure occurs and the exposed individual then develops symptoms, the person should be transferred to a medical facility with BSL-4 containment capabilities until proper assessment of the individual is accomplished.
    - If an individual show signs of a general infection (i.e. elevated temperature, increased white blood cell count, etc.) and there is no evidence of an exposure, the individual should be treated as appropriate for the symptoms, and monitoring should continue as prescribed by the Draft Protocol.

In addition, there is the question of how long to continue monitoring of SRF personnel after the completion of life detection and biohazard testing: several factors may need to be considered in this decision, such as the protection of the workers versus the protection of the general population.

5. Techniques and Technologies of analyses for Life Detection and Biohazard

There are a wide number of proposed approaches on the techniques to use in order to investigate the presence of life and biohazard in returned samples.
As an example it is possible to define a list of scientific devices as reported by Wilson *et al.* (1999):

- **Optical Microscope**, with a range from medium to high power, to cover sample selection through to high-resolution observation of larger 'fossil'-like structures and associated minerals.
- **IR or Raman Spectrometer**, in conjunction with the microscope system, to analyse individual grains and any associated organic material.
- **Atomic Force Microscope**, allied to the stage of the optical microscope, to examine in 3D in the nm-range selected elements of the microscope field containing possible fossils.
- **Alpha-Proton-X-ray Spectrometer**, to carry out elemental analysis (excluding H), on minerals associated with 'fossil' sediments and on any associated carbonaceous material.
- **Gas Chromatograph/Mass Spectrometer**, to analyse the volatiles from carbon residues, to provide elemental, molecular, and isotopic abundances, and compositions.
- **Ion Microprobe**, in conjunction with the microscope, to analyse regions of samples associated with 'fossils' and to give structurally defined 13C/12C ratios for organic traces.
- **Homochirality detector**, to search for optical activity. This may be a dedicated optical instrument using a liquid sample or it may be simply a chiral column included in the gas chromatograph.

Another interesting approach - that was the outcome of a dedicated workshop as reported by Kminek *et al.*(2014) - provides some suggestions on how to organize the sequence of sample analysis (Table 1) and to focus on life detection (Table 2).

Table 1. Sequence of steps for sample analyses (Kminek *et al.* 2014).

Step	Sample condition	General type of analysis
1	Sample acquisition	Remote and in-situ analysis to characterise sample type and geological context
2	Solid material outside of sample container	Solid sample analysis: full sequance
3	Head space gas	Gas sample analysis: full sequence
4	Solid samples in container	Solid sample analysis: non-destructive & non-invasive
5	Solid samples removed from the container	Solid sample analysis: non-destructive & minimal invasive
6	Fluid inclusions from solid samples removed from the container	Liquid sample analysis: full sequence
7	Solid samples removed from the container	Solid sample analysis: non-destructive & minimal invasive, destructive

Table 2. Analytical techniques grouped according to their invasiveness (Kminek *et al.* 2014)

Invasiveness	Solid sample analysis	Gas sample analysis	Liquid sample analysis
Non-destructive & non-invasive	• 3D X-ray micro-tomography • Surface imaging and spectroscopy	Not applicable	Not applicable
Non-destructive & minimal invasive	• Microscopy • Fluorescence • IR, visible, UV, deep UV spectroscopy • SEM	• IR, visible, UV, deep UV spectroscopy	• Microscopy • Fluorescence • IR, visible, UV, deep UV spectroscopy
Destructive	• SEM, TEM, nano-X-ray tomography • XRD, XANES • GC-MS, GC-IRMS, FTIR-MS, LC-MS, TOF-SIMS, Nano-SIMS • Target independent biopolymer sequencing	• GC-MS, GC-IRMS, FTIR-MS, LC-MS	• GC-MS, GC-IRMS, FTIR-MS, LC-MS, TOF-SIMS, Nano-SIMS • Target independent biopolymer sequancing, flow citometry

Finally another study suggested a long list of techniques that are able to measure biosignatures according to their general characteristics: global, morphological, mineralogical, organic chemistry, molecular and biochemical, isotopic analyses (Race *et al.* 2009). Sensitivity, limitation and development needed are also reported for each technique.
Summarizing all the cited studies, as well as others of the same type not shown in this report, lead to a proposed list of techniques suitable for life detection along with details about the field of application, their efficiency and limits. Nevertheless, what is actually missing is a general, critical approach able to make a comparison between the techniques in terms of effectiveness, in order to find a ranking to prioritize techniques.

