DEVELOPMENT OF PROTOCOLS FOR MICROBIOLOGICAL CONTROL IN ALTAMIRA CAVE

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ABSTRACT

Art Caves are threatened by uncontrolled growth of microorganisms, especially fungi. At the same time, in spite of the extreme ambiental conditions, they are inhabited by very diverse microorganisms. Altamira Cave contains one of the best collections of parietal art and is also threaten by potential microbial deterioration. In order to preserve the state of paintings, after a research project developed in 2013-2014, came out the "Plan de Conservación Preventiva" (PCP) for the Altamira Cave. Among the objectives of the PCP is the systematic control of the cave microbiota, and methods investigated here will become standard for these purposes.

Cultivation of bacteria from natural habitats shows that only a small fraction of the bacteria present in such environments, can be cultivated under regular laboratory conditions and clearly indicate that alternative methods are required for quantitative purposes. Therefore, we have used alternative culture methods, as well as other quantitative methods based on fluorescent staining of microbes such as direct counting by fluorescence microscopy and flow cytometry. These techniques have been applied to water, air and soil samples and the results compared and combined with qualitative biodiversity analysis performed by 16S rDNA sequencing from isolated colonies or directly from the cave samples.

INTRODUCTION

Microbial activity in caves has been object of increasing interest within the past decade. Caves provide environmental extreme conditions, which are under energetically unfavorable and nutrient-limited conditions for individual species and microbial communities. There are different type of caves: 1) Karst caves, such as limestone caves, the most common type of show caves, where rain reacts with CO_2 forming carbonic acid that will dissolve soluble rocks. 2) Volcanic caves, originated from flowing lava and the effects of volcanic gases. 3) Sea caves, generated by the action of the waves that bump into the rocks and cliffs. 4) Ice caves, formed by the wind and water that erodes against the surface and freezes. 5) Sandstone caves, formed by the exposure of the rocks to the water and wind during time.

In these severe ecosystems, microbes remain as free living-cells in the air, as plankton in water, associated with ceiling or wall surfaces or inhabiting in the soil. As they are the primary producers in cave ecosystems and support a wide range of organisms, they need to get nutrients and energy to grow and survive. General lifestyles of microbes within caves can be: photoautotrophs which acquire energy from light and use CO_2 as principal source of carbon, chemoautotrophs that get energy from the oxidation of chemical compounds and fix CO_2, and heterotrophs that get energy from oxidation of organic matter.

Therefore, biotic influence plays a pivotal role in the geological, physicochemical and ecological development of the cave as they are able to colonize rock surfaces, water and air and use the organic matter within them to growth and interfere in the natural biogeochemical processes of the cave such as mineral crystallization processes. In the analysis and identification of these complex microbial populations are in first lane phylogenetic studies which are critical in order to reveal knowledge for novel and unknown bacterial communities such as for example profiling phylogenetic marker genes as 16rRNA gene that has been successfully applied. The classic method consists in amplifying the 16S rDNA region with specific primers and cloning it into a host vector to generate a 16S rDNA library. Then, selected clones carrying the DNA fragment of interest are sequenced for identification and at the end a taxonomic classification of bacterial species has been made. Through this method, *Proteobacteria* has been reported to be the dominant phylogenetic group of bacteria in current carbonate caves as we can see in Table 1(Zhou et al., 2007).

With these molecular analysis, it has been revealed that $CaCO_3$ crystals are bioinduced by the action of a dense network of microorganisms. Bacteria uses, as a source of fuel, the dioxide of carbon from inside the cave to dissolve the carbonate and produce calcite crystals. Cuezva et al. (2012) reported the biogeochemial role of *Proteobacteria* and *Actinobacteria* in the formation of $CaCO_3$ crystals by 16rRNA analysis

Table 1. Phylogenetic diversity of bacteria in different karstic caves.

Citations	Nullarbor Caves (Holmes *et al.,* 2001*)*	Altamira Caves (C.Schabereiter-Gurtner *et al* ,. 2002)	Llonín and La Garma Caves (C.Schabereiter-Gurtner et al,. 2003)	Lechuguilla and spider Caves (Northup D. *et al* ,. 2003)	Lower Kane Cave (Engel *et al.,* 2003*)*	Niu Cave (Zhou et al,. 2007)	Herrenberg Cave (Rusznyák*et al,.* 2011*)*	Kartchner Caverns (M.ortiz *et al,.* 2014)	Pnahkyndeg Cave (S.De Mandal *et al,.* 2015)
Proteobacteria	40.0	52.3	41.1	34.9	92.7	42.6	44.7	28	12.03
Acidobacteria		23.8	16.5		5.6	18.6	9.4	2	
Plantomycetes	5.7	4.8				9.0	3.2	4	12.41
Bacteroidetes	8.6	9.5	5.9		1.7	2.1	11.3		
Chlorofexi		4.8	1.2			7.5	3.2	3	29.97
Nitrospirae	5.7		3.5	15.5		8.0	6.5	2	
Actinobacteria	5.7	4.8	20	11.6		6.4	3	7	22.55
Firmicutes			10.6	37.9			4	3	
Gemmatimonadetes						2.7	0.8		
Cyanobacteria								2	
Verrucomicrobia							1.1		
Fibrobacteres							0.3		

More recently, 16S rRNA gene analysis is performed by high-throughput Next Generation Sequencing (NGS) techniques that are strongly emerging in the microbiology field. NGS provides deeper insights into the number and potential function of genes within the microbial community. Furthermore, NGS has the power to obtain in a single run hundreds of thousands of sequence reads providing huge amount of sequence information data.

While sequencing methods improved significantly, quantification of bacteria it is still a real challenge, and even more to cultivate samples taken from natural habitats in agar plates. Only a small fraction of the bacteria in natural habitats can usually be cultivated under standard laboratory conditions. This difference between total cell counts and cultivable cell counts is known as the "great plate count anomaly". Growth media with solid agar is the most used approach to culture bacteria but is not sufficient. Generally the visible colonies of bacteria that can be found in a cave consist of a mix of different types of bacteria growing together and most of them do not growth *in vitro* if separated. Certain bacterial types that do not growth in conventional media may have slow-growth, need codependency with other species for metabolic cooperation or crossfeeding or just some signaling molecules that are present within natural habitat. Also it has been described that these uncultivated bacteria's demand explicit growth requirements such as specific nutrients, pH and temperature conditions or levels of oxygen and humidity. The best alternative is to stimulate their natural environment *in vitro* in order to get such growth promotion. But till now, the many limitations and laboriousness of isolation work, undermine the efficiency of microorganism cultivability. In addition, accessing to this missing microbial diversity is critical for two reasons: it can play important roles in the biogeochemical of the cave and represents an untapped mine of bioactive compounds.

Plate count improvements

It is well known that the number of bacteria in a sample able to form colonies on standard solid media is only a small proportion of the total number of bacteria present in that sample. For complex natural samples it is not possible that all components grow under a single culture condition. Furthermore we know that bacteria may undergo periods of decreased biological activity and may be also be in non culturable states (viable but not cultivable, persistors, etc). It is then necessary to understand that plate counts will always be partial and the results provided by these methods must be considered as an indicator of total bacterial population. At most, using exactly the same culture conditions along time, we can assume that the proportion of colony counts also remains constant over the time.

Several methods have been devised that allow some growth of naturally occurring bacteria, but these find application in Biotechnology for the discovery of novel natural compounds, but are not useful as rapid counting methods to substitute standard plate counting. A few reports in the literature suggested changes in culture methodology amenable to practice. Tanaka et al., (2014) published that some inhibitors could be formed in culture media if agar was autoclaved together with salts and other compounds in the culture media and suggested that some increase in plate count could be obtained if nutrients, salts and agar were autoclaved by separate and mixed after. Replacing agar with another solidifying agent such as gellan gum has been also proposed to improve colony forming capability of bacteria in natural samples. Also, adding catalase, cyclic AMP or lactones to solid medium to increase culturability has also been reported in the literature. However, these additions are directed to specific bacterial groups, either showing Quorum Sensing, or requiring specific growth factors. Furthermore, the additives needed are expensive and were not considered with a general value to improve colony recovery. In these experiment, we tried to autoclave the agar separately from the phosphate as reported but no significance in growth promotion was detected. Counting viable cells is usually done by diluting the original sample onto an appropriate culture medium such as TSA or BA (Figure1). After incubation, total number of cells, referred as colony-forming units (CFUs), are calculated. The equation would be:

$$\boldsymbol{CFUs = \frac{1}{V_p} \cdot n_b \cdot d \cdot v_o}$$

Where;

n_b = total number of bacteria

V_o = starting volume

V_p = plated volume

d= dilution

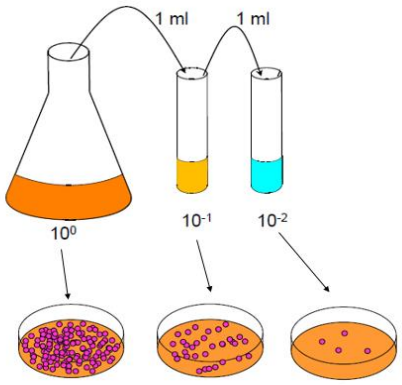


Figure1. Schematic representation of standard plate count

As any other technique, SPC has its advantages and disadvantages. It doesn't measure the entire bacterial population but rather those who grow in particular growing conditions. Furthermore, frequently colonies can be clumped together increasing error in reporting. These drawbacks make the technique useless when characterize or count the total number of bacteria in complex microbial ecosystems.

Fluorescence microscopy

As plate counts are deficient in estimating accurately and reliably the total number of living bacteria and also general growth conditions provided in the laboratory create selective pressures that act against microorganism upgrowth, new alternative direct counting approaches appeared, such us microscopy or flow cytometry. In fact, more and more researchers nowadays combined indirect methods with direct methods, for enumerating viable microbial populations.

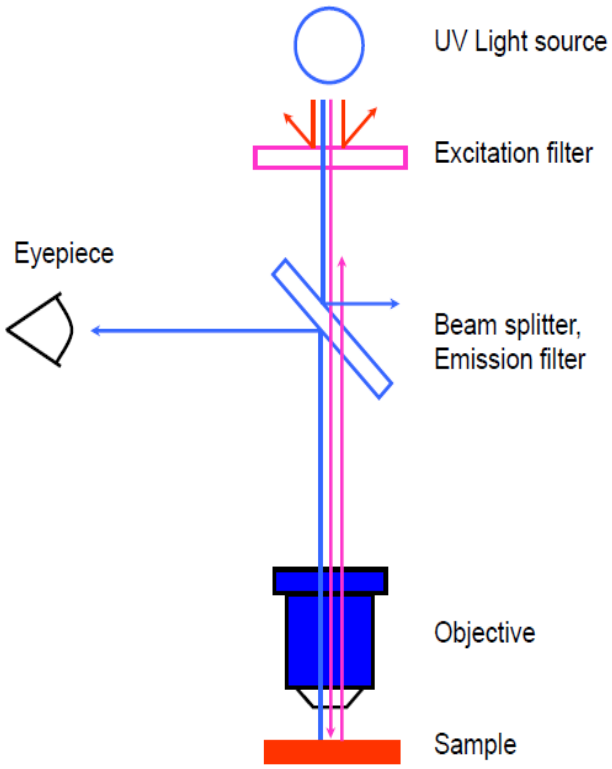
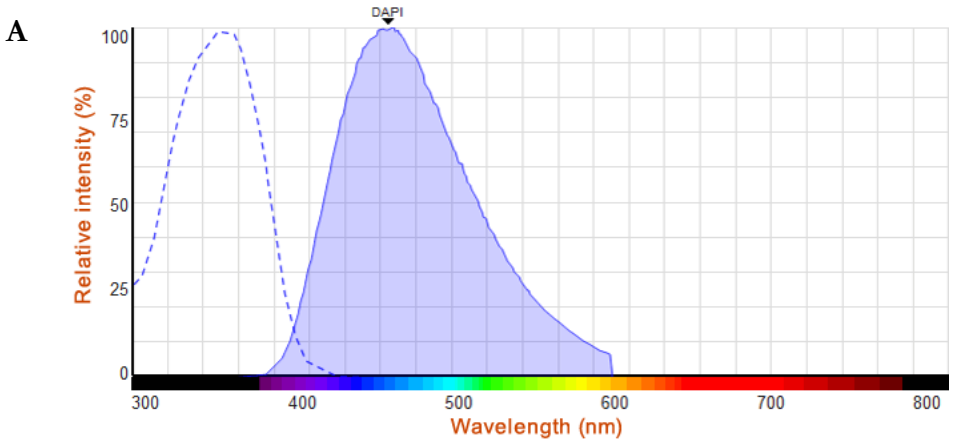


Figure 2. Simplified diagram of the operation of a fluorescence microscope

Epifluorescence microscopy (EFM) consists in irradiating the sample with correct wavelengths for the stain that has been selected through a wavelength selective excitation filter. Then, selective wavelengths reflect though a dichromatic mirror (beam splitter) that split a beam of polychromatic light in various monochromatic beams with different wavelengths that impinges upon the sample once passed the microscopy objective acting as a condenser (Figure 2). Subsequently, the sample emits its own light that passes back to the beam splitter and is filtered by the emission filter which

discard the unwanted emitted fluorescence. Finally, the microscopy objective serves as the image-forming light gatherer that reaches the eyepiece or detector.

The effective separation and detection of excitation and emission spectra of the dye is crucial for obtain high efficiency illumination of the sample. Epifluorescence microscopy on 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) stained samples is recently prefered for quantification of total bacteria counts. DAPI has excitation peak approximately 350nm (UV) and fluorescence detection through a 450/50nm bandpass filter (Figure 4). DAPI emits blue fluorescence upon binding to AT regions of DNA samples. With DAPI is common to have several of background fluorescence problems like unbound DAPI or DAPI bound to non-DNA material that may fluoresce a weak yellow. For soil samples, which consisting in highly heterogeneous environments containing varied proportions of water and organic and inorganic materials, has made it difficult to enumerate bacteria without masking effects. The optimal concentration of fluorochrome used to stain bacteria and the masking effect of particles collected in filters that are not bacteria are technical issues of great importance in obtaining accurate estimates. Also, as our samples are constituted of a variety of different bacteria, each species of bacteria displayed a different fluorescence/cell count relationship. SYBR dyes have also proved to be very efficient nowadays such us SYTO BC. This stain is a mixture of the best SYTO dyes for bacterial staining, permeable to all cell membranes and show a large fluorescence enhancement upon binding nucleic acids. When bound to DNA, SYTO BC has excitation and emission spectra of 485 and 500 nm (Figure 4).



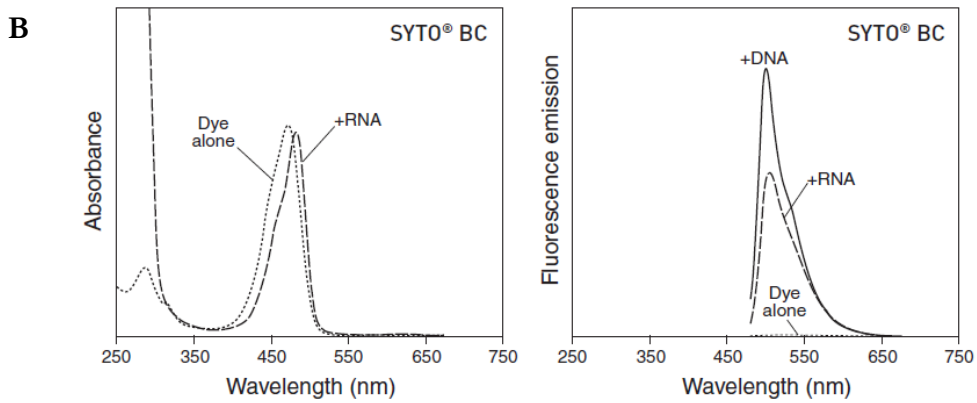


Figure 4. Excitation and emission spectra of (A) DAPI and (B) SYTO BC stains.

