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Mineralogical and geomicrobiological investigation of phosphorite from Ervenik, Croatia

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ABSTRACT

Phosphate minerals hydroxylapatite, fluorapatite and crandallite were identified in nodules within phosphorites from Ervenik, Croatia. The minerals were identified using optical microscopy, XRD, SEM and EDS analyses. The presence of fungi was recognized only in association with phosphate-rich phases. Fungal activity resulted in the dissolution of apatite, producing hollow crystals, particularly in hydroxylapatite–enriched zones. A substantial number of hyphae were observed on the surface of phosphate minerals, in addition to saprophytic bacteria and bacterial spores. Induced activity of phosphate-accumulating bacteria in an aquatic environment caused dissolution of the phosphate minerals. The aqueous phase contained increased concentrations of several elements, including Ca, Sb, U, V and As. These elements are important constituents of minerals of the apatite group. As a consequence of the crystallization of apatite, the concentration of phosphate decreases with a corresponding increase in aluminium concentration, resulting in the prevalence of crandallite as the stable phase, forming the outer sector of the spherulites.

Keywords: phosphorite, hydroxylapatite, fluorapatite, crandallite, fungi, bacteria

1. INTRODUCTION

Phosphorite deposits commonly occur in caves, caverns and other karst phenomena (DAHANAYAKE & SUBASINGHE, 1989; and references therein). They were identified in limestones near the village of Ervenik, in Croatia during a 2009 field campaign. It is generally assumed (IVANOVIĆ et al., 1973) that these limestones are of Cretaceous age, while the exact age of the deposits is unknown, but assumed to be Pleistocene. The source of phosphorus could be the phosphate-rich guano material originating from bats and birds inhabiting the surrounding caves (MARKOVIĆ, 2002). Several outcrops of these deposits were found at the foot of the SE part of Mt. Velebit near Ervenik, Croatia (POSILOVIĆ et al., 2010).

Phosphates are considered distinct in that they are able to incorporate more than half of the elements of the periodic table into their atomic structures (PASERO et al., 2010). The apatite group minerals and crandallite are assumed to be secondary alteration phases and the main phosphorus-bearing minerals.

The aim of this study was to define the paragenesis of phosphorite from Ervenik, Croatia and to study bacteriological influence to this phosphorite.

2. MATERIALS AND METHODS

2.1. Sampling and preparation of minerals

Phosphorite samples were collected at a location close to the village of Ervenik (Fig. 1). The former phosphorite mine is no longer operational, so the samples were collected from old mine dumps. Thin sections were prepared for optical investigation. The collected rock samples were crushed, sieved and the particle size of 0.212–0.500 mm was prepared in the laboratory for separation and bacteriological experiments, as well as other analyses.

2.2. Experimental methods

A polarising microscope was used for optical investigations. Mineral phase determinations of the bulk samples were done using a Philips X'pert powder diffractometer, with CuKα radiation filtered with a graphite monocrystal monochromator running at 40 kV and 40 mA. An X-ray diffraction data set was collected from 4 to 65º2θ. Powder diffraction patterns were identified and indexed using X'Pert HighScore Plus v. 2.1. software (PANALYTICAL, 2004), and ICDD Powder diffraction file database (PDF 2, ICCD, 2004). Unit cell parameters were calculated using "Unitcell" software (HOLLAND & REDFERN, 1997). Crushed samples of phosphorite were examined by scanning electron microscopy (Tescan Vega TS 5136), coupled with an INCA 250 system for elemental composition analysis. The water content in the minerals was determined by drying a 1.000 g aliquot of the mineral at 105°C/4 h.

In sieved samples, the number of natively present bacteria was established. Since the mineral samples were originally found under aerobic conditions and were maintained in an appropriate environment until analysis, the number of aerobic/facultatively anaerobic bacteria was checked. The number of natively present heterotrophic bacteria either viable or in the form of spores was determined in each mineral. The presence of faecal streptococci was tested in order to check the possible anthropogenic influence in the form of faecal contamination in the minerals (BOUVY et al., 2010). In order to check whether the minerals facilitated bacterial growth in contact with water, bacteriological tests were performed (1) after 3 min, and (2) after 4 days of incubation.