The major drivers we are taking into account are to:

- Define which technique is really important and what can be considered as optional;
- Rationalize the entire activity flow inside the curation;
- Provide a support for the evaluation of the design choices of the curation.

Starting from this idea, we focused on the building of a correlation matrix where to correlate the biosignatures with the techniques. It is known that a number of techniques can detect each biosignature and, at the same time, each technique can be applied for a number of biosignatures. Using the correlation matrix method (Akao 1994 and Cohen *et al.* 2009), it is possible to summarize all this information at a glance. It is also possible to give an extra-value to the matrix, trying to be more critical: the idea is not only to determine the correlations between the biosignatures and the techniques, but also to define how strong each correlation is. To do it, in case a correlation exists, a value was given. In particular, the scale used for the correlation value was:

- **9** - Very specific technique for the biosignature, with high resolution;
- **3** - Technique suitable for the biosignature, although not specific, and/or with medium resolution;

- **1** - No specific technique for the biosignature but still usable and/or with medium/low resolution

Another extra-value was chosen to quantify how substantial is each biosignatures, rating it in a scale from 1 to 4.
The results are shown in Table 3, where:

- The biosignatures are organized per area: morphological, chemical, biochemical, isotopic analysis, mineralogical;
- An importance value is given for each techniques;
- A correlation value is given, if the correlation exists;
- The list of techniques is organized in order to have a quasi-diagonal matrix, where, for each biosignature, the higher correlation value techniques (9, in green) are written in the first available columns

Table 3. Biosignatures and analytical techniques correlation matrix used for life detection

BIOSIGNATURES		IMPORTANCE	Optical microscopy	Electron microscopy - SEM	GC-MS	LC-MS	MALDI-TOF	Fluorescence microscopy	Raman spectroscopy	High Performance Liquid Chromatography (HPLC)	Polymerase Chain Reaction (PCR)	Enzyme-linked immunosorbent assays (ELISA)	Fluorescenct in-situ hybradization (FISH)	Sequnecing	Chromatography	Protein microarray / Marker Chip	SIMS	Isotope Ratio Mass Spectrometry (IRMS)	13C-NMR	SEM-EDX	XRF	X-Ray CT	XRD	Electron microscopy - TEM	NMR	FTIR	Marker chip with antibody	Capillary Electrophoresis (CE)	MC-ICP-MS
Morphological	Size of single cell - Size of targets	4	9	9				3																3					
	Numbers of single bacteria - Number of targets	4	9	9				3																3					
	Population size (colonies)	2	9	9																									
Chemical	Chemical composition	4			9	9	9																		1	3			
	Chirality	4			9	9	3								1														
	Organic molecules	4						9	9	9							1			1				3		3			
Biochemical	DNA, RNA	4									9	9	9	9															
	Organic pigments	4					3		9	3					9											1		3	
	Protein	4					9					9		9		9													
Isotopic analysis	Isotopes, Isotopologues	3			3												9	9							1			3	
	Isotopomers	2																	9										
Mineralogical	Elemental analysis	4																		9	3								
	Structure, Mineralogy	4	3					1	3											1	9	9	9			3			

TECHNIQUES

It is important to recognize that some of the measurements designed to satisfy the requirements of planetary protection are also of extreme high importance to the preliminary characterization of the samples and scientific research objectives. It should also be highlighted that the elements of matrix (biosignatures, techniques, correlation and given values) are the results of a collegial work done by the working group of EURO-CARES Work Package 2.

The important aspect that we would like to underline is that by using the correlation matrix method progression towards a quantitative evaluation approach in selecting the suitable techniques for LD, able to deal with numbers and indicators was achieved.

The correlation matrix has not to be considered as a firm milestone, as it has to be considered that future developments or enhanced technique's sensitivity can lead to a new version. This is "photography" of the state-of-art of what is available now to detect life and possible biohazard.