Another way to improve image visualization is related to the membrane filters. Previously, polycarbonate membrane filters were stained with Irgalan Black or Sudan Black to prevent background fluorescence. Nowadays, there are available commercially black polycarbonate membrane filters avoiding the above steps. These polycarbonate membranes contains uniform cylindrical pores that provide the sample distribution in one plane across the entire exposed membrane surface. Several considerations when preparing samples for microscopy are usually relevant. First consideration is to place the filter without wrinkles over the microscope slide is important to prevent bacteria to be situated in different Z or planes. Second consideration is the mounting media itself, each media has a particular refractive indice used with specialized objectives to achieve the highest numerical aperture and resolution possible. In these experiment, sample was filtered vacuum onto black polycarbonate membrane filter and immersion oil has been placed at the interfaces between the microscope slide and the sample preparation and between the cover slip and the sample (Figure 5).

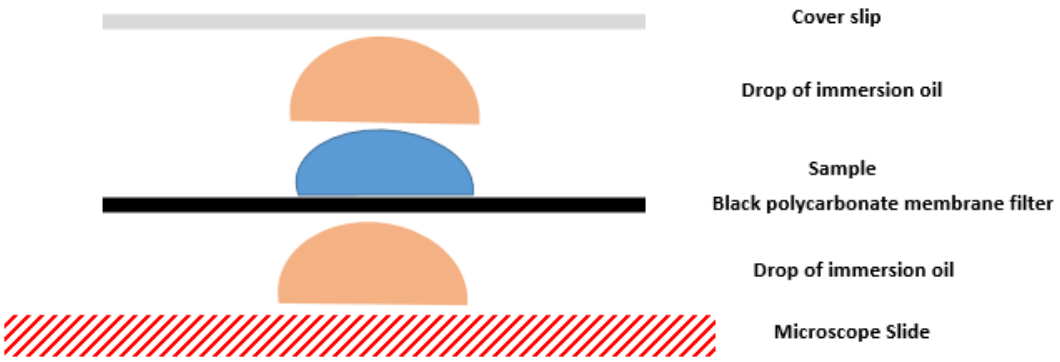


Figure 5. Slide preparation in the fluorescence microscopy.

Another problematic challenge in these technique is bacteria counting. Kichman et al., 1982 has determined that the optimal scheme for enumerating bacteria is to increase the number of fields per filter at least more than seven to decrease sample variance. Otherwise, if the number of bacteria drops below 20 per field then the coefficient of variance increases. Thus, increasing the number of fields per filter decreases the problem of the uneven distribution of bacteria. Our approach to direct counting consisted in counting 9 fields per filter per sample. For measuring the number of cells per filter, we calculate the total area of the field divided by the total number of the total area of grids in a view (Figure 6). Thus, there are 3,03 X 10^4 fields /filter. Total number of bacteria / filter can be obtained by multiplying 3,03 X 10^4 fields /filter by the total average of bacteria counts per 9 fields for each sample.

$$A_f = \pi \cdot r^2 = \pi \cdot 12{,}5^2 = 490{,}9mm^2$$

$$A_G = 0{,}12749 \cdot 0{,}12723 = 0{,}0162mm^2$$

$$\frac{A_f}{A_G} = \frac{490{,}9}{0{,}0162} = 3{,}03 \cdot \frac{10^4 \text{fields}}{\text{filter}}$$

$$\boldsymbol{\frac{bacteria}{filter} = \bar{x} \cdot 3{,}03 \cdot \frac{10^4 field}{filter}}$$

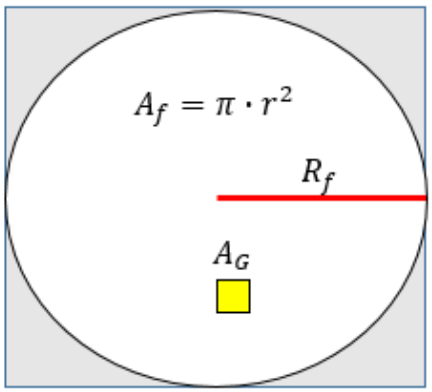


Figure 6. Cell density calculations.

Where;

A_f = Area of a field
A_G =Area of grid in field view
$\bar{x}$ = Average bacteria per 9 fields

Flow Cytometry

Flow cytometry (FCM) is another counting technique that has been successfully applied for detection of mixed populations in environmental samples based on their cytometric characteristics. FCM provides the measurement of size and complexity of each bacteria and also the fluorescence emission produced by the laser beam. Scattered light (SS) is related to cell size and forward light scatter (FS) is related to the complexity of bacteria meaning that larger objects will refract more light than smaller ones. This parameters are intrinsic to the sample but fluorescence it is not. The basis of this technique consists in injecting the sample in a liquid called the sheat fluid. As the sheath fluid moves, narrows the chamber creating a single stream of particles that passes through one or more beams of light. Both light scattering and fluorescence emission, refer also as detectors, and provides information about the particle's properties (Figure 7).

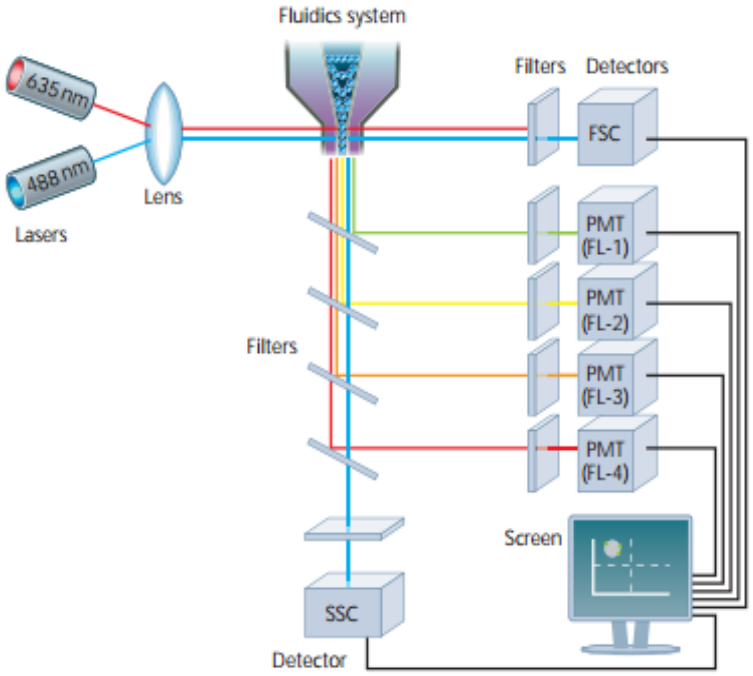


Figure 7. Schematic view of the flow cytometry method.

Regarding fluorescence, as EFM, there are specific fluorochromes that exhibit fluorescence when binding to nucleic acids such us SYTO BC. The wavelength of excitation and emission are also crucial for the analysis of total bacterial cell counts. Counting was done adding a known amount of reference beads within the sample. This process is not sometimes so accurate because it can get contaminated and needs prior sonication to avoid aggregation, otherwise provides an internal standard for quantification. It could happen that two particles can pass together as one and this phenomenon is known as coincidence. This effect is much higher when handling concentration level of $2{,}5 \cdot 10^6$ cells ml^{-1}. Then, is very helpful to dilute samples that present high concentrations in water or buffer before counting. So calculation of total number of bacteria ml^{-1} is done by multiplying total number of bacteria estimated through FCM by the known amount of beads (10^6), and by the total number of beads within the sample.

$$\frac{\boldsymbol{bacteria}}{\boldsymbol{mL}} = \frac{(\boldsymbol{n_b} \cdot \boldsymbol{10^6})}{\boldsymbol{n_m}}$$

Where;

$$n_b = number\ of\ selected\ events$$

$$n_m = total\ number\ of\ microspheres$$

Altamira Cave

Altamira is the most important Palaeolithic art site of Spain. It has over 260 valuable paintings and engravings and it was declared as UNESCO world heritage. The Altamira Cave was closed to the public in 1977 due to the presence of deterioration in paintings produced by decades of visitors. During the last years, it suffered several openings and closings with studies of research groups in between. Professor Villar and his team did studies in the 80's proposing an access of 11.000 people per year distributed in groups of 5 and 20. Later studies lead to suspension of the visits in 2002 due to variations of air temperature and CO_2 concentration in the cave halls produced by visitors groups could damage the paintings. To date, researchers have developed a preventive conservation plan (PCP) for the preservation of Altamira Cave paintings. The PCP objective is to design and implement systematic work methods and define response mechanisms against risk situations that endanger the preservation of the Altamira Cave. This strategy is based on the identification of the risk detected during study, in the definition of actions to avoid, impede or minimize it and in the implementation of monitoring and control procedures. The actions of PCP gather biodeterioration control, monitoring of radon gas concentration and ambient conditions, checking of moisture and drip points, reporting of the conservation paintings state and study of geological-structural stability.

Altamira Cave is situated between the municipalities of Santillana del Mar and Reocín (Cantabria) at 158 meters above sea level, only 4 km from the Cantabrian Coast. Altamira has a length of 270 m, along we can find prehistoric prints and paintings. It has an average depth of 8m, minimum 5 and maximum 22m. The cave is divided in several halls that correspond to the following denomination: Vestibulo, Cocina, Policromos, Pasillo de Policromos, Cruce, Sala de los Muros Pasillo Gran Sala, Hoya, Gran sala, Sala del Pozo and Cola de Caballo. Sala de Policromos is where most of the paintings concentrate.

Ambiental conditions of the cave are generally well known and have been extensively described (Soledad Cuezva, tesis doctoral 2008).

Humidity, either from drip infiltration of rainwater or natural condensation from different season. It is close to saturation most part of the year. Temperature is also fairly constant between 14-15ºC. CO_2 concentration is much higher than in the exterior, may reach 4000-5000 ppm at some times of the year. All these ambient parameters, Radon gas concentration, water chemical analysis, etc show typical yearly variations already well stablished.

Main risks for the paintings identified so far included:

- Pigment loss caused by water dripping from the ceiling of the Polychromes hall, either of infiltration or condensation origin.

- Biodeterioration from microorganism proliferation.
- Human impact

All of these factors can cause serious hazard to the preservation of the Altamira Cave and its paintings. These factors have a connection among them. For example, the colonization and spread of microorganism depends on exchange rates of aerobiological and atmospheric gases from the exterior of the cave. In Altamira, the higher concentrations of airborne bacteria and fungi are found in the halls and galleries closer to the entrance (Sala de Polícromos, Vestibulo and Cruce) where there is a connection with the exterior. Despite of being the farthest hall, Sala de los Muros is also confirmed to have increasing numbers of bacteria perhaps because of a putative entry from the outer atmosphere that favors the entry and dissemination of microbial colonization's (García Antón et al., 2013).

The management and conservation of show caves has to be carefully studied and monitoring the rates of microbiota inside the cave is critical because if these levels exceeded they can cause severe problems in the conservation of the cultural cave heritage. The case of Lascaux Cave is the best well known example so far of microbial crisis. Lascaux Cave was discovered in 1940 and was closed in 2001 by a *Fusarium solani* plague (Bastian et al., 2010). First, a treatment with benzalkonium chloride eliminated *F.solani* but also selected clonal populations of *Ralstonia* and *Pseudomonas* highly resistant to the biocide. Later on, these resistant clones were replaced by an outbreak of *Scolecobasidium tshawytschae* isolated from the outbreak of black stains on the walls. Apparentely, degradation of the biocide and the presence of other carbon sources led the appearance of *S. tshawytschae.* It is has been described many times the correlation between bacteria, fungi and arthropods. In Lascaux cave the presence of the collembolan arthropod species *Folsomia candida* was observed to feed mostly on *F. solani* but also on *S. tshawytschae. F. candida* produced black faecal pellets that act as vector of entomopathogenic fungi (Bastian et al., 2009)

Regarding Altamira Cave, the principal biological deterioration risk comes from the presence of three types of visible colonies through all the cave that has been described previously in literature (Soledad Cuezva, tesis doctoral 2008). Yellow colonies between 2-5 mm found in the wall of the entrance and are composed principally of *Proteobacteria*, *Acidobacteria, Actinobacteria* and *Firmicutes*. Grey colonies (2,5-1 mm) established around ceiling cracks and walls of Polychrome access corridor, entry, corridor of Cruce hall and sala de Muros and consisted of *Proteobacteria* and *Actinobacteria.* Finally, white colonies (3-1 mm) composed mainly by *Proteobacteria* and *Planctomycetes* and are located on the ceiling of Polychrome and entry.

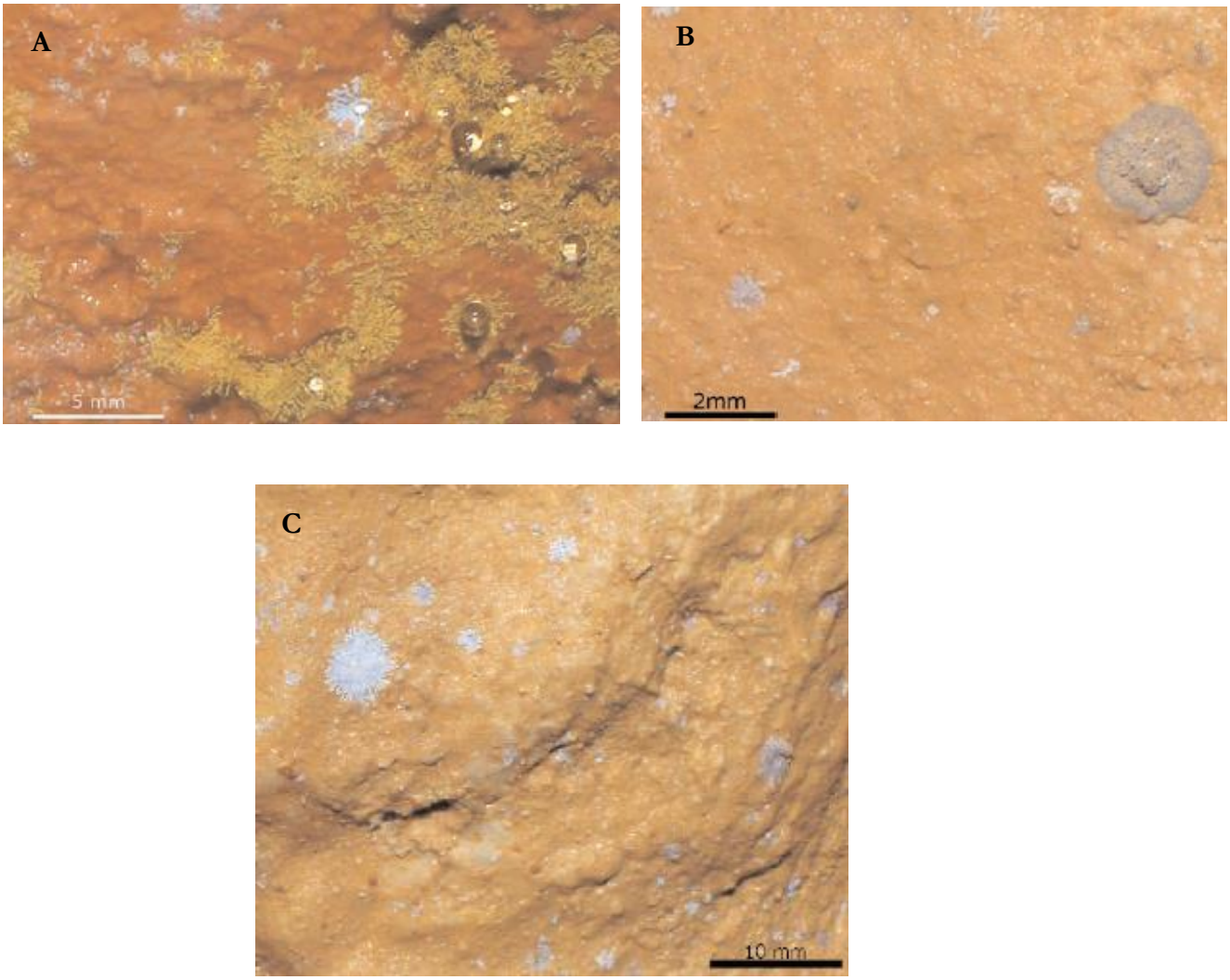


Figure 8. General aspect of the three types of colonies that we can find in a wall of Altamira. A Representation of yellow colonies located in the projection of a roof. B Representation in detail of grey colonies. C Representation in detail of white colonies. (Soledad Cuezva, tesis doctoral).