In order to check if the phosphate-accumulating bacteria use the minerals as the source of phosphorus, the performance of phosphate-accumulating bacteria *Acinetobacter junii* DSM no. 1532 was tested (HRENOVIĆ et al., 2010a).

For determination of the presence of bacteria in minerals, a 1.0 g aliquot of each mineral was placed in a tube containing 9 mL of 0.05 molL^{-1} NaCl. Samples were vigorously shaken on a mechanical shaker (45Hz/3min, Kartell TK3S) in order to detach the adsorbed bacteria from the mineral (DURHAM et al., 1994). The number of aerobic/facultatively anaerobic bacteria was determined in the supernatant. The number of faecal streptococci was determined on Slanetz-Bartley agar (Biolife, Italy) after 3-day incubation at 37°C. The number of heterotrophic bacteria and bacterial spores was determined on the nutrient agar (Biolife, Italy) after 3-day incubation at 22 °C. In order to determine the number of bacterial spores, the minerals were pasteurized at 80 °C for 10 minutes.

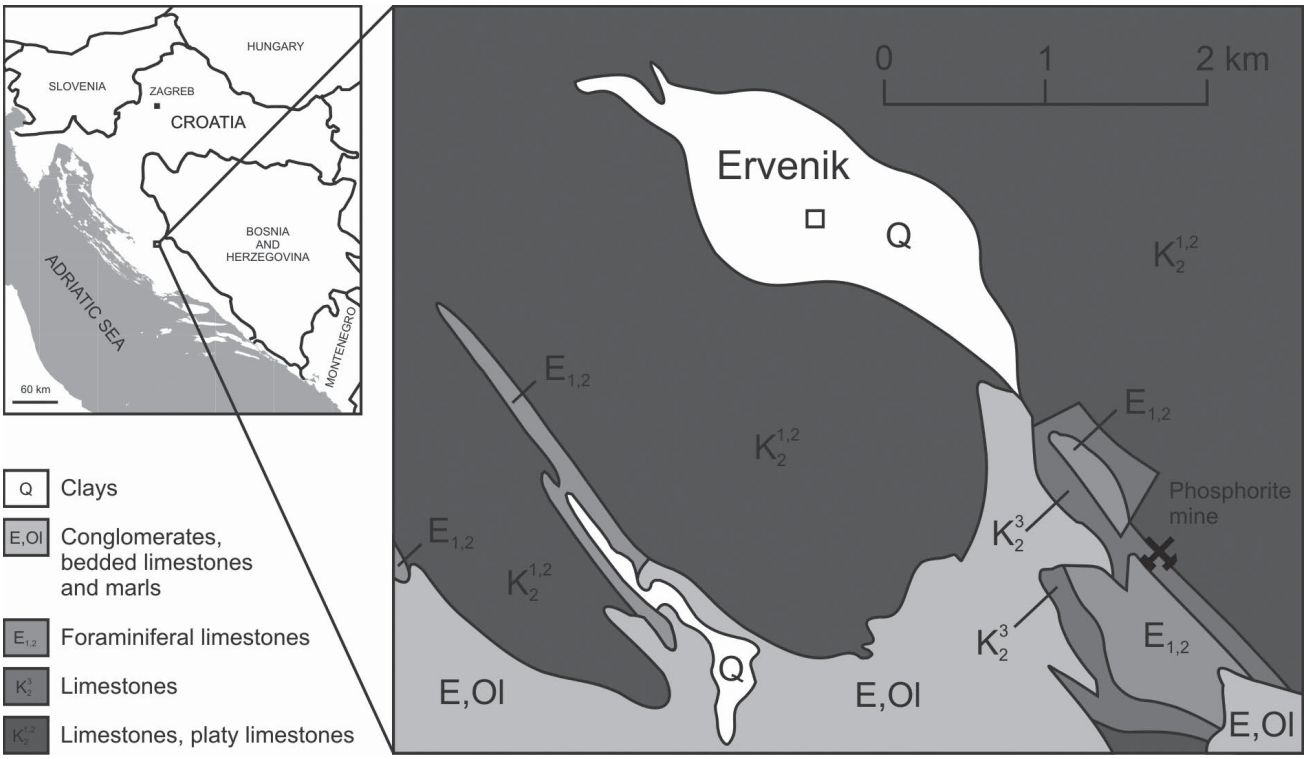

Figure 1: Location map and geological setting of phosphorite occurrence near Ervenik, Croatia. Basic lithologies are simplified after IVANOVIĆ et al. (1973).