Analysing the correlation matrix it is now possible to state some thoughts:

- The initial list includes 27 different techniques;
- The number of high-correlation techniques (given a value of 9, in green) are 20;
- There are 8 techniques able to identify 2 or more biosignatures;
- If only the high-correlation techniques are considered, the minimum number of techniques needed to identify all the biosignatures is 9. This number decreases to 7, if also the medium correlation (given value 3) techniques are considered;
- If only the high-correlation techniques are considered, the minimum number of techniques needed to identify all the high-importance (given value 4) biosignatures is 7. This number decreases to 6, if also the medium correlation (given value 3) techniques are considered.

At this stage of the analysis, the result can help to define the major drivers, cited at the beginning of this paragraph:

- The matrix allows to select the most important techniques;
- This leads to defining the procedures to be performed inside the curation, that are strictly related to the techniques;
- Starting from the obtained results it is possible to facilitate the design choices: choosing a technique that allows a better evaluation of curation dimensions (depending on the size and position of the instrument, etc.) and layout (depending on its position, the compatibility with other instruments, the need of auxiliary gear, etc.).

A further analysis can be done where four more indicators are included:

- Biosignature occurrence: shows the number of times that the each biosignature is detected by a different techniques (i.e. organic molecules can be detected by 9 different techniques);
- Techniques occurrences: shows the number of biosignatures that can be detected by a single techniques (i.e. FTIR can detect 5 different biosignatures);
- Non Destructive/Destructive Coefficient: it gives an added value to disentangle destructive and non-destructive techniques, (1 if the technique is destructive, 1.1 if partially destructive, 1.2 if partially destructive/non destructive , 1.3 if not destructive);
- Technique mean value is the technique mean correlation with the detected biosignatures (i.e. FTIR, that has 5 occurrences with biosignatures, has a low technique mean value equal to 2.2);
- Techniques importance rating is calculated, for each column (technique), as the sum of the products of the biosignatures importance, the correlation value and the non-destructive/destructive coefficient (i.e. the optical microscopy technique has the higher value).

Among the previous indicators, the most important is the techniques importance rating, which at the same time is dependent on the biosignatures, the importance, the correlation value, and the techniques occurrences. The new correlation matrix is reported in Table 4.

The use of this enhanced analysis can help determine the choice of the techniques for life detection and biohazard assessment and similarly, can be applied for the instruments and techniques used for the samples preliminary characterization procedures.
A further study is needed in order to assess other techniques feature, such as the compatibility with clean rooms and BSL-4 laboratories, the presence of waste, the easiness of samples preparation. At this moment, we still are acquiring the data to perform this analysis, and results will be presented in next WP2 reports.

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Table 4. Biosignatures and analytical techniques correlation matrix used for life detection, where further parameters are considered: biosignatures occurrence, techniques occurrence, non-destructive/destructive coefficient, technique mean value and techniques importance rating.