In general there is a gradient in the amount of colonies, especially yellow colonies, being more abundant near the cave entrance and decreasing as we move inwards. The Polychrome Hall shows some colonization but it does not affect significantly main figures. Furthermore, artificial structures in the corridor leading to the Polychrome Hall seems to represent a barrier for colony advance.

Another visible form of deterioration is the formation of carbonate deposits of different forms produced by the interplay of biologic agents and natural carbonate equilibrium in carbonate caves. If these carbonate formations, known as speleothems form on paintings, they represent a risk for conservation. In karstic environments, microbial growth is typically found associated with mineral biofabrics of $CaCO_3$ as it has been mentioned above. In Altamira, grey and white colonies have been observed to be associated with microcrystalline $CaCO_3$ aggregates. Calcium carbonate minerals which

are composed by three different atoms (calcium, carbon and oxygen), can be dissolved by water easily and forms deposits of calcium and dolomite in several steps:

1st reaction: $H_2O + CO_2 = H_2CO_3$

(water + carbon dioxide = carbonic acid)

2nd reaction: $CaCO_3 + H_2CO_3 = Ca_2 + 2 (HCO_3)$

(limestone + carbonic acid = calcium + carbonic acid)

3rd reaction: $Ca_2 + 2 (HCO_3) = CO_2CaCO_3 + H_2O$

(calcium + carbonic acid = calcite +water)

3rd reaction: $Ca^{2+} + Mg^{2+} + 4\ HCO_3^- = MgCa[CO_3]2 + 2CO_2 + 2\ H_2O$

(calcium + magnesium+ carbonic acid=dolomite + carbon dioxide+water)

These calcium deposits named as speleothems can form four different limestone formations:

-dripstone, mineral formations caused by rain water that can that grow upwards from the cave floor (stalactites) or downward from the cave roof (stalagmites). Also, these formations may extend producing columns that can go either up and down. Straw is another form of dripstone forming hollow tubular stalactites.

- flowstone, mineral deposits that resemble a thin sheet by precipitation from flowing water such us shawls when the water reaches the roof.

-Pore deposits when water enters through pores and cracks depositing layers of calcite crystals.

-Pool deposits that are calcite crystals built up within cave pools such us rimstone, where the calcite is deposited at the edges of cave pools.

OBJECTIVES

Control of microbial activity is important for preserving cultural heritage in caves because of their biodeteriorative potential. Accurate determination of total bacteria is a bit tricky but can be accomplished by direct or indirect techniques. Culture dependent methods as SPC are selective and shown to systematically underestimate numbers of total bacteria. Moreover, no universal conditions are settled for enumerating all microorganism by viable plating. On the contrary, direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. The objectives of this study will be to:

1. **Control the risk of biodeterioration of the paintings in Altamira Cave.**
2. Develop and standardize protocols for systematic control of Altamira Cave microbiota.
3. Evaluate different counting techniques for microbial quantification.
4. Analyze the culturable microbial diversity in Altamira Cave.

MATERIALS AND METHODS

SAMPLING

Altamira cave is located between the municipalities of Santillana del Mar and Reocín (Cantabria) at 158 meters above sea level. The cave has an average depth of 8m, minimum 5 and maximum 22m. Samples of different sites of the cave were obtained between the January-April months of 2015 using different techniques. Sites of samples are indicated with blue letters in the map in Figure 9.

Aerobiology samples.

Air samples for aerobiological analysis were taken at all the points indicated. Two different sampling procedures were used.

For direct plate counting a SAS-duo sampler system was used. The system has two sampling heads for standard 90 mm Petri Dishes. The unit was operated to collect 500 L air per head (and per plate) in a period of three minutes. At each point six plates were collected. Two TSA plates. Two *Brucella* agar Plates and two Sabouraud-chloramphenicol plates.

The Coriolis-μ air Sampler (Bertin Technologies, France) was used to collect air microorganisms suspended in sampling buffer. The Coriolis sampler was set to collect a single 3 m^3 air sample at each sampling point in a time of 10 minutes. Collecting cups, and collecting buffer were those recommended by the supplier.

The SAS and Coriolis samples are taken simultaneously at the same sampling points. Thus sampling time is around 12 minutes per point. This value is important in order to minimize the time spent in the cave for sampling.

Water samples.

Water was collected from dripping points in Vestibulo, Cruce and Pozo. A volume of at least 5 mls was taken in sterile containers.

Water was also aseptically taken from the collecting containers placed in the Policromos hall.

Soil and support (walls)

Samples of soil and support (around 1 g) were taken from the sampling sites in sterile Falcon Tubes. All samples were transported in isothermal containers to the laboratory and appropriately manipulated or conserved, usually in hour after sampling.

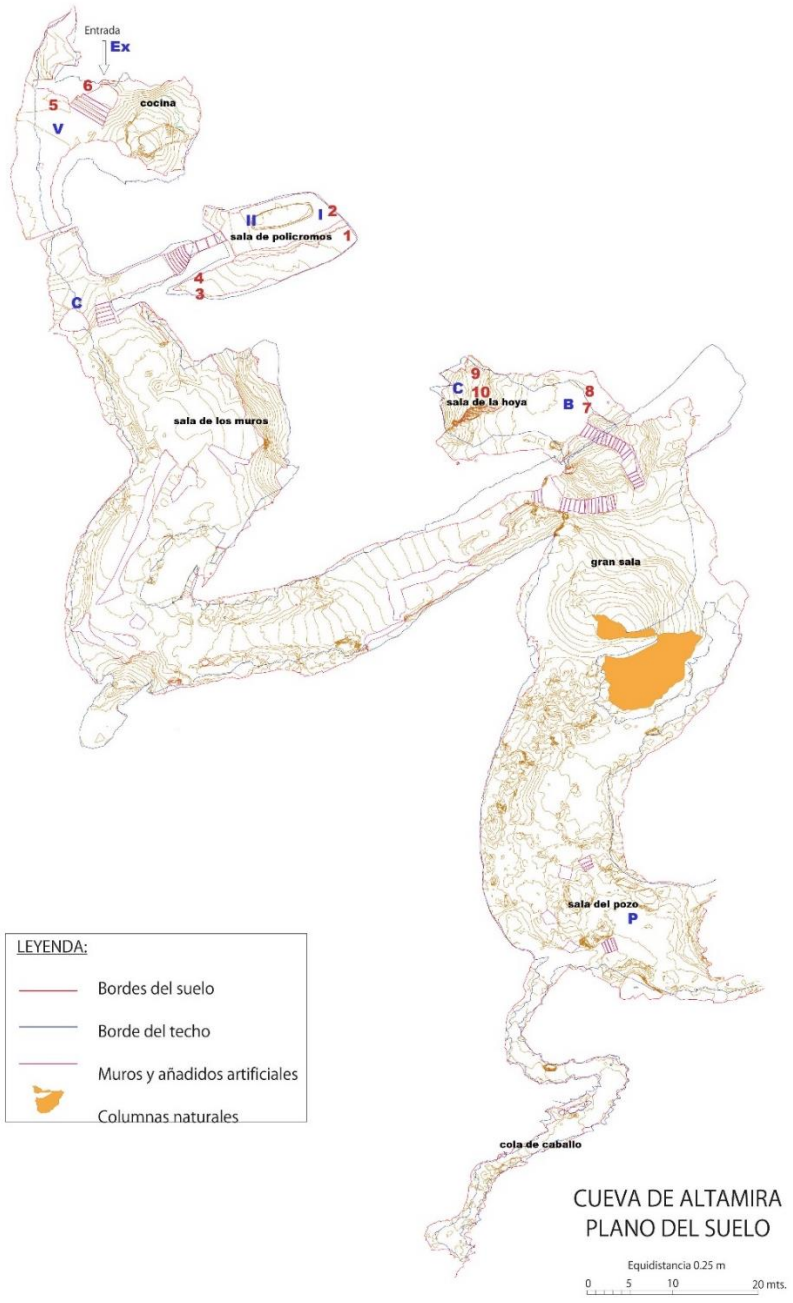


Figure 9. Map of the Altamira Cave. The map indicates the location of the different halls and the sites sampled are marked with blue capital letters as follows: Ex: exterior. V: Vestíbulo. C: Cruce. I: Polícromos I. II: Polícromos II. B: Hoya Bisonte. C: Hoya Cabra. P: Pozo.

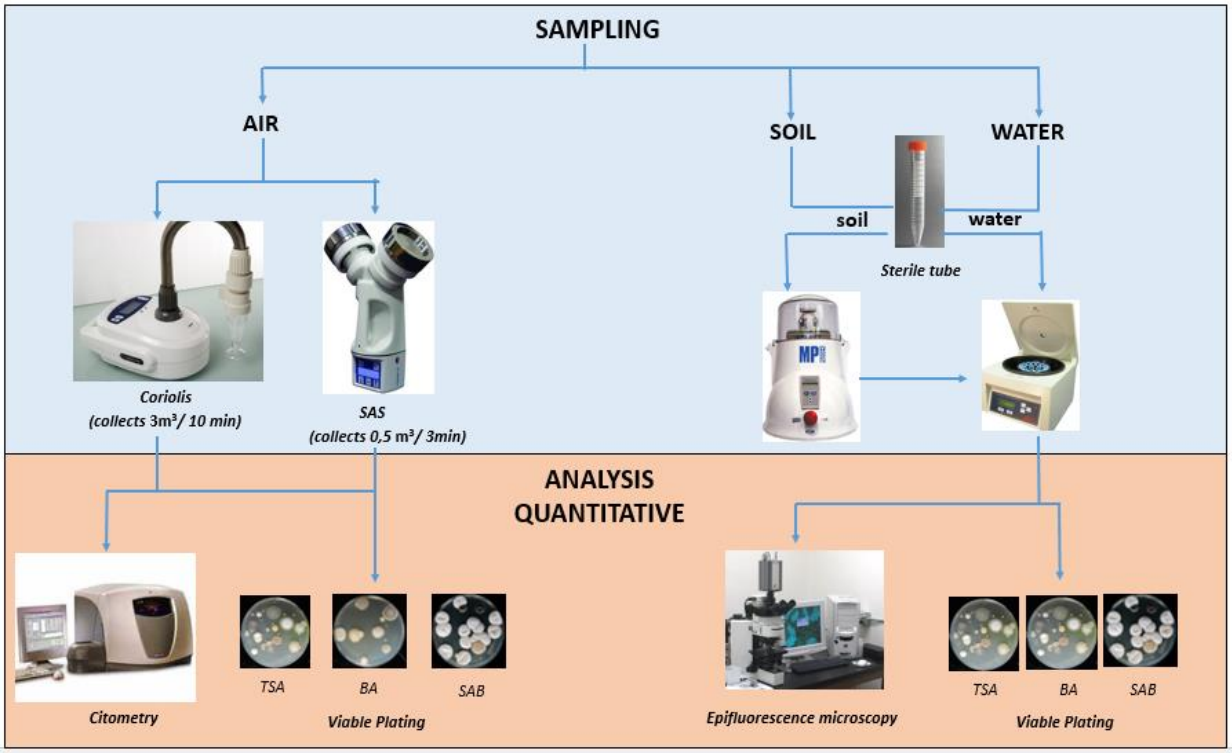


Figure 10. General drawing of the different methods of sampling and counting techniques for air, soil and water sample.

CULTURE CONDITIONS

All culture media were from Pronadisa, (Laboratorios Conda, Spain). Media were prepared according manufacturer conditions.

TSA plates were incubated at 30º C and colonies counted at 24 and 48 hours.

Brucella agar plates (BA) were incubated at 37º C, one in regular and the other under 5% CO2 atmosphere. Colonies were count after 24 and 48 hours.

Sabouraud plates were incubated at 26º C, and colonies counted after 72 hours incubation.

1 g of soil was weighed into a 15 mL Falcon tube and 5 mL of PBS was added. Samples were vortexed during 5 min, placed on ice 5 min and again vortexed 5 min. Later, soil samples were clarified during 2 min at 1500 x g. Serial dilutions were done to plate it in TSA for bacteria and SAB for fungus and incubated at 30ºC and 26 ºC as indicated above.

1mL of water was centrifuged at 15000 g during 2 min. Then, top 900 µL were carefully discarded and pellet was resuspended in the remaining liquid. Serial dilutions were done as required before plating in TSA and SAB and incubated as indicated above.

FLOW CYTOMETRY

Samples for flow cytometry were air samples collected with the Coriolis, soil samples processed as indicated or water samples.

1mL of sample was placed in an eppendorf tube (1.5 ml). Samples were simultaneously stained with 1 μL de SYTO BC from the counting bacterial kit (life technologies, UK). Samples were vortexed and incubated 37ºC during 5 min. Microspheres calibration suspension (6 μm) from the counting bacteria kit were sonicated during 10 min and added 10 μL to each sample (10^6 beads). Samples were vortexed and analysed by FC 500 (Coulter, Inc, Madrid, Spain) after 30 min incubation at room temperature. The argon ion laser was tuned to an output of 15 mW at 488 nm. Excitation of SYTO BC at 485 nm produces green fluorescence emission at 500 nm. Forward-angle light scatter (FSC) and side-angle light scatter were used and FL1 was used to detect fluorescence at 525 nm.

Data acquisition and processing:

The parameters FSC, SSC and FL1 were recorded and 60,000 events for each measure were stored in list modes. FSC and SSC were plotted in a logarithmic scale and data analyzed with CXP software. Bacteria counts were finally obtained by multiplying total number of bacteria detected by FC to the number of microspheres mL^{-1} and divided by the total number of beads detected by FC.

EPIFLUORESCENCE MICROSCOPY

Water samples and processed soil samples were used for microbial quantification by Fluorescence microscopy.

1mL of sample was mixed with 4 mL of PBS 1X filtered previously by Millipore 0,2 μm filter (Millipore, Bedford, USA) and stained with SYTO BC at a concentration 1 μg mL^{-1} during 20 min in the dark. After staining, samples were vacuum filtered onto black polycarbonate membrane filter having a pore size of 0,2 μm (Whatman, NY, USA). Afterwards, the filters were dried and mounted on a glass slide on a minimum of immersion oil (Zeiss, Oberkochen, Germany). More immersion oil was added to the filter surface and a glass cover slip was placed over the filter and firmly pressed down. A drop of immersion oil was placed on top of the cover slip to be analyzed by epifluorescence microscopy (DMA500; Leica Microsystems).

