Development of protocols for microbiological control in Altamira Cave

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ABSTRACT

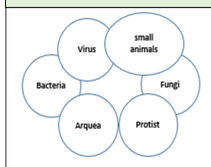
Art Caves are threatened by uncontrolled growth of microorganisms, especially fungi. At the same time, in spite of the extreme ambiental conditions, they are inhabited by very diverse microorganisms. Altamira Cave contains one of the best collections of parietal art and is also threaten by potential 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

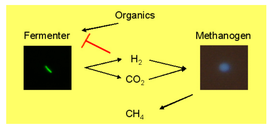
MICROBIAL ACTIVITY

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

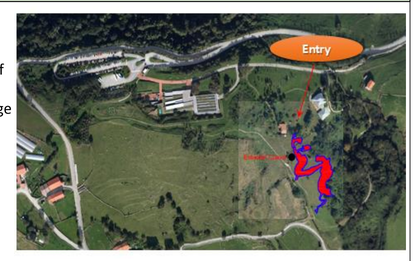


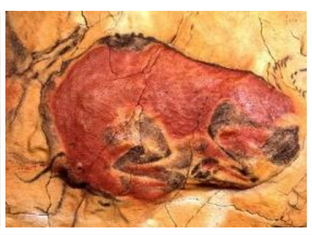
ALTAMIRA CAVE:

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



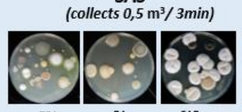


SAMPLING

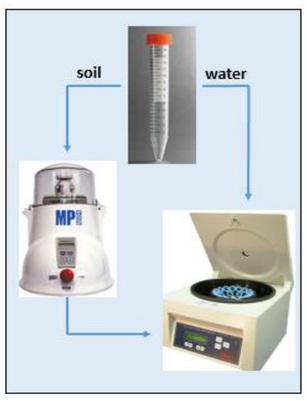
AIR

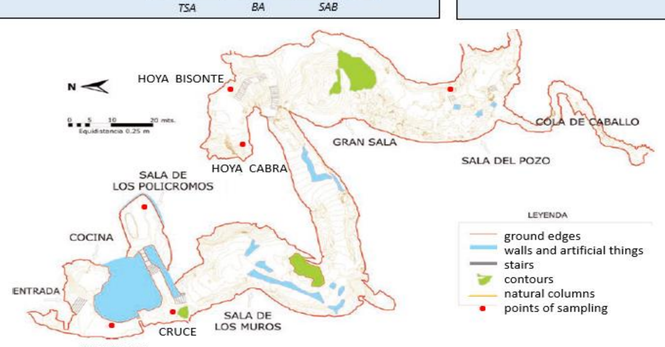
Coriolis *(collects 3m³/ 10 min)*

SAS *(collects 0,5 m³/ 3min)*



SOIL AND WATER





ANALYSIS QUANTITIVE

CULTURE

- Air (SAS)
- Water
- Soil

Extraction procces

Plating

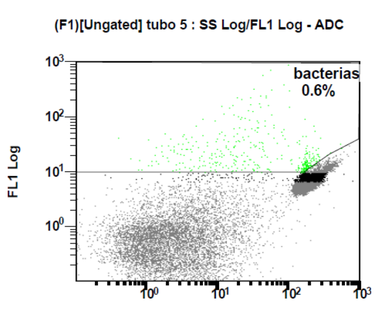
TSA 30ºC | BA 37ºC | BA + CO2 37ºC | SAB 26ºC

Culture improvements *(Tanaka et al.)*

FLOW CYTOMETRY

- Air (Coriolis)
- Water

Bacterial Counting kit (Life Technologies)



EPIFLUORESCENCE

- Soil
- Water

Counting procedure

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

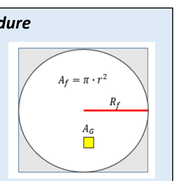
$A_G = 0{,}12749 \cdot 0{,}12723 = 0{,}0162\,mm^2$

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

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

Where:

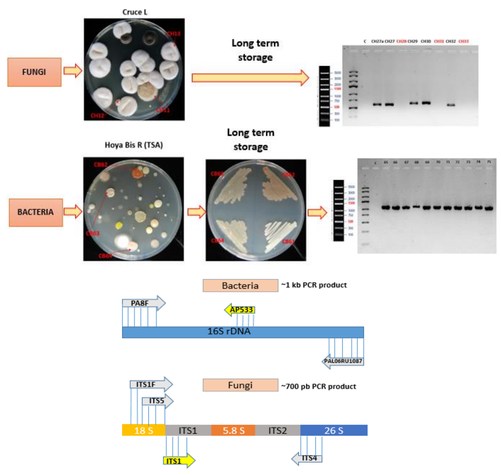
A_f = Area of a field
A_G = Area of grid in field view
$\bar{x}$ = Average bacteria per 9 fields



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

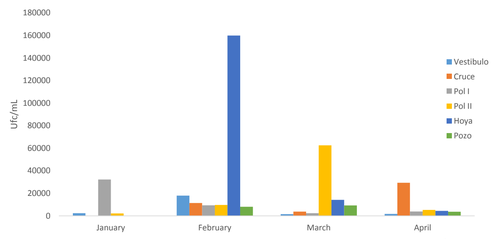


Figure 2. Total bacteria counts by flow cytometry (bacteria/m3) in air samples taken with the Coriollis sampler from different halls of Altamira Cave.

WATER

PLATE COUNTING

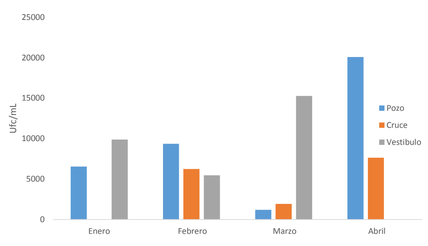


Figure 3. Monthly cell counts by plating (cfu/mL) in water samples from Altamira Cave. Water samples were diluted and incubated in TSA at 30ºC 36h.