In order to establish the likelihood of multiplication of the bacteria present on mineral surfaces in contact with water, a 1.0 g aliquot of each mineral was placed in a tube which contained 9 mL of 0.05 molL^{-1} NaCl and incubated for 4 days in the dark at 22 °C, with continual stirring (70 rpm) without additional aeration. The bacteriological analysis was performed after this incubation period.

In order to check if the phosphate-accumulating bacteria *A. junii* use the minerals as a source of phosphate, a 1.0 g of autoclaved (121°C/20 min) mineral was placed in a tube which contained 9 mL of 0.05 molL^{-1} NaCl. A suspension of *A. junii* was added (1.06±0.04 x 10^7 CFU/mL) to each tube and these were aerobically incubated in darkness for 24 h at 30°C with stirring (70 rpm). After incubation, samples were vigorously shaken on a mechanical shaker in order to detach the adsorbed bacteria from the mineral. The number of *A. junii* was determined from the supernatant on the nutrient agar after incubation at 30 °C/24 h. The Neisser stain was utilised to confirm the presence of polyphosphate granules in the cells of *A. junii* while the Gram stain was used to confirm the shape of bacteria and immobilization of the cells on the mineral surfaces. All measurements were done in triplicate. The final pH of the suspension was measured using a WTW 330 pH-meter.

A multi-element analysis of water samples after the dissolution experiments was done by high resolution inductively coupled plasma mass spectrometry (HR-ICPMS), on the sector-field instrument Element 2 (Thermo, Bremen, Germany). The samples were diluted and an indium internal standard (1 μL^{-1}) added. The measurements of selected isotopes were performed at three different resolutions: low resolution (^{7}Li, ^{85}Rb, ^{98}Mo, ^{111}Cd, ^{120}Sn, ^{121}Sb, ^{133}Cs, ^{205}Tl, ^{208}Pb,^{238}U), medium resolution (^{23}Na, ^{25}Mg, ^{27}Al, ^{42}Ca, ^{51}V, ^{52}Cr, ^{59}Co, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{86}Sr, ^{138}Ba) and high resolution (^{39}K, ^{75}As). External calibration was used for quantification. Standards were prepared by appropriate dilution of a multi-element reference standard (Analytika, Czech Republic). Details of the analytical method are described elsewhere (FIKET et al., 2007).

Statistical analyses were carried out using Statistica Software 9.1 (STATSOFT, 2009). The numbers of bacterial CFU were logarithmically transformed beforehand to normalize the distribution and to equalize variances of the measured parameters. The comparisons between samples were done using the one-way analysis of variance (ANOVA), and subsequently the post-hoc Duncan test was performed for calculations concerning pair-wise comparisons. Statistical decisions were made at a significance level of $p<0.05$.

3. RESULTS

3. 1. Mineralogical analyses

The phosphate mineral occurrences found in caves are usually compact and reddish or greyish in colour, with a high Al_2O_3 and FeO content, but low in carbonate. The minerals occur as white to greyish spherulitic aggregates or single crystals <2 mm in size. Optical microscopy and SEM of spherulitic aggregates showed at least two different phosphate phases.

Thin sections of phosphate containing samples show clear zonality. In the inner core of the spherulites, apatite crystallises in the form of crystal sprays up to 1.5 mm in length; the outer parts of the spherulites contain very finely grained radial to fibrous crandallite. Larger spherulites are banded and composed of alternating apatite and crandallite layers.

Samples of phosphorite recently collected at the mine dumps, left during the 1950's were crushed to grains, up to 2mm diameter and separated into three fractions named: Ervenik_white, Ervenik_pink and Ervenik_mix.

On the basis of combined SEM, EDS and XRD analyses, the phosphate phases were identified as hydroxylapatite, fluorapatite, and crandallite.