Counting procedure:

The images with ≈4 μm thick Z-stacks (10-12 planes of 0.45 μm Z-step size) were acquired using a 63x 1.3NA objective and specific filters for SYTO BC (Exc. 495/15; Em. 530/10). Fluorescence and brightfield images were sequentially acquired with a DU8285_VP camera (Andor Technology; 0.16μm pixel size). Images are presented after selection of the plane with the shaper focus and

adjustment of brightness to maximize signal. In all cases, exposure time, sensor gain, and digital adjustments were the same for control and experimental samples. Nine fields per filter were used from each sample and Z-stacks were obtained from each field and projected in a unique image processed by J-image program. Subsequently, local maxima of fluorescence from each image were found using a fixed value of noise. Then, the average of the maxima values obtained by counting discarding the noise were calculated between the nine fields per filter to obtain a total bacteria count.

Samples obtained with Coriolis were counting by epifluorescence and cytometry following the protocols mentioned previously. Colonies obtained with SAS were object of sequencing and identification by amplification of 16 rRNA for bacteria and ITS1 for fungi.

IDENTIFICATION OF CULTURED MICROORGANISMS

Extraction of DNA:

Isolated colonies obtained at 30ºC in TSA plates for bacteria and at 26ºC for fungus were picked and partly used for long term storage and for isolation of DNA. Bacterial samples were resuspended in 100 µL of distilled H_2O and in the case of fungus 100 µL of PrepMan Ultra Sample Preparation Reagent (Life Technologies, Texas, USA) were used instead. Samples were vortexed for 10 to 30 seconds and heated in a heat block settled to 100ºC for 10 min. Subsequently, sample tubes were cooled to room temperature for 2 min and centrifuged at the 15000 g for 2 min. Finally, 50 µL were transferred to a new eppendorf tube and used as raw DNA preparation for sequencing.

***PCR amplification of 16S rRNA and ITS1 rRNA fragments*:**

2,5 µL of the DNA extract were used as template for amplification with specific primers. PCR was performed using KAPA HiFi HotStart ReadyMix PCR kit (Kapabiosystems, Boston, United States). 16 rDNA fragments were amplified from bacterial samples with the reverse primer PA8F (5'AGAGTTTGATCCTGGCTCAG3') and the forward primer PL06RU1087 (5'CGCTCGTTGCGGCTCAG3'). Fungal samples were amplified with the reverse primer ITS4 (5'TCCTCCGCTTATTGATATGC3') and one of the two designed forward primers ITSF1 (5'CTTGGTCATTTAGAGGAAGTAA3') or ITS5 (5'GGAAGTAAAAGTCGTCGTAACAAGG3') as we can see in figure 11. PCR reaction was conducted as follows: 3 min initial denaturation at 95ºC, followed by 25 cycles of denaturation 20 s at 98ºC, annealing at 57ºC for 15 s and extension at 72ºC for 15 s and finally 1 cycle of extension at 72ºC for 7 min after amplification was completed. PCR products were analyzed by gel electrophoresis. 1,2 % agarose gels in Tris Borate buffer were run for 1h at 100 V.

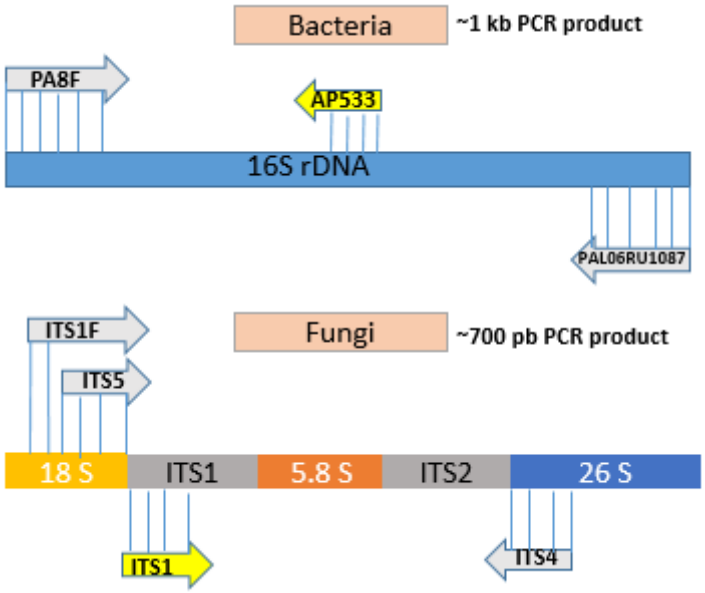


Figure 11. Primers used for amplify and sequencing 16rDNA (bacteria) and ITS1region

Sequencing of 16S rDNA and ITS1 rDNA amplicons:

PCR products were purified with the DNA Clean & Concentrator™-5 kit (Zymo Research, CA, USA). 10 µL (>20 ng) of purified DNA were used as a template in cycle sequencing reactions containing 3 µL of primer ITS1 (fungus) and AP533 (bacteria) at a concentration of 10 pmol μL^{-1} (Figure 10). DNA sequencing was performed by the YouPlateit protocol in StabVida(Portugal)

RESULTS AND DISCUSSION

To perform this study we used different counting techniques due to the variability and limitations that carry each method. Also, with this results we do not pretend to obtain exact microbial counts, but at least a trend about relative abundance and biodiversity of Altamira microbiota.

Samples were collected from the halls: Policromos, Vestibulo, Pozo, Cruce and Hoya. In each hall, samples were obtained from water, soil, wall and air and analyzed for microbiota quantification. For each sample, the most appropriates techniques were applied depending on the complexity and the cleaning of the sample.

CULTURE IMPROVEMENTS

Before starting with counting analyses we tried to make some improvements in the medium culture in order to increase the number of cells cultivated from our environmental samples. As mentioned before, Tanaka et al., (2014) published that some inhibitors could be formed in culture media if agar was autoclaved together with salts and other compounds in the culture media. Then, we did some trials autoclaving together and separately agar and phosphate. Site samples were different places of the IBBTEC (terrace of -1 floor, snack bar of 0 floor, hallway stairs of 1 floor, laboratory 02.16 of 2 floor and corridor of 3 floor). In each sample, TSA and BA medium were applied and samples were collected by SAS micro air sampling. Bars in blue represent cfu obtained in medium autoclaved together and bars in yellow on medium autoclaved separately (Figure 12). Contrary to expectations, total colony counts were slightly higher when autoclaved together all the components of the media. From this moment, all plates were prepared from singles dehydrated medium.

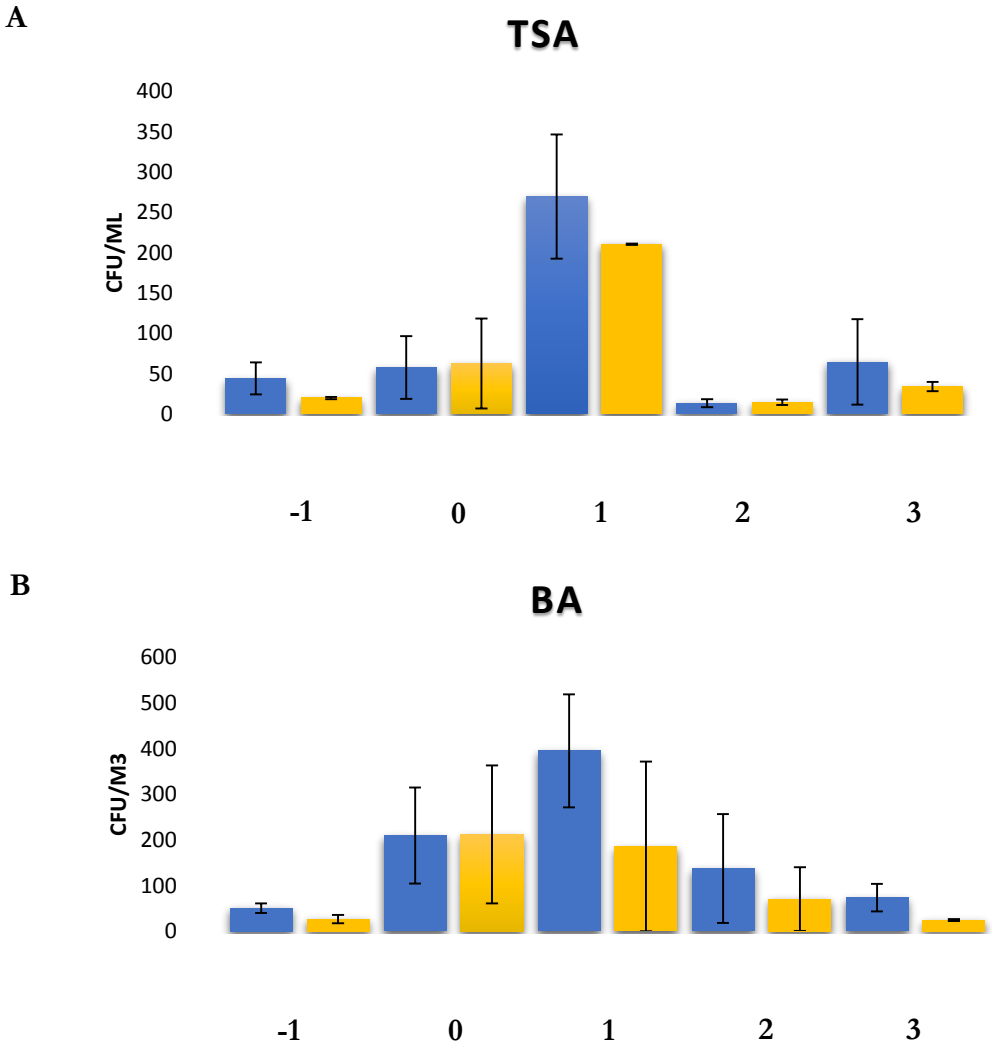


Figure 12.Cell counts (cfu/m3) in air samples from different halls of IBBTEC. Air samples were collected by SAS and incubated in TSA at 30ºC 36h (A) and BA at 37ºC 36h (B).Data represent independent plates Bars in blue are medium autoclaved together and bars in yellow is autoclaved separately

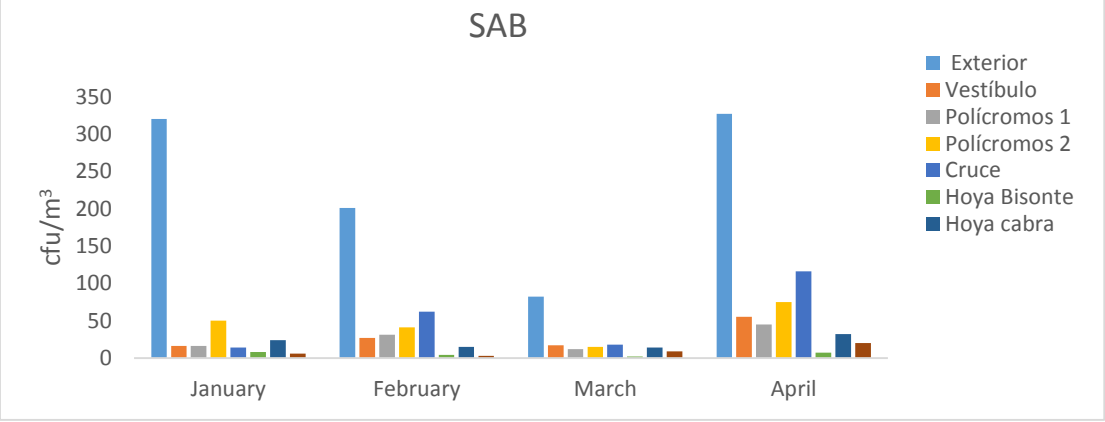
AEROBIOLOGY COUNT

Air samples were gathered with 2 different air sampler apparatus and analyzed by two counting techniques (viable plating and flow cytometry) and then compared to observe differences between both methods. Samples which were collected with Coriollis μ air sampling were derived for subsequent analysis of counting with the flow cytometry. Samples collected with SAS air sampling in TSA and BA medium for bacteria and in SAB for fungi were counted by viable plating. TSA and SAB were incubated at 30ºC and 26 ºC respectively during 36 h and 72 h. Furthermore, for the molecular analysis a total of 32 air fungi colonies were selected according to morphology, aspect, color and size and for bacteria 35 air colonies also selected following the same parameters and sequenced.

Aerobiology viable count: SAS

Samples were taken monthly in different halls of Altamira Cave as well in the exterior. Fungi were representatively much higher outside where there is a major variety on woody substrates, soils, leaf litter, dead animals, and animal exudates as they are generally parasites or decomposers, than inside of the cave as it is considered low energy ecosystems. Regarding inside, more counts were obtained in Vestibulo, Policromos and Cruce. In these halls, the high content of fungi is related to the presence of paintings and its nearest position to the entrance. As we move inside into the cave fungi counts decreases. However, total fungi counts are between 30-300 cfu/m^3 meaning that it doesn´t reach 1 colony per liter (Figure 13). On the contrary, bacteria cell counts are higher inside the cave than outside as they are not influenced by susceptibility of the host material to be colonized as fungi are (Gorbushina, 2007). The constancy of temperature, humidity and darkness seems to act in favor for cave bacteria life. As fungi, we detected a higher number of culturable microorganisms in Policromos, Cruce and Hoya. The presence of paintings (most in Policromos and less in Cruce and Hoya) that have been altered chemically and physically by the visits of many years provide niches for evolution of complex microbial communities. Furthermore, bacteria cfu's were in range from 30 to 250 cfu/ m^3 (Figure 12).

A



B

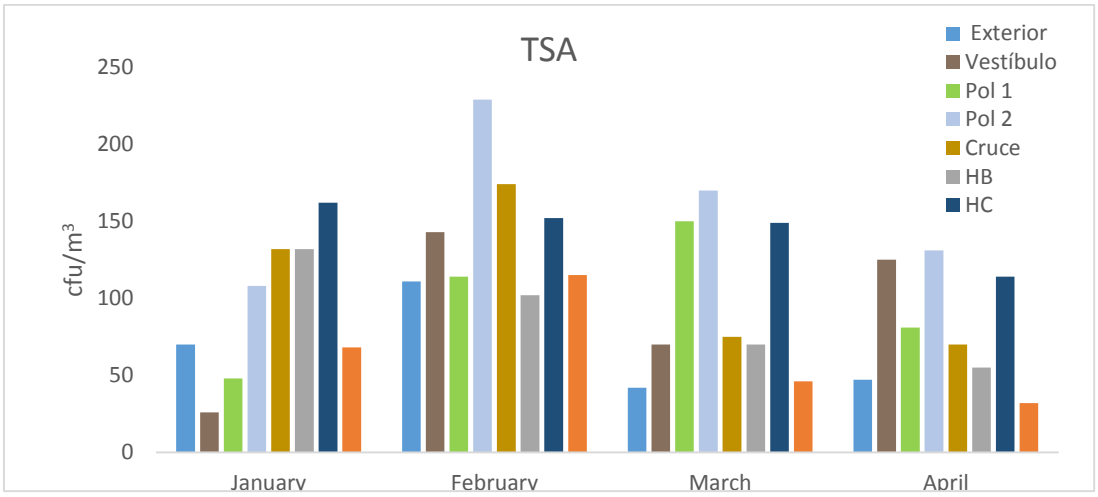


Figure 13. Monthly cell counts by plating (cfu/m3) in air samples from different halls of Altamira Cave. Air samples were collected by SAS and incubated in TSA at 30ºC 36h (A) and SAB at 26ºC 72h (B).