FLOW CYTOMETRY

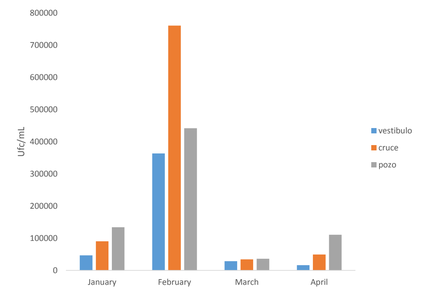


Figure 4. Total bacteria counts by flow cytometry (bacteria/mL) in water samples from Altamira Cave.

SOIL

PLATE COUNTING

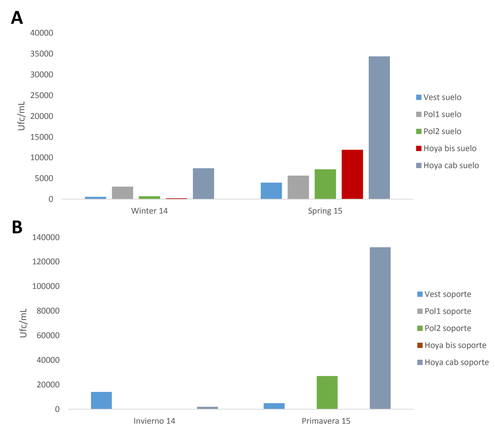


Figure 5. Seasonal cell counts by plating (cfu/g) in soil and wall samples from Altamira Cave. TSA plates were incubated at 30ºC 36h.

EPIFLUORESCENCE MICROSCOPY

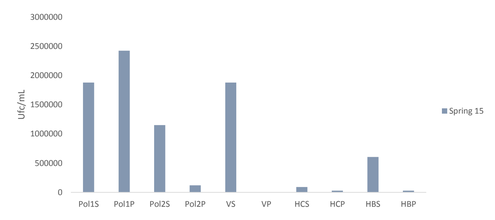
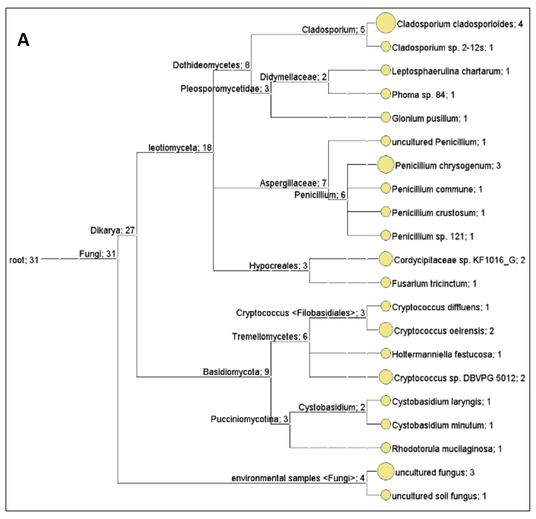


Figure 6. Total cell counts by epifluorescence microscopy of soil and Wall samples from Altamira Cave.

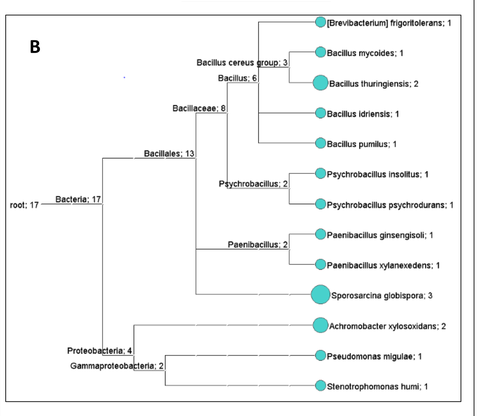
SEQUENCING

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

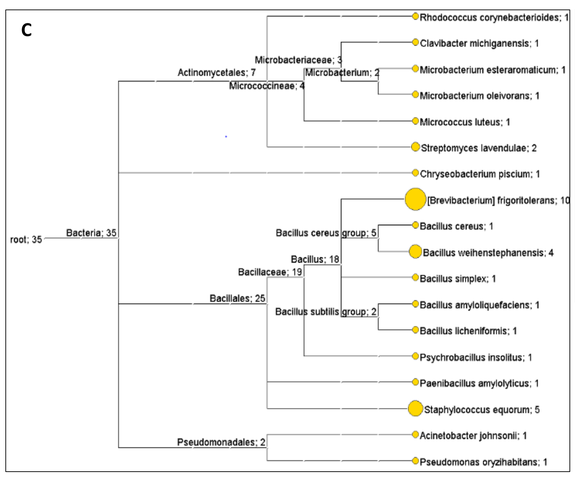
AIR FUNGI



SOIL BACTERIA



AIR BACTERIA



WATER BACTERIA

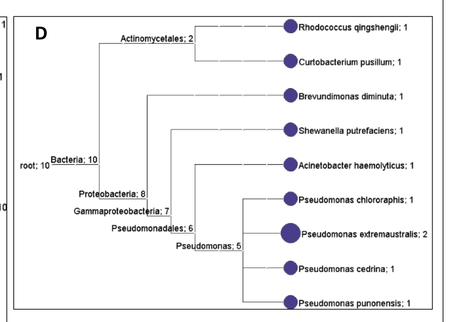


Figure 7.Phylogenetic diversity of the Altamira sequences computed by Megan. The number of sequences assigned at each taxon is indicated in the cladogram tree branches.

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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