The sample Ervenik_white has been identified as an apatite group mineral (predominantly fluorapatite), based on these unit cell parameters: $P6_3/m$, a = 9.423(2) Å, c = 6.878(2) Å, and V = 529.0(2) Å^3) (Fig. 2A, Table 1). In the sample Ervenik_pink, two different phosphate phases were identified: an apatite group mineral (predominantly hydroxylapatite) based on unit cell parameters: $P6_3/m$, a = 9.342(2) Å, c = 6.893(2) Å, and V = 520.9(2) Å^3) and crandallite ($R\bar{3}m$, a = 7.006(3) Å, c = 16.19(1) Å, and V = 688.2(6) Å^3) (Fig. 2C, Table 1). As expected, in the sample Ervenik_mix, a mixture of the aforementioned samples was identified (Fig. 2B, Table 1). A very good fit of the observed unit cell parameters with database information, enabled us to identify the mineral phases without additional chemical characterization.

Broken samples of phosphorite were used for SEM investigations. Idiomorphic apatite crystals were found in the cores of the nodules. The crystal habit is a simple combination of hexagonal prisms and basal faces. They are <50 μm long and <35 μm wide (usually 25 x 10 μm). Some crystals show parallel growth, while others have more irregular growth patterns (Fig. 3). Some crystals are hollow. The smaller ones have empty cores, but larger crystals have a hexagonal core within the hollow outer part of the crystal. Tiny fibres connecting the core and crust of the crystals can occasionally be seen (Fig. 3a).

A closer look at the fibres identified them as fungal hyphae that grow on the surface of apatite crystals and influence these with their metabolic processes (Fig. 4).

The outer zones of nodules consist of smaller crystals of crandallite. Crandallite crystals are of rhombohedral habit <5 μm in size. They usually have small basal faces (Fig. 5), and are mutually separated by fine-grained anhedral bauxitic material.

Compositions of apatite and crandallite were confirmed by EDS analyses. EDS analyses of apatite, in the cores of nodules, commonly show the presence of Ca, P, and O. This is a strong indication for hydroxylapatite (Fig. 6A). In contrast to hydroxylapatite, the EDS spectrum of crandallite shows the additional presence of aluminium (Fig. 6B).