Flow cytometry

Air samples obtained monthly taken from the Halls: Vestibulo, Cruce, Pol1, Pol2, Hoya, Pozo were analyzed by FC. For each sample, FC acquired data for cell size (FS), internal heterogeneity (SS) and fluorescence (FL1). Cells stained with SYTO BC were mixed with a known concentration of microspheres calibration suspension and analyzed. Data obtained from the FC is represented in two plots as we can see in figure 14, in the first plot is represented cell size (FS) and internal heterogeneity (SS) of the bacterial population that are in the sample and the microspheres calibration suspension while in the second is displayed the fluorescence of such bacterial population that were selected in the plot before. The counts obtained have to be filtered from the noise that usually produces the buffer due to salts and air bubbles. Also, buffer coriolis contains triton that is a detergent, and can make bubbles when placing in the FC tubes which can be also counted as a bacteria.

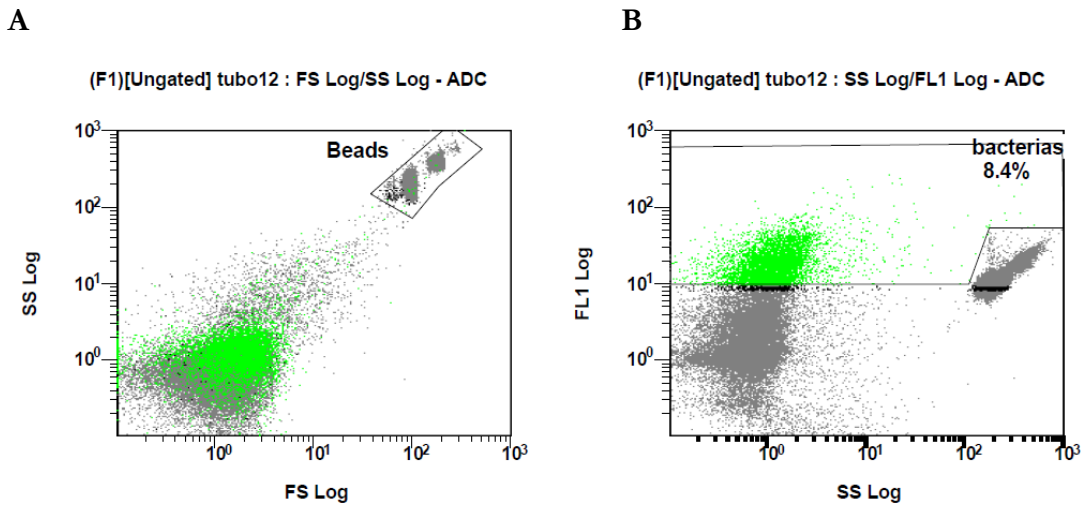


Figure 14. Example of plots obtained in a FC measure. A shows the parameters of cell size (FS) and internal heterogeneity (SS) of the bacterial population that are in the sample. The green color represents the fluorescence population detected in the second plot. Cells were stained with SYTO BC that emits at 500 nm (green chanel). B shows the fluorescence bacterial population registered within the first plot. Microspheres calibration suspension (6 μm) were used to determine the density of the cells.

In FC measures there is always a sample point that deviates from the rest. In despite of this, airborne bacteria were more abundant in February month. Regarding counts per hall, bacterial concentrations were significantly higher in Policromos and Hoya due to the presence of paintings as there is a strong correlation between biodeterioration of cultural heritage art work and bacteria content (Ciferri, 1999). Also as we move into the cave bacteria cell counts decreases (Figure 15). Higher counts of microorganism in the halls closest to the entrance as Vestibulo can be explained by the existence of exchange rates of aerobiological and atmospheric gases from the exterior of the cave that facilitates the entrance of nutrients and dispersion of bacteria.

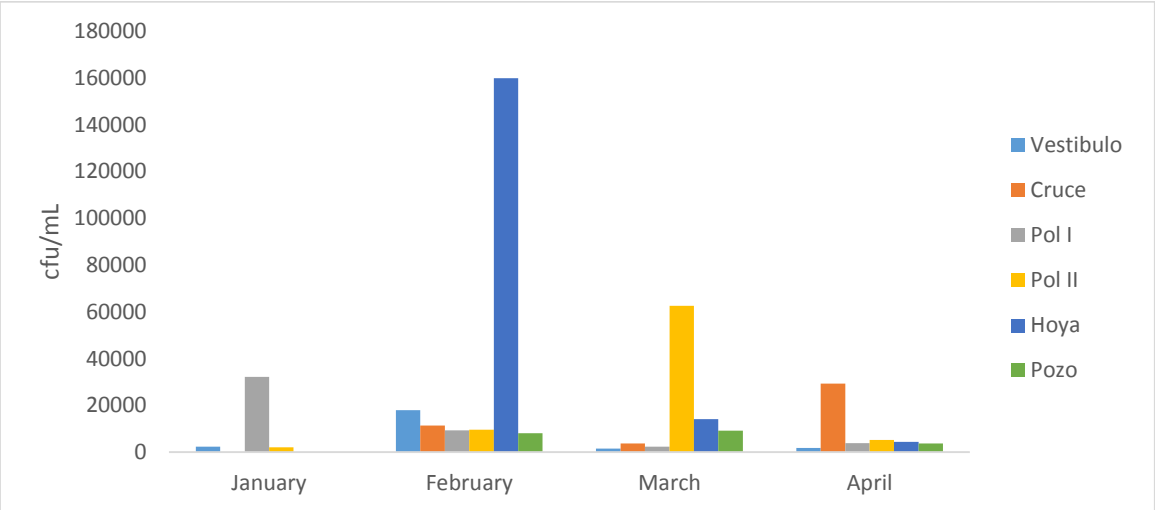


Figure 15. Total bacteria counts by flow cytometry (bacteria/m3) in air samples taken with the Coriollis sampler from different halls of Altamira Cave.

When comparing results obtained by plating and flow cytometry, we can observe in table 2 that cytometry counts 100 times more than plating. Sampler and location significantly influenced bacterial counts and also, as SAS as well as Coriollis air samplers use different flow rates, they would have different collection efficiency, as we can see in table 2. Furthermore, parameters as incubation time, sampling duration and media may also favor these differences. These results can verify the phenomenon by viable plating method in which doesn't grow more than 1% of the total viable cell present in the sample. Also, we can cloncude that except the sampling points that escapes in each month, flow cytometry is a more precise method for quantifying bacteria in this type of samples.

In any case, this is a very limited dataset and conclusions drew from them have limited significance.

Table 2. Comparison of cell counts in air samples by plating and flow cytometry.

	JANUARY			FEBRUARY			MARCH			APRIL			
	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	
Vestibulo	26	2336	89,85	143	18004	126	70	1510	22	125	1776	14	
Cruce	132		0	174	11416	66	75	3796	51	70	29349	419	
Pol1	48	32232	671,5	114	9398	82	150	2320	15	81	3896	48	
Pol2	108	2137	19,79	229	9652	42	170	62564	368	131	5267	40	
Hoya B	132		0	102	0	0	70	14177	203	55	4426	80	
Pozo	68		0	115	8104	70	46	9278	202	32	3706	116	
Media			130,19			64			143			120	114,40

WATER COUNT

Previous studies in dripping waters in Altamira cave reveals the important role of bacteria in biogeochemical process of the cave (Laiz et al., 1999). But taking samples from water is not so simple as with air and soil because it depends on the weather, if rains or not and also site samples are still in need of improvement. However, water samples were collected in a sterile tube and processed as is indicated in materials and methods to be analyzed by viable plating and flow cytometry. Furthermore, for the molecular analysis a total of 11 colonies obtained from water on TSA plates were selected according to morphology, aspect, color and size.

Viable plating

Generally in water samples we get more growth of culturable microbial populations, in comparison with those obtained in air, soil and wall samples with a bacteria content of <20000 of cfu/mL (Figure16). This is because water provides better conditions for bacterial development (high humidity, relatively low and stable temperature, water pH close to neutrality and nature of the organic matter). Samples missing in figure 16 are because we didn't have enough sample as indicated above. However, significantly higher percentages of bacteria were found in Vestibulo as it is the nearest hall to the entrance and where more water filtration are present. Pozo have elevate numbers of bacteria despite of being the farthest hall of all and this could be perhaps of putative entries from the outer atmosphere that has been described recently by García Antón et al (2013) and favors the entry and dissemination of microbial colonization's.

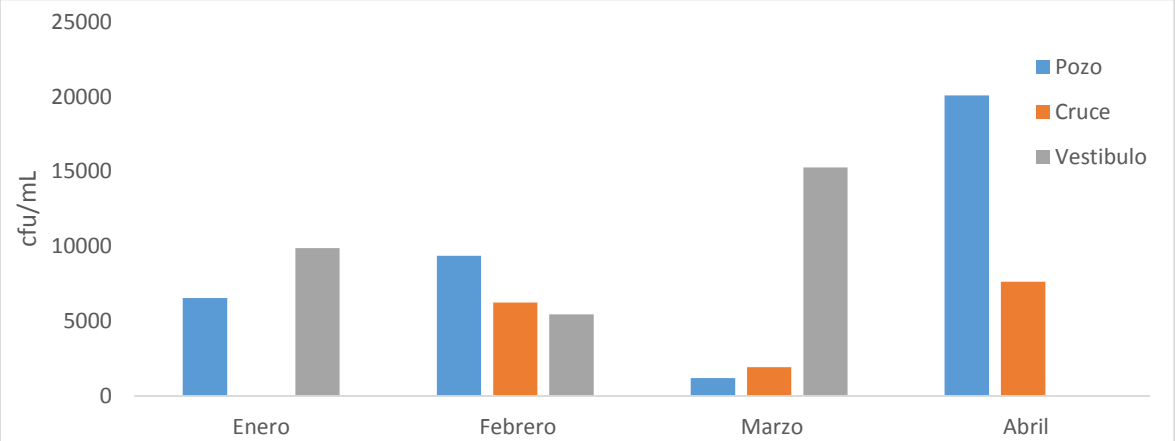


Figure 16. Monthly cell counts by plating (cfu/mL) in water samples from Altamira Cave. Water samples were diluted and incubated in TSA at 30ºC 36h.

Flow Citometry

In FC measure, we have an opposite context regarding plating. February is the month with more counts while in the rest decreases significantly. More bacteria cell counts were obtained as we moved inside the cave (Figure 17). Except February month, bacteria seems to increase its numbers as we move inside the cave and cell counts remains between 50.000-150.000 cfu/mL.

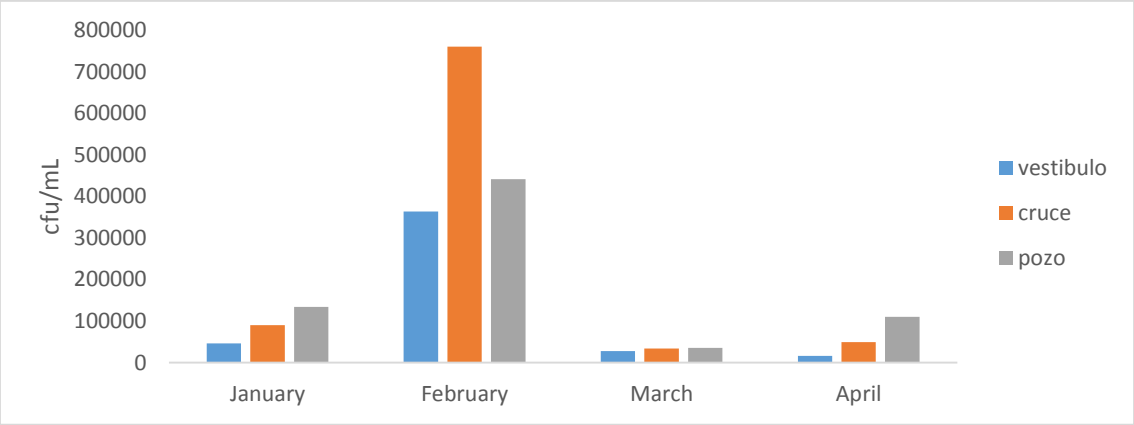


Figure 17. Total bacteria counts by flow cytometry (bacteria/mL) in water samples from Altamira Cave.

As we can see comparing results from both techniques, cytometry counts 28 times more than viable plating (Table 3). This again verifies the phenomenon that doesn't grow more than 4% of the total viable cell present in the sample and that flow cytometry is a more reliable technique than viable plating for total bacterial counting.

Table 3. Comparison between total cell counts by viable plating and flow cytometry in water samples.

	JANUARY			FEBRUARY			MARCH			APRIL			
	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	
Vestibulo	9880	46.385	5	5.460	363.648	67	15.280	28.329	2	0	16.152		
Cruce	0	90.385		6240	761.120	122	1920	34.228	18	7.640	49.316	6	
Pozo	6540	134.132	21	9.360	441.287	47	1.200	35.980	30	20.080	110.660	6	
Media			12,60			79			17			6	28,43

SOIL AND WALL COUNT

Bacteria in Altamira Cave are more detectable at human eye in wall and ceiling forming biofilms composed of aggregated microcolonies of mixed bacterial populations attached to rock surfaces resulting in the three types colonies (yellow, grey and white) mentioned before the introduction part (Soledad Cuezva, tesis doctoral 2008). These colonies are so abundant in Vestibulo hall and increasingly moving inwards to the point that they are starting to be seen in Policromos Hall. For this reason is important to have a control and study them because they could represent a potential risk for the conservation of the cave and its paintings (Portillo et al., 2008, Portillo et al., 2009). Then, soil and wall samples were collected in a sterile tube and processed as is indicated in materials and methods to be analyzed by viable plating and epifluorescence microscopy. In this type of samples, we couldn't use FC because the soil and wall samples are so dirty that could obstruct the cytometer.

For the molecular analysis, a total of 17 soil and wall bacteria colonies were selected according to morphology, aspect, color and size.

Viable count

Soil and wall samples were collected seasonaly instead monthly. Spring is the season where bacteria cell counts are higher and also, as we move inside the cave, counts increase. On the contrary, this doesn't happened with wall samples because it barely grows nothing even though walls are colonized by biofilms of the three types of colonies (Figure 18). This is explained by the so called phenomenon sintrophy where bacteria in order to grow needs the nutritional associations with other partners that provides him substrates and nutrients (Lueders et al., 2004).