BIOSIGNATURES		IMPORTANCE	Optical microscopy	Electron microscopy - SEM	GC-MS	LC-MS	MALDI-TOF	Fluorescence microscopy	Raman spectroscopy	High Performance Liquid Chromatography (HPLC)	Polymerase Chain Reaction (PCR)	Enzyme-linked immunosorbent assays (ELISA)	Fluorescenct in-situ hybradization (FISH)	Sequnecing	Chromatography	Protein microarray / Marker Chip	SIMS	Isotope Ratio Mass Spectrometry (IRMS)	13C-NMR	SEM-EDX	XRF	X-Ray CT	XRD	Electron microscopy - TEM	NMR	FTIR	Marker chip with antibody	Capillary Electrophoresis (CE)	MC-ICP-MS
		TECHNIQUES	4	4	3	5	5	9	4	6	4	5	1	2	8													
		TECHNIQUES OCCURRENCES	4	3	3	2	4	4	3	2	1	2	1	2	1	2	1	1	1	3	1	1	4	2	5	1	1	1
		TECHNIQUES MEAN VALUE	7,5	9,0	7,0	9,0	6,0	4,0	7,0	6,0	9,0	9,0	9,0	9,0	5,0	9,0	5,0	9,0	9,0	3,7	4,3	9,0	9,0	3,0	1,0	2,2	3,0	3,0	3,0
		TECHNIQUES IMPORTANCE RATING	102	90	81	72	96	64	84	48	36	72	36	72	40	36	31	27	18	44	52	36	47	55	8	43	12	12	9
		NON DESTRUCTIVE/DESTRUCTIVE COEFFICIENT	1,3	1,1	1	1	1	1,3	1,2	1	1	1	1	1	1	1	1	1	1,3	1,1	1	1,3	1,3	1,3	1,3	1,3	1	1	1
		TECHNIQUES IMPORTANCE RATING (*ND/D)	133	99	81	72	96	83	101	48	36	72	36	72	40	36	31	27	23	48	52	47	47	55	10	56	12	12	9
Morphological	Size of single cell - Size of targets	4	9	9																								
Morphological	Numbers of single bacteria - Number of targets	4	9	9																								
Morphological	Population size (colonies)	2	9	9																								
Chemical	Chemical composition	4			9	9	9																		1			
Chemical	Chirality	4			9	9	3								1													
Chemical	Organic molecules	4						9	9	9							1		1		1			3		3		
Biochemical	DNA, RNA	4									9	9	9	9														
Biochemical	Organic pigments	4					3		9	3					9					1				3			3	
Biochemical	Protein	4			3		9				9	9	9	9		9												
Isotopic analysis	Isotopes, Isotopologues	3															9		6									
Isotopic analysis	Isotopomers	2																										
Mineralogical	Elemental analysis	4																		9	3							
Mineralogical	Structure, Mineralogy	4	3					1	3											1	9	9	9			3		

6. Sample selection

Rummel *et al.* (2002) presented the first draft protocol for LD on Mars Sample Return samples including both destructive and non-destructive methodologies. In principle it could be assumed that sample used for LD will not be available for further research analyses anymore, i.e. sample preparation and analytical techniques for LD are all considered destructive. However, instruments for LD can use invasive techniques or not and can be grouped according to their capability to preserve or not the samples in their original state.

A sample collected on Mars would have a complex structure, which will depend on the site where the sample was collected. Samples are expected to show great heterogeneity according to the site where they will be collected that will reflect the scientific objective of the mission. Thus samples will have a complex aggregated structure, which depends on physical, chemical and potentially, biological interactions between mineral particles and organic matter. The aggregated structure of samples creates heterogeneous microenvironments with highly different liquid, gas and organic concentrations, pH and oxidation-reduction conditions. Before the fraction of the sample is selected to be used for LD, the sample should be categorized on the basis of different physical and chemical properties. Prior knowledge or at least approximate estimates of the spatial variation and structure of the target are also highly valuable when designing sampling procedures. The proportions of mineral and organic fractions would differ extensively among different samples collected from Martian soils with properties that can range from layered sedimentary rocks, hydrothermal rocks, breccia or regolith.

The number of independent sub-samples required to assess LD depends on the precision with which biosignature is being measured and it is depended on the spatial scale i.e. research questions might be different at different scale. For its nature, the selection of sub-samples for LD is based on statistical approach. Statistical studies will address the spatial autocorrelation of sample microbiological properties at different spatial scale. This approach is, again, the requirement for a quantitative measuring procedure. The range of spatial autocorrelation regulates the threshold distance at which sub-samples can be considered to be independent in terms of biosignatures and, thus, sampled.

In Rummel *et al.* (2002) LD and BH tests were described and they proposed as rough estimate a 10% as a reasonable amount of sample to be used for LD and BH assessment. The same figure was used by DeVincenzi and Bagby (1981). Furthermore sample sizing calculations were assumed by MEPAG E2E-iSAG (2011) for planetary protection aspects and the mass of 1.5 g of each sample was assumed reasonable amount to conduct LD and BH tests. However, according to the techniques chosen for life detection, the amount of samples needed has to be evaluated accordingly. With

our approach it is possible to provide a prioritization of the techniques used for LD-BH. Thus, an actual amount of sub-sample can be evaluated from those techniques that are selected on the basis of prioritization. The final amount of sub-sample is the result of the technique sensitivity, fraction of variable observable (molecule, polymer, cell, etc.), number of analysis repetition.

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