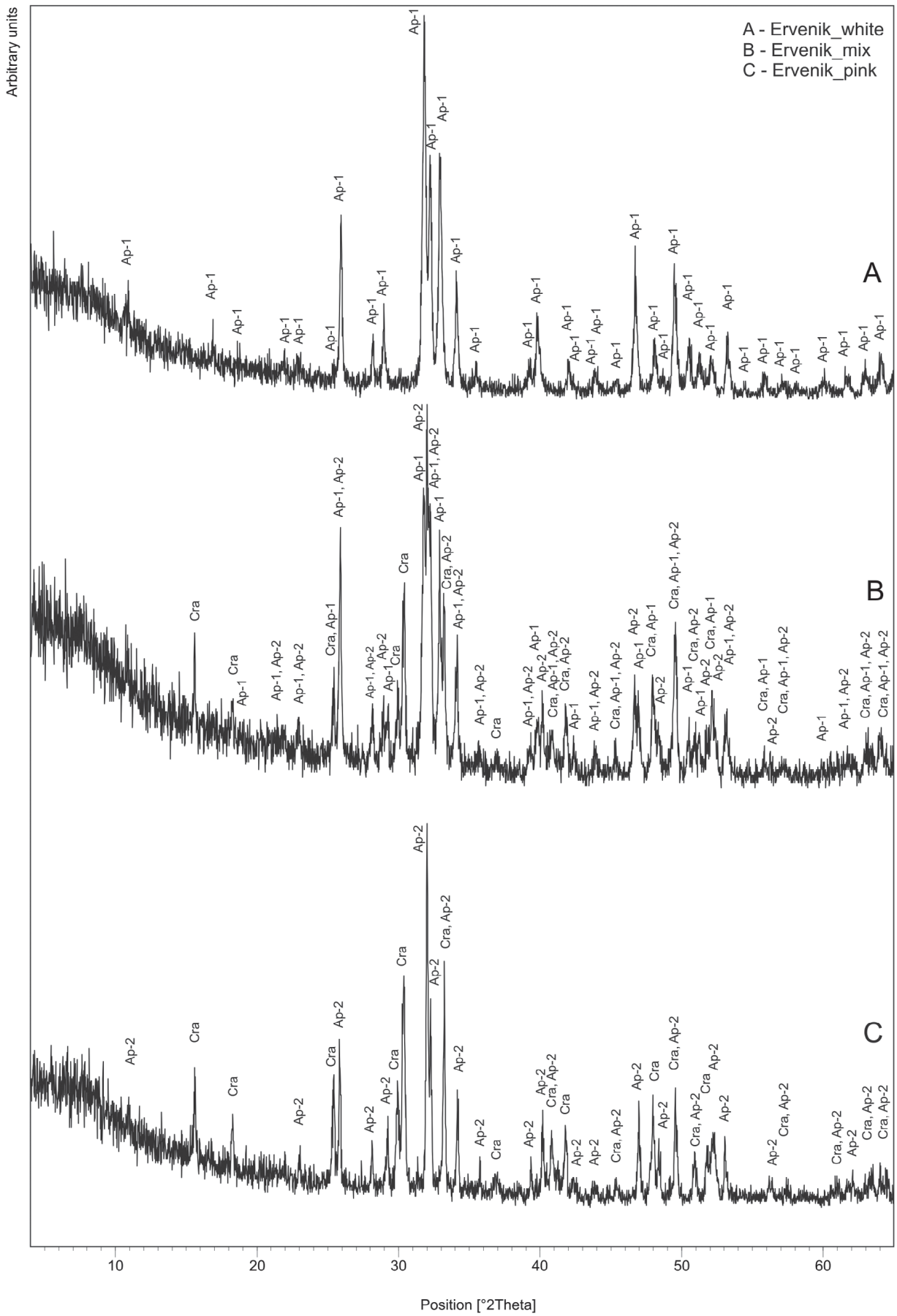

Figure 2: X-ray diffraction patterns of samples from Ervenik. (A) Sample Ervenik_white; (B) Ervenik_mix; (C) Ervenik_pink; Ap-1 = apatite group mineral (probably fluorapatite); Ap-2 = apatite group mineral (probably hydroxylapatite); Cra = crandallite.

Table 1: Unit cell parameters for the identified phases.

MINERAL	s.g.	a / Å	c / Å	α	γ	V / Å^3
Apatite group mineral Ervenik_white	$P6_3/m$	9.423(2)	6.878(2)	90.00°	120.00°	529.0(2)
PDF 01-070-0796 (fluorapatite)	$P6_3/m$	9.4215	6.8739	90.00°	120.00°	528.41
Apatite group mineral Ervenik_pink	$P6_3/m$	9.342(2)	6.893(2)	90.00°	120.00°	520.9(2)
PDF 01-086-0740 (hydroxylapatite)	$P6_3/m$	9.3520	6.8820	90.00°	120.00°	521.26
Crandallite	$R\bar{3}m$	7.006(3)	16.19(1)	90.00°	120.00°	688.2(6)
PDF 01-070-2069 (crandallite)	$R\bar{3}m$	7.0050	16.1920	90.00°	120.00°	688.09