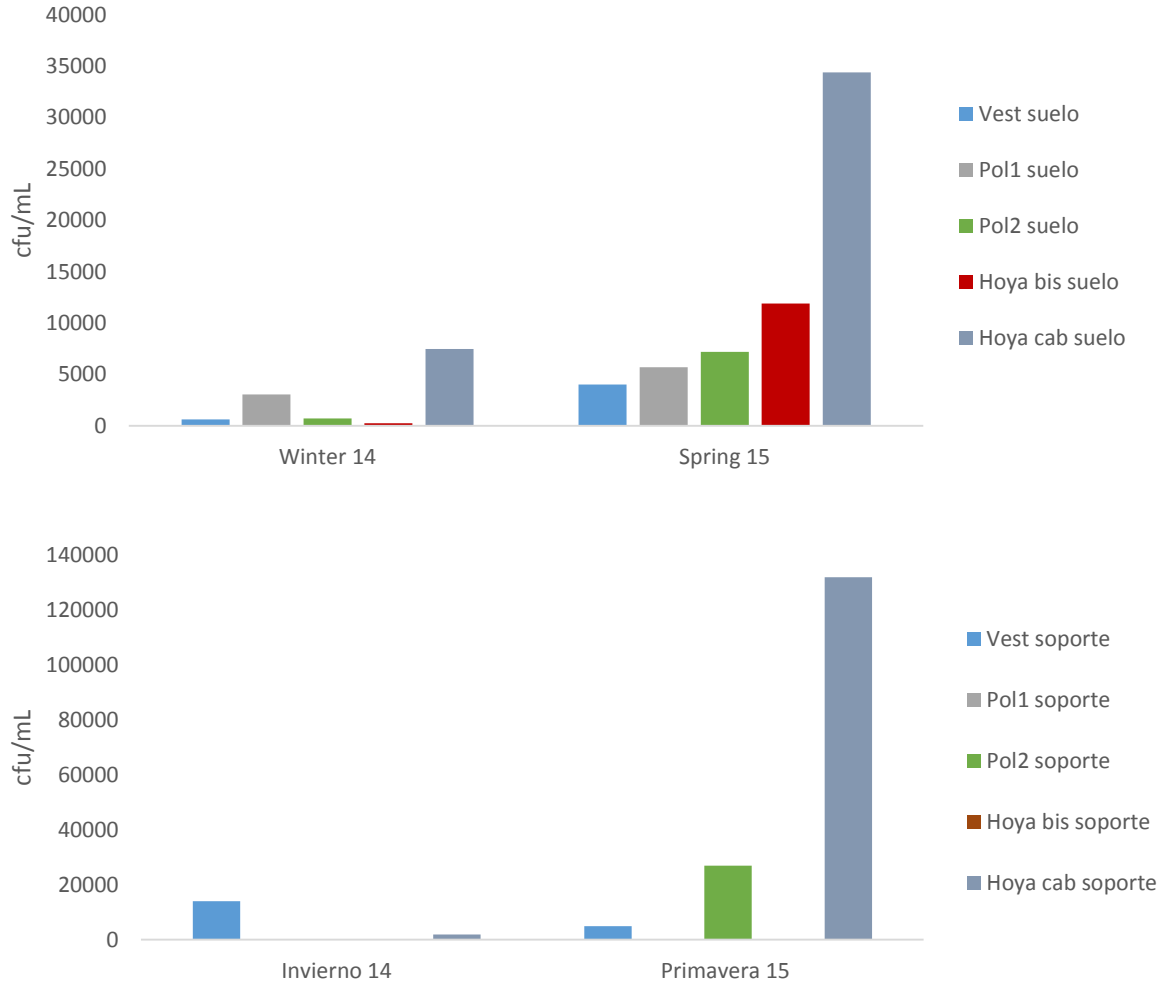


Figure 18. Seasonal cell counts by plating (cfu/g) in soil and wall samples from Altamira Cave. TSA plates were incubated at 30ºC 36h.

Epifluorescence microscopy

For epifluorescence microscopy, only spring season was analyzed because it was the last technique that we handled and we did some trials previously. Samples were stained firstly with DAPI as is the most widely stain used for these type of experiments. DAPI is specific for nucleic acids and also many other things that wasn't bacteria resulting in dirty images that were difficult to be analyzed and counted (Figure 19). Then, we tried with SYTO BC (the same stain that we use in FC) that is also specific for nucleic acids and provides cleaner images. Also, with both stains we had some problems in order to analyze wall samples because they seem to be much dirtier than soil samples resulting in murky images where you can't estimate anything (Figure 18).

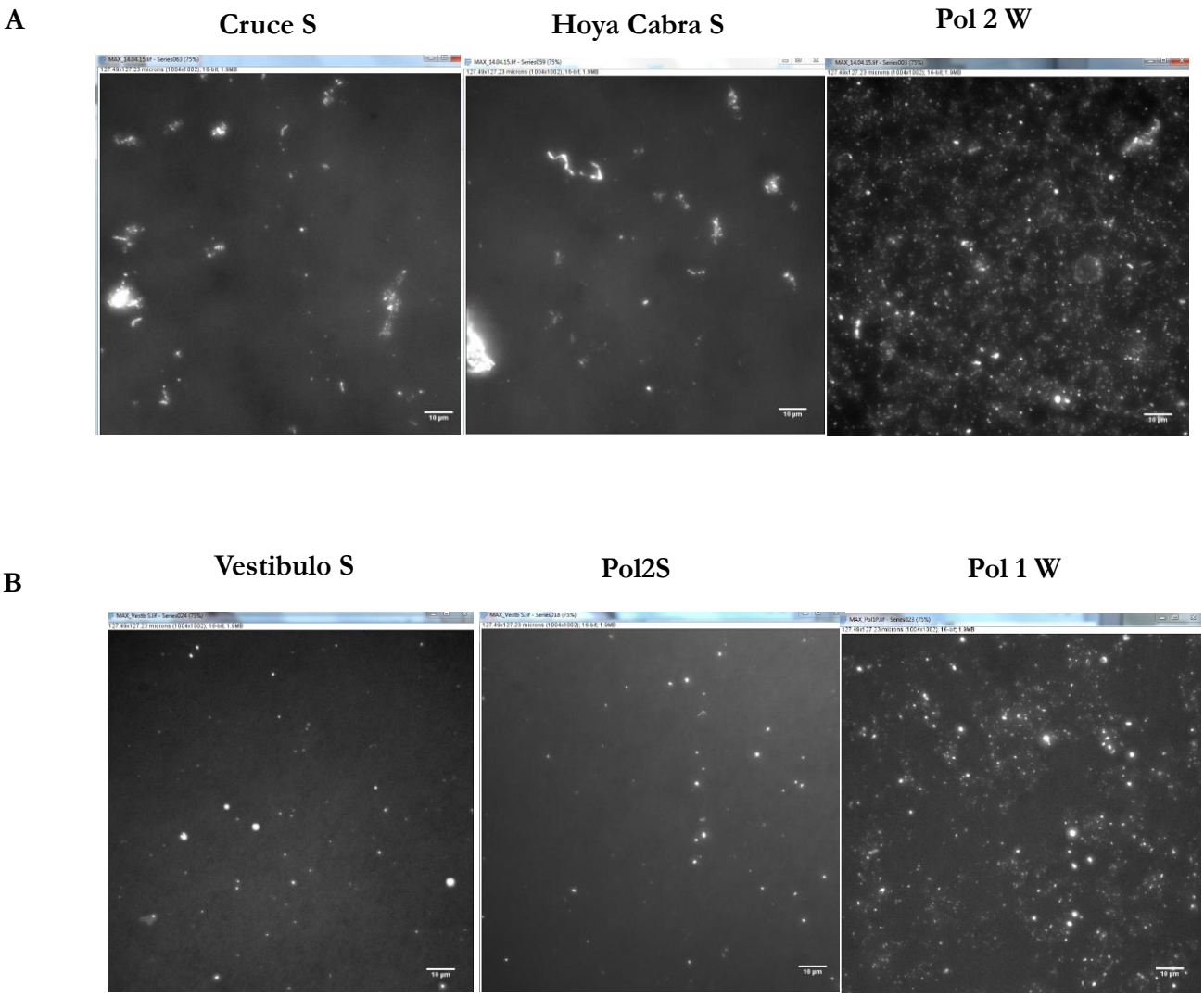


Figure 19. Epifluorescence microscopy images of soil (S) and Wall (W) samples stained with DAPI (A) and SYTO BC (B).

Regarding cell counts, soil and wall samples with SYTO BC were counted by ImageJ program used for analyzing microscopy images. Finally, cell bacteria counts were obtained by removing the noise produced by the bottom. Then, Policromos is undoubtely the hall with more bacteria cell counts as we have been observed before with other samples but microscopy barely counts for the rest of samples (Figure 20). This is because despite of being cleaner images additional improvements and good controls has to be done for a better estimation of bacterial population.

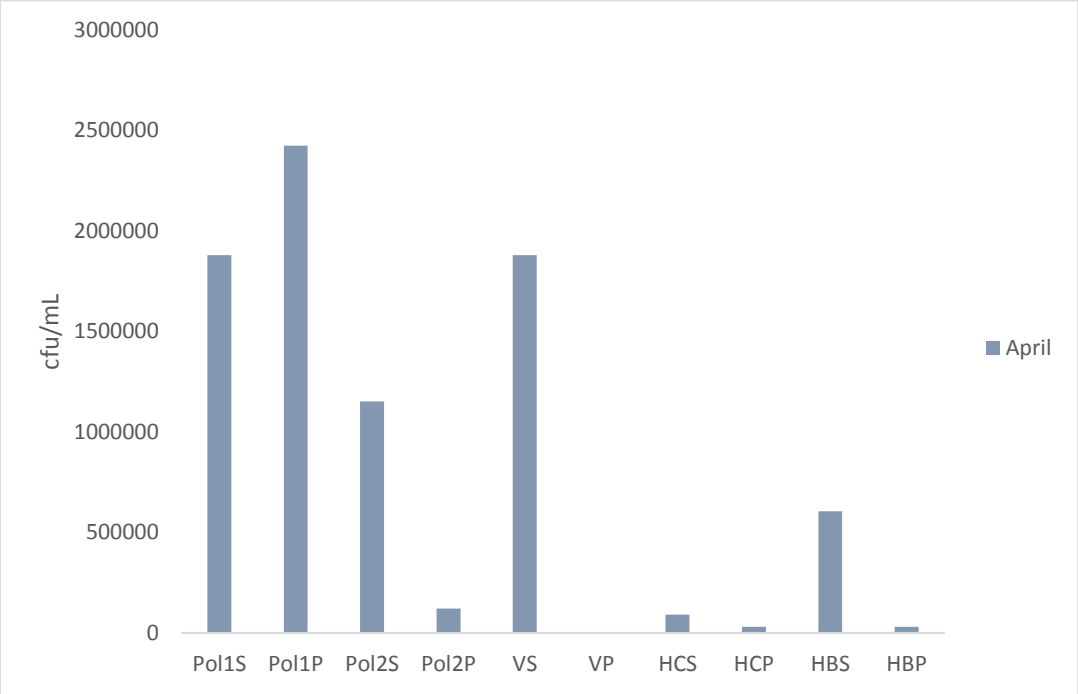


Figure 20. Total cell counts by epifluorescence microscopy of soil and Wall samples from Altamira Cave

When comparing results from both techniques, epifluorescence microscopy counts 105 times more than viable plating (Table 4). This result, as above, again verifies the plate count anomaly. Despite of this, additional improvements may be done in our protocols and especially for the microscopy counting.

Table 4. Comparison between total cell counts by viable plating and flow cytometry in water samples.

	APRIL		
	CC	**EF**	**Ratio**
POL1S	5.700	1878600	330
POL1P	0	2424000	0
POL2S	7.200	1151400	160
POL2P	27000	121200	4
VS	4.000	1878600	470
VP	4.900	nc	
HCS	34400	90900	3
HCP	131900	30300	0
HBS	11.900	606000	51
HBP	0	30300	0
Media			105

The microscopy counting results are promising but additional improvements are needed. In addition to more samples to analyze, cleaning of the samples, live-dead staining, etc can be used for ameliorating microscopy results significance.

SEQUENCING

Air fungi

Fungi colonies for sequencing were selected from air samples and bacteria from air, soil and water samples. A total of 31 air fungi colonies were selected according to morphology, aspect, color and size and for bacteria 35 (air), 11 (water) and 17 (soil) samples from the PCR amplification products. Sequences, about 500 bp were compared against 16S microbial database (bacteria), or nr database (fungi). Finally, taxonomical analysis was done with Megan 5 (version 5.10.3) that produces assemblies of BLAST alignments with the taxonomic NCBI classification and represent it in cladograms.

MEGAN assigned 87% of the fungi sequences and most frequent OUT's was *Penicillum* (23 %) at genus level and especially at species level *Penicillum chrysogenum* (10%) considered a possible penicillin strain producer (Figure 21). *Cladosporium* (19%) was the second frequent genus in which *Cladosporium cladosporoides* (13%), a fungal plant pathogen that affects wheat, was the most abundant specie. In Jarkowicka cave, recently discovered in 2012, 22 species of fungi were found in which *Cladosporium* spp. were the fungi most frequently isolated (Pusz et al., 2015). *Leptosphaerulina chartarum* (3%) was another

hit and has been reported that produces xylanolytic enzymes which have been demonstrated to improve the resistance and immunity of plants and have great potential use for industrial and agricultural applications (Wu et al., 2013). *Cryptococcus* (7%) is a fungi often dwelling soil and its presence in air could be due by the spread of spores produced by the fungi. The genus *phoma* (3%), also sequenced, has been described as plant pathogenic specie. The uncultivated fungis (13%) that we get from MEGAN results is because when doing a BLAST for all the sequences together, you can select parameters and make the program choice the first hit that sometimes it is an uncultivated specie that has the same coverage and identity as the other first hits. In this case, one cultivated fungi is an *Epicoccum nigrum* (10%) that is also a plant pathogen, which produces colored pigments that can be used as antifungal agents against other pathogenic fungi (Fávaro et al., 2012). *Fusarium* species (3%) are also frequently found associated with biodeterioration of paintings in show caves as the case of *F. solani* in Lascaux Caves (Bastian et al., 2010).

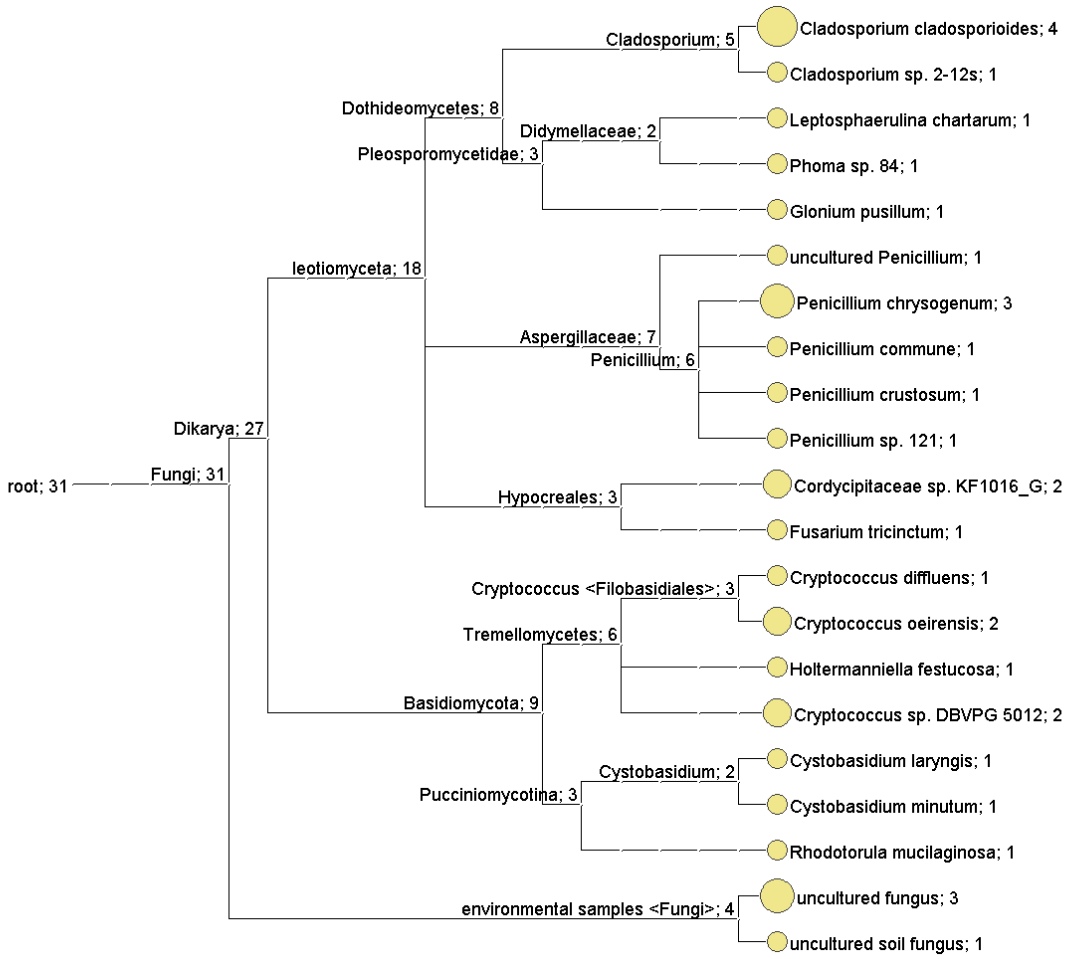


Figure 21. Phylogenetic diversity of the Altamira air fungal ITS sequences computed by Megan. The number of sequences assigned to each taxon is indicated in the cladogram branches.