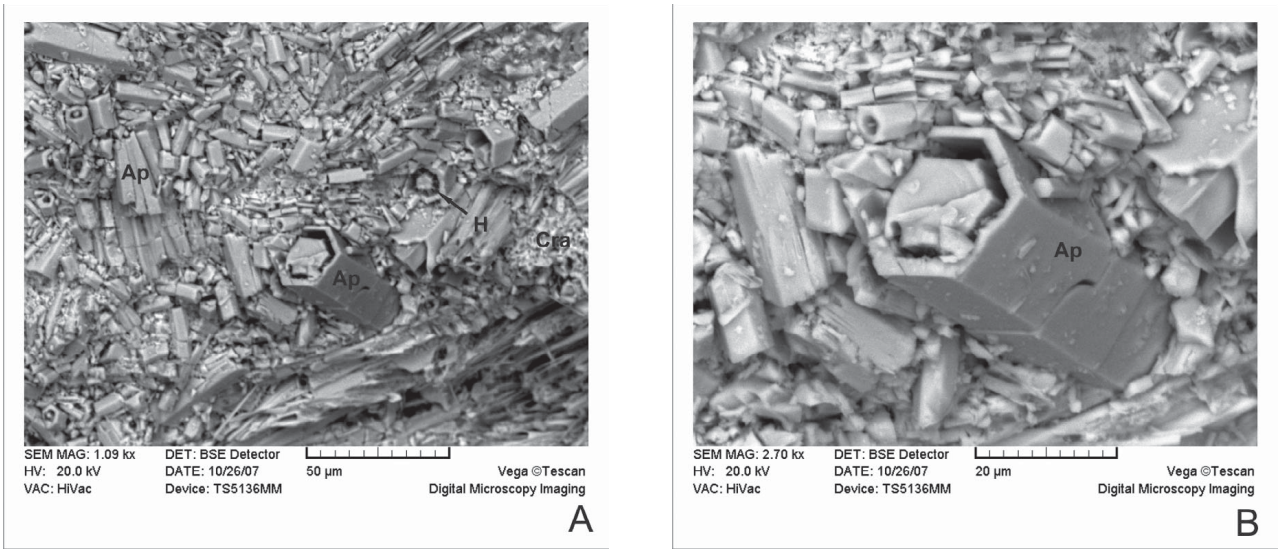

Figure 3: (A), (B) SEM BSE photograph of phosphorite from Ervenik, Croatia. Apatite crystals (Ap) are <50 µm long. Most of them are hollow with central zone preserved.

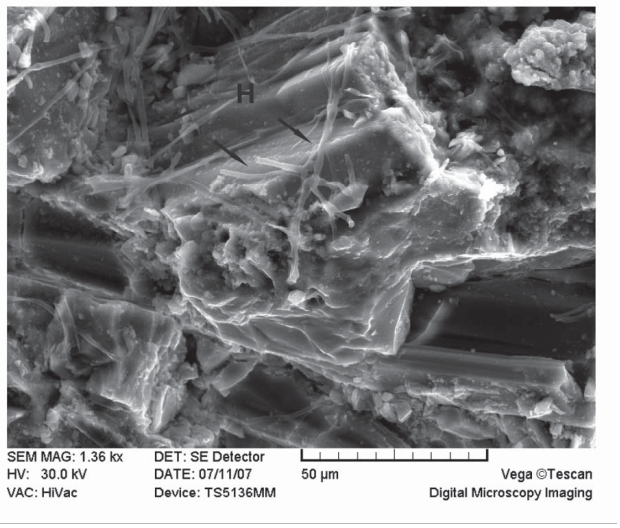

Figure 4: The partial dissolution of an apatite crystal is a result of fungal activity on zonal apatite crystals (solid solution of hydroxylapatite and fluorapatite). SE mode allows better view of the organic hyphal tissue (H).

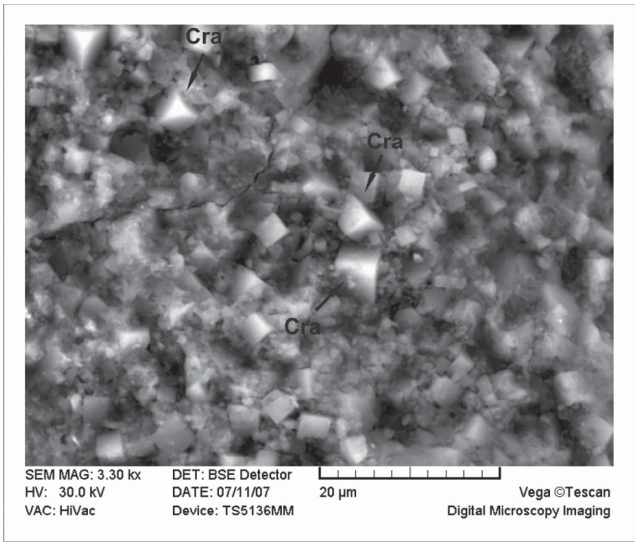

Figure 5: Crandallite crystals (Cra) from phosphorite are developed on the contact with the bauxitic host rock. The crystals are of rhombohedral habit and up to 5 µm in size. SEM BSE image.

3.2. Bacteriological analyses

The absence of faecal streptococci (bacteria of the genus *Enterococcus*), used as surrogates for human and animal pathogens in assessing water quality (BOUVY et al., 2010), confirmed that the minerals were not contaminated with municipal wastewater (Table 2).