Air bacteria

For air bacteria, MEGAN assigned 100% of all sequences to the genus level. The most represented phyla were *Firmicute*s with 25 isolates out of 35, followed by the *Actinobacteria* (7) and *Proteobacteria* (gamma) with only 2 cases. Among the Firmicutes, *Bacillus* (51%) was the most frequent genus (Figure 22) and *Brevibacterium frigoritolerans* (29%) at specie level which strains belonging to this species were reported to be isolated from dairy animal or human skin and marine water or marine fish (Groth et al., 1999). However, *Bacillus* species are spore forming, and have been demonstrated often in soils and have the ability to be plant-growth-promoting bacteria (Nautiyal et al., 2013). Furtehermore, *Bacillus* strains have been previously isolated like other *Pseudomonas* species in aerobiology studies from Lascaux Cave (Martin-Sanchez et al., 2014). *Staphylococcus equorum* (14%) is another abundant specie within the *Firmicutes* group. This specie is frequently isolated from fermented food products as cheeses and sausages (Irlinger et al., 2012). Probably, it also represents a type of ambient bacteria.

Soil bacteria

In soil samples, MEGAN assigned 100% of all sequences to the genus level. In this case, they belong to the phyla firmicutes and the proteobacteria. No actinobacteria were obtained in soil cultures, even when these are typical soil bacteria. In our case either the small sample size (only 17 isolates analyzed) or a poor recovery of actinobacteria for our soil culture procedure, could explain this result.

Among the soil firmicutes, *Bacillus* (36%) is the most abundant genus and *Sporosarcina globispora* (18%) at specie level (Figure 23).

Furthermore, air and soil bacteria are very similar because some bacteria, for example *Bacillus spp*, remains in a vegetative state in the soil and produces airborne spores. Selected actinobacterial isolates as *Rhodococcus* (3% air) and *Streptomyces* (3% air) were not obtained from soil. However this taxa has been reported to play a role in the formation of mineral deposits in karstic caves.

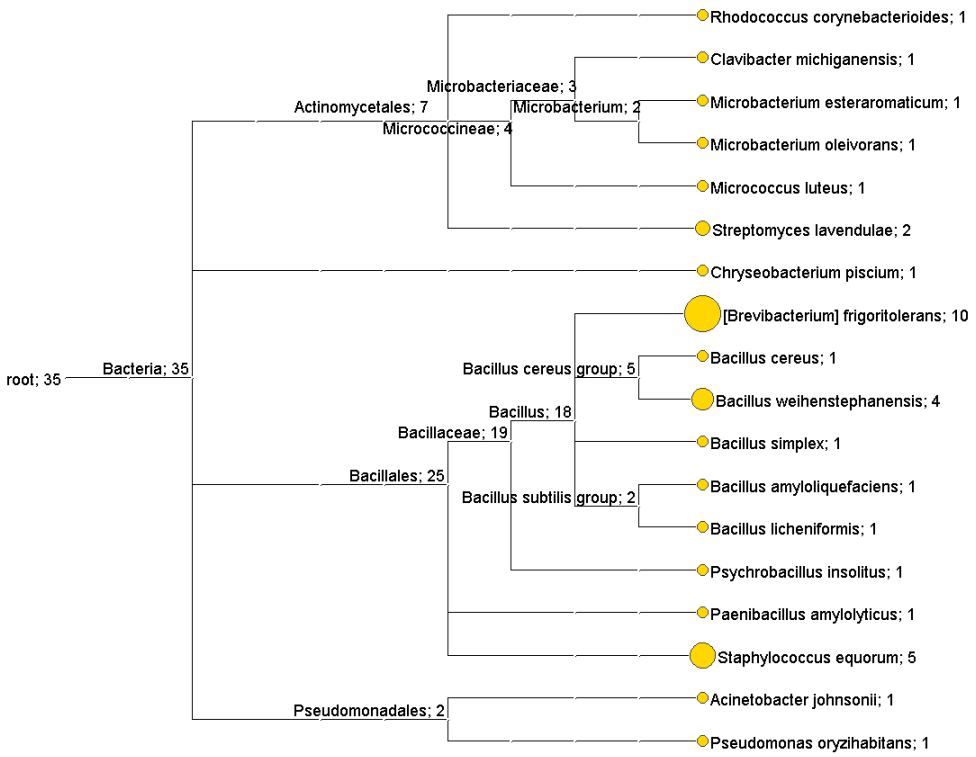


Figure 22. Phylogenetic diversity of the Altamira air bacteria sequences computed by Megan. The number of sequences assigned at each taxon is indicated in the cladogram tree branches.

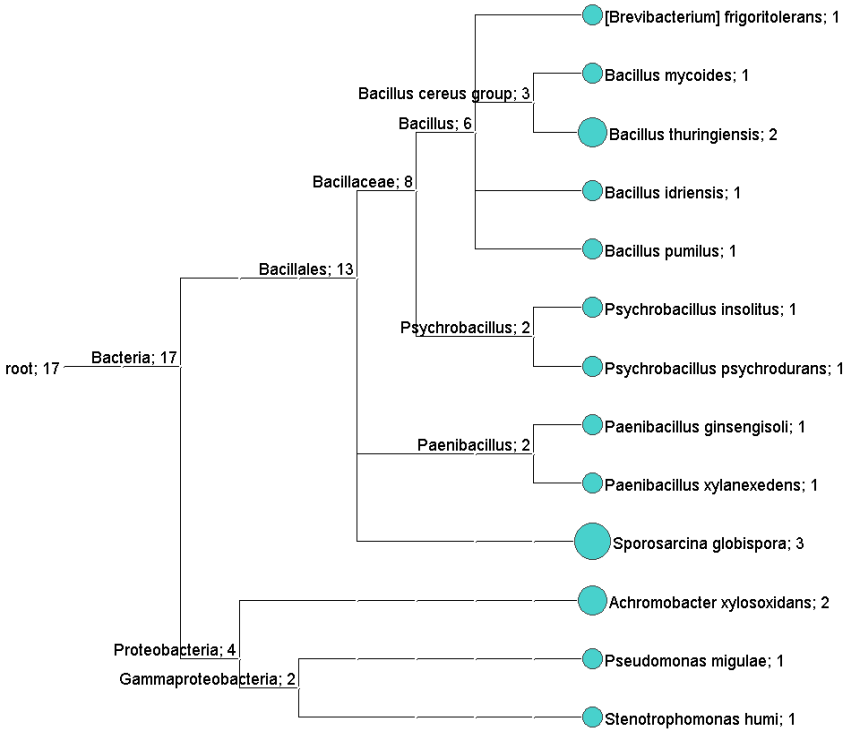


Figure 23. Phylogenetic diversity of the Altamira soil bacteria sequences computed by Megan. The number of sequences assigned at each taxon is indicated in the cladogram tree branches.

Water bacteria

Regarding water samples, MEGAN assigned 100% of the sequences to the genus level. In these samples we do no obtain *Firmicutes*. The *Proteobacteria* was the most abundant phylum (80%) followed by the Actinobacteria. *Pseudomonas* (50%) was the most abundant genus and *Pseudomonas extremaustralis* (20%) at species level (Figure 24). *P. extremaustralis* have been demonstrated to produce polyhydroxyalkanoates that have a role in the tolerance and degradation of environmental pollutants. *Brevundimonas diminute* (10%) has been reported to be associated with calcium carbonate precipitation (Rodriguez-Navarro et al., 2012). The presence of *Actinomycetes* (20%) could be also due to the stable temperature of the water (10-15 Cº) that seems to favor the growth of heterotrophic bacteria.

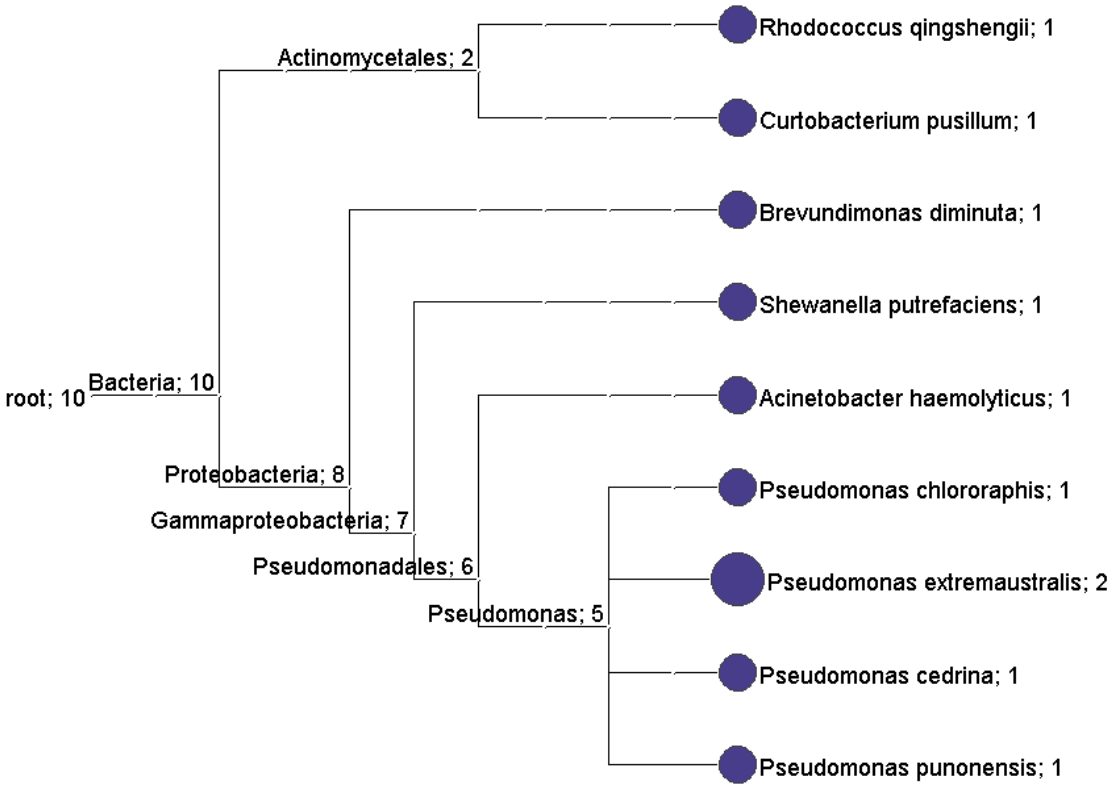


Figure 24. Phylogenetic diversity of the Altamira water bacteria sequences computed by Megan. The number of sequences assigned at each taxon is indicated in the cladogram tree branches.

CONCLUSIONS AND FURTHER RESEARCH

Taken together the data presented here, we can conclude that estimating the number of bacteria present in the samples cannot be accurately determined by traditional approaches. Each method gives us a side view of the total. The cell enumerations reported here by the flow cytometry or epifluorescence microscopy shows results 20-100 higher than by viable plating revealing the significant differences present between different counting techniques. Also, to this we must add technical characteristically factors for each method which may have a potential to over- or underestimate the 'real' values of bacteria. However, we do not need to obtain very accurate quantitative results. The aim of the final study was to provide a reference value representing normal, non-alarming levels of bacterial colonization. Above those, a risk must be considered and appropriate measures undertaken.

The results of this study highlight the importance of combining different counting techniques in order to understand the geomicrobiology of karstic caves, and especially in Altamira Cave.

A program based on the methods described here: Air sampling for direct plate counting, combined with the Coriolis sampler that allows quantification by Flow cytometry and microscopy gives a reasonable aerobiological control. Furthermore Coriolis samples allow the application of high throughput sequencing methods to air samples.

Culture, flow cytometry, and microscopy may be applied also to soil and water samples.

Systematic application of this workflow to samples taken periodically from the cave should provide a robust image of microbial situation in Altamira Cave.

Another alternative quantitative technique for enumerating bacteria is quantitative PCR. If universal bacterial or fungal primers are used, it would be possible to obtain another estimation of the number of total bacteria or fungi. By now, we have not implemented this method, however we keep it in mind to evaluate single species of bacteria or fungi in the case that an unusual proliferation appears.

A final objective in this project is the development of methods to control the eventual proliferation of microbes menacing or damaging seriously the paintings. This happened already in another caves and the lesson we have learned is that control measures often produce unpredictable effects worsening the deterioration problem.

Conservative control methods could be directed to cut the energy or nutrient inputs supporting microbial proliferation. This type of control requires first to identify the nutrients and energy flow for the cave microbiota. Extensive microbiological characterization is needed before such control methods can be implemented.

Colony sequencing gave insights of the culturable microbial diversity in Altamira Cave. In previous molecular studies, *Proteobacteria* and *Acidobacteria* were the dominant phylum taken from the wall painting material of Policromos Hall (Schabereiter-Gurtner et al., 2002) and *Proteobacteria* on dripping water (Laiz et al., 1999). In this study, the results revealed that Altamira community was dominated by *Firmicutes* phylum such us *Bacillus* genus on the air as well as on the soil and wall, whereas *Proteobacteria* such us *Pseudomonas* genus was the dominant as previous mentioned studies on dripping waters.

The results of this study will be taken into account to modify the standard protocols for microbial characterization in the microbiological part of the PCP. Preventive conservation requires the definition of standard protocols, which should be applied periodically over the time. The results of these measurements need to be validated according the technical specifications of the protocols. Then, the results should be carefully analyzed and used to define the normal values for the different microbiological parameters analyzed and to detect risk and alarm situations.

Finally from all the studies performed we must try to find countermeasures to be taken to control a biodeterioration alarm. It is however important keep in mind lessons learned from similar situations in other art caves. Any control measure should be carefully analyzed, applied only in extreme cases and designed according the ecological equilibrium of microorganisms dwelling in the cave (Sterflinger and Piñar, 2013).

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Development of protocols for microbiological control in Altamira Cave

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ABSTRACT

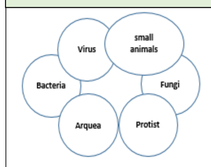
Art Caves are threatened by uncontrolled growth of microorganisms, especially fungi. At the same time, in spite of the extreme ambiental conditions, they are inhabited by very diverse microorganisms. Altamira Cave contains one of the best collections of parietal art and is also threaten by potential microbial deterioration. In order to preserve the state of paintings, after a research project developed in 2013-2014, came out the "Plan de Conservación Preventiva" (PCP) for the Altamira Cave. Among the objectives of the PCP is the systematic control of the cave microbiota, and methods investigated here will become standard for these purposes.