The original minerals contained higher numbers of viable saprophytic bacteria than the bacterial spores (Table 2).

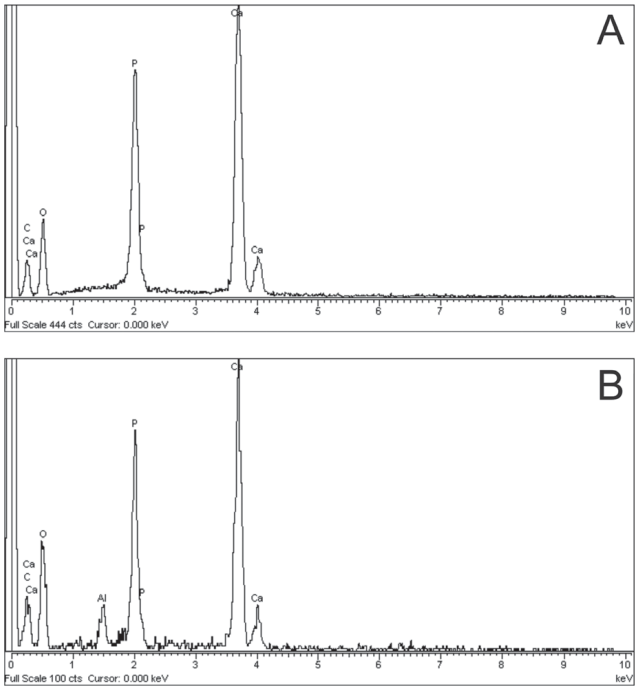

Figure 6: (A) EDS spectrum of hydroxylapatite. (B) EDS spectrum of crandallite.

The presence of viable bacteria in the materials may be explained by the 0.92–0.98% water content (which was lost at 105 °C/4 h) in the minerals, which was sufficient to maintain the bacteria. The sample Ervenik_pink naturally contained a significantly lower number of viable saprophytic bacteria than the other two samples. After 4 days of incubation, the number of viable saprophytic bacteria significantly increased in samples Ervenik_white and Ervenik_mix, but not in sample Ervenik_pink. The sample Ervenik_pink still contained the lowest number of viable saprophytic bacteria. The multiplication of saprophytes in samples decreased in order Ervenik_white = Ervenik_mix > Ervenik_pink (Table 2). It seems that the number of viable saprophytic bacteria and its multiplication was dependent of the content of crandallite in the minerals. In particular, the Al contained in crandallite can have a negative influence and a toxic effect against bacteria (HRENOVIĆ et al., 2010b).

The number of bacterial spores (Table 2) in the samples did not change on incubation of minerals in 0.05 mol L^{-1} NaCl for 4 days. This suggested that the minerals in the presence of nutrient depleted water were not suitable for induction of the germination of bacterial spores.

The final pH values (Table 2) of the aqueous media became alkaline after 3 min of contact of minerals with 0.05 mol L^{-1} NaCl, and slightly decreased to neutral after 4 days of incubation. Although significantly higher in the aqueous medium which contained the sample Ervenik_pink than Ervenik_white or Ervenik_mix, the maximum difference was only 0.19 pH units. Therefore, the pH value of the medium can be eliminated as a cause of different bacterial numbers in minerals.

In experiments with phosphate-accumulating bacteria, all inoculated bacteria *A. junii* were immobilized onto minerals during 24 h of incubation (Fig. 7). The number of immobilized bacteria per mass of dry mineral was 1.04±0.09 x 10^7 CFU/g. There was no significant change among the initial and final numbers of *A. junii* in the tubes. It is probable, that the mineral and nutrient depleted water failed to provide enough sources of nutrients for multiplication of bacteria which were isolated from activated sludge treating the wastewater (HRENOVIĆ et al., 2003). All inoculated bacteria survived and accumulated polyphosphate inside the bacterial cells. The phosphate accumulated inside the cells of *A. junii* in the form of polyphosphate granule was confirmed by the Neisser stain (Fig. 8). This experiment confirmed that the phosphate-accumulating bacteria *A. junii* were able to use the minerals as the source of phosphate