Cultivation of bacteria from natural habitats shows that only a small fraction of the bacteria present in such environments, can be cultivated under regular laboratory conditions and clearly indicate that alternative methods are required for quantitative purposes. Therefore, we have used alternative culture methods, as well as other quantitative methods based on fluorescent staining of microbes such as direct counting by fluorescence microscopy and flow cytometry. These techniques have been applied to water, air and soil samples and the results compared and combined with qualitative biodiversity analysis performed by 16S rDNA sequencing from isolated colonies or directly from the cave samples.

OBJECTIVES

- Control the risk of biodeterioration of the paintings in Altamira Cave.
- Develop and standardize protocols for systematic control of Altamira Cave microbiota.
- Evaluate different counting techniques for microbial quantification.
- Analyze the culturable microbial diversity in Altamira Cave.

INTRODUCTION



-Air
-Water
-Ceiling
-Wall
-Soil

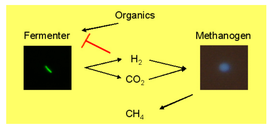
MICROBIAL ACTIVITY

1.photoautotrophs→ energy from light (photosynthesis). Fix CO2.

2.chemoautotrophs→energy by oxidation of chemicals compounds. Fix CO2

3.heterotrophs→gain energy from existing organic compounds

Synthrophy

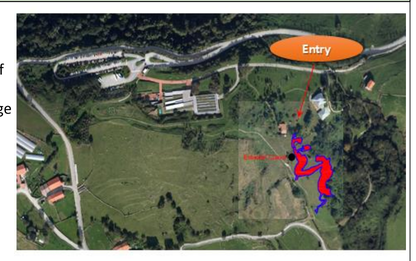


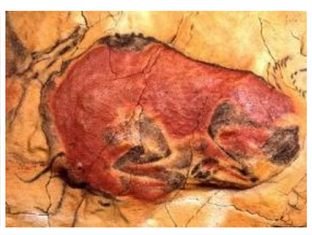
ALTAMIRA CAVE:

1.Karst Cave
2. Most important Palaeolithic site of Spain with 260 valuable paintings.
3. Declared as UNESCO world heritage

BIODETERIORATION

"any undesirable change in the properties of a material caused by the vital activities of living organisms" Hueck et al. 1965



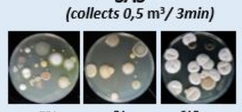


SAMPLING

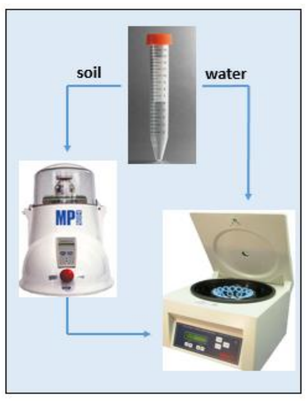
AIR

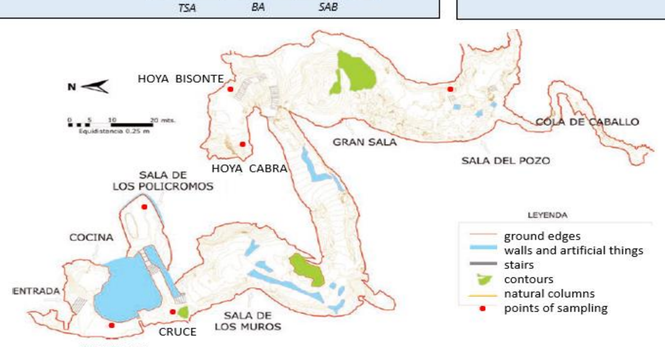
Coriolis *(collects 3m³/ 10 min)*

SAS *(collects 0,5 m³/ 3min)*



SOIL AND WATER





ANALYSIS QUANTITIVE

CULTURE

- Air (SAS)
- Water
- Soil

Extraction procces

Plating

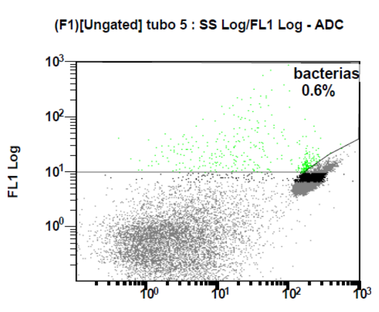
TSA 30ºC | BA 37ºC | BA + CO2 37ºC | SAB 26ºC

Culture improvements *(Tanaka et al.)*

FLOW CYTOMETRY

- Air (Coriolis)
- Water

Bacterial Counting kit (Life Technologies)



EPIFLUORESCENCE

- Soil
- Water

Counting procedure

$A_f = \pi \cdot r^2 = \pi \cdot 12{,}5^2 = 490{,}9\,mm^2$

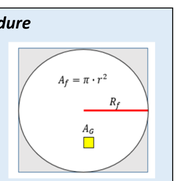
$A_G = 0{,}12749 \cdot 0{,}12723 = 0{,}0162\,mm^2$

$\frac{A_f}{A_G} = \frac{490{,}9}{0{,}0162} = 3{,}03 \cdot \frac{10^4 fields}{filter}$

$\frac{bacteria}{filter} = \bar{x} \cdot 3{,}03 \cdot \frac{10^4 field}{filter}$

Where:

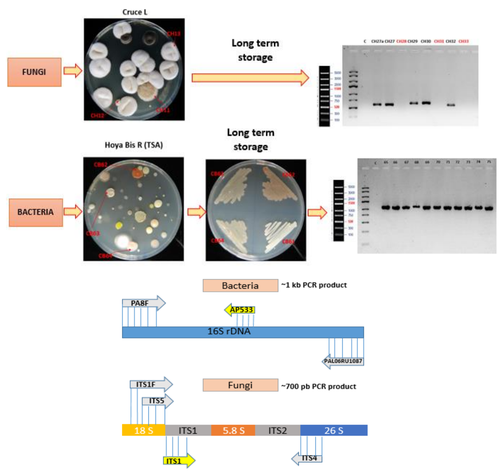
A_f = Area of a field
A_G = Area of grid in field view
$\bar{x}$ = Average bacteria per 9 fields



MOLECULAR ANALYSIS

SEQUENCING

- Air (SAS)
- Water
- Soil



Sequences, about 500 bp were compared against 16S microbial database (bacteria), or nr database (fungi). Taxonomical classification was done with Megan 5 (versión 5.10.3).

RESULTS

AIR

PLATE COUNTING

Figure 1. Monthly cell counts by plating (cfu/m3) in air samples from different halls of Altamira Cave. Air samples were collected by SAS and incubated in TSA at 30ºC 36h (A) and SAB at 26ºC 72h (B).

FLOW CYTOMETRY

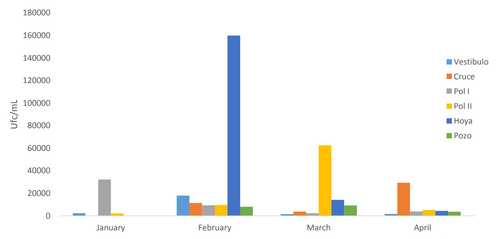


Figure 2. Total bacteria counts by flow cytometry (bacteria/m3) in air samples taken with the Coriollis sampler from different halls of Altamira Cave.

WATER

PLATE COUNTING

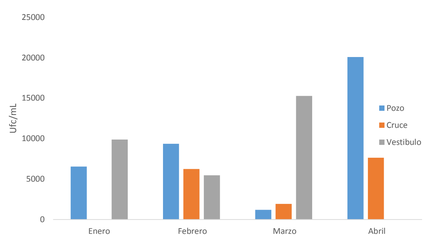


Figure 3. Monthly cell counts by plating (cfu/mL) in water samples from Altamira Cave. Water samples were diluted and incubated in TSA at 30ºC 36h.

FLOW CYTOMETRY

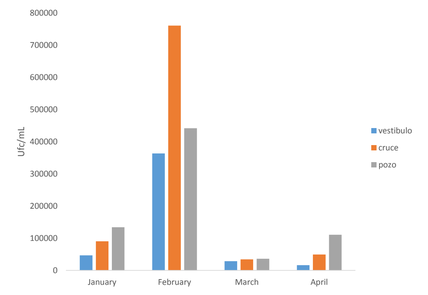


Figure 4. Total bacteria counts by flow cytometry (bacteria/mL) in water samples from Altamira Cave.

SOIL

PLATE COUNTING

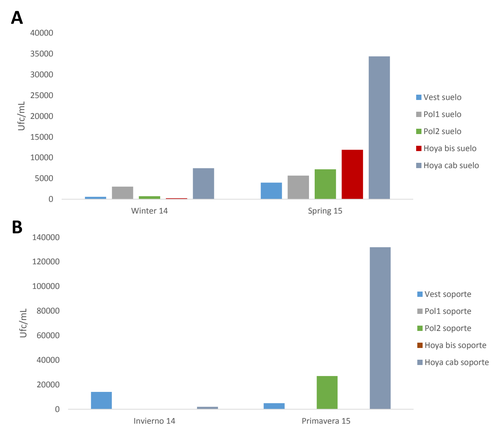


Figure 5. Seasonal cell counts by plating (cfu/g) in soil and wall samples from Altamira Cave. TSA plates were incubated at 30ºC 36h.

EPIFLUORESCENCE MICROSCOPY

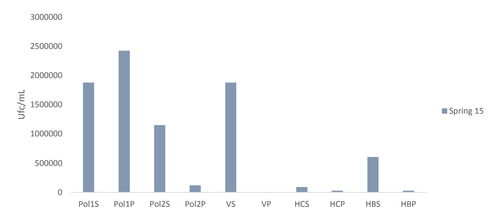
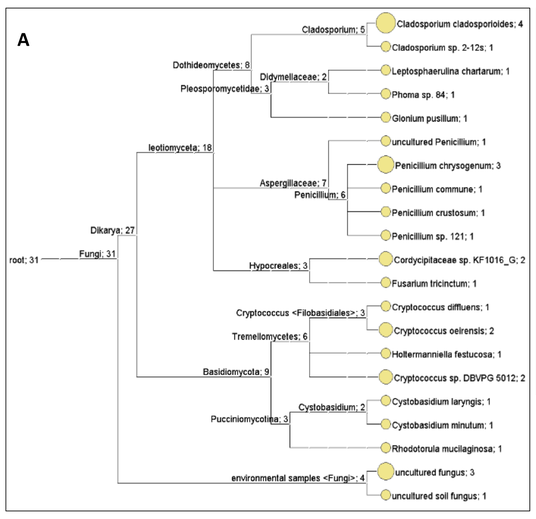


Figure 6. Total cell counts by epifluorescence microscopy of soil and Wall samples from Altamira Cave.

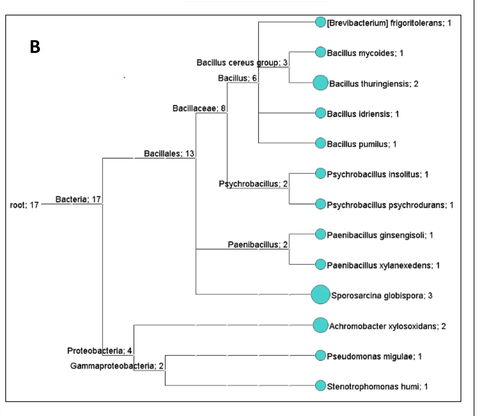
SEQUENCING

93 colonies were sequenced with success: 31 fungi and 62 bacteria. From these, 35 were from air, 17 from soil and 11 from water.

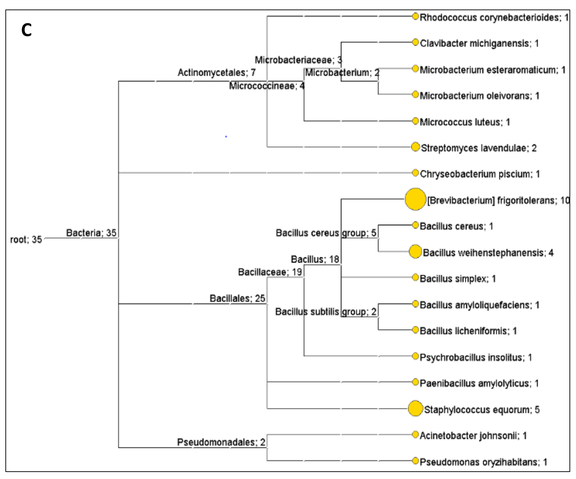
AIR FUNGI



SOIL BACTERIA



AIR BACTERIA



WATER BACTERIA

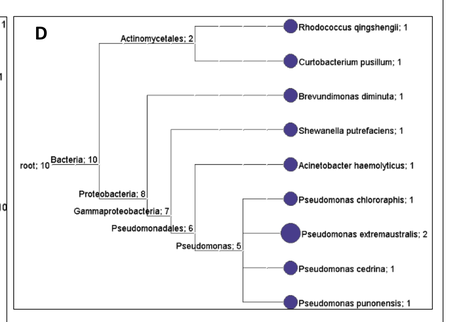


Figure 7.Phylogenetic diversity of the Altamira sequences computed by Megan. The number of sequences assigned at each taxon is indicated in the cladogram tree branches.

PLATE COUNTING VS FLOW CYTOMETRY IN AIR SAMPLES

Table 1. Comparison of cell counts in air samples by plating and flow cytometry.

	JANUARY			FEBRUARY			MARCH			APRIL		
	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio
Vestibulo	26	2336	89,85	143	18004	126	70	1510	22	125	1776	14
Cruce	132			174	11416	66	75	3796	51	70	29349	419
Pol1	48	32232	671,5	114	9398	82	150	2320	15	81	3896	48
Pol2	108	2137	19,79	229	9652	42	170	62564	368	131	5267	40
Hoya B	132			102	0		70	14177	203	55	4426	80
Pozo	68			115	8104	70	46	9278	202	32	3706	116
Media			130,19			64			143			120

114,40

PLATE COUNTING VS FLOW CYTOMETRY IN WATER SAMPLES

Table 2. Comparison of cell counts in water samples by plating and flow cytometry.

	JANUARY			FEBRUARY			MARCH			APRIL		
	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio	P	FC	Ratio
Vestibulo	9880	46.385	5	5.460	363.648	67	15.280	28.329	2	0	16.152	
Cruce	0	90.385		6240	761.120	122	1920	34.228	18	7.640	49.316	6
Pozo	6540	134.132	21	9.360	441.287	47	1.200	35.980	30	20.080	110.660	6
Media			12,60			79			17			6

28,43

PLATE COUNTING VS EPIFLUORESCEN MICROSCOPY IN SOIL SAMPLES

Table 3. Comparison of cell counts in soil samples by plating and epifluorescence microscopy.

	SPRING 15		
	CC	EF	Ratio
POL1S	5.700	1878600	330
POL1P	0	2424000	0
POL2S	7.200	1151400	160
POL2P	27000	121200	4
VS	4.000	1878600	470
VP	4.900	nc	
HCS	34400	90900	3
HCP	131900	30300	0
HBS	11.900	606000	51
HBP	0	30300	0
Media			105

CONCLUSIONS

1. Culture dependent methods are selective, therefore are biased and, as a consequence, they have been shown to systematically underestimate numbers of total bacteria.
2. Direct counting procedures (Flow cytometry and microscopy) are rapid but have the disadvantage that they do not discriminate between living and dead cells. Additional improvements may be done in our protocols.
3. Depending on the sample, plating efficiency can be less than 1%.
4. Colony sequencing provides a rapid overview of culturable microbial diversity of air, water and soil samples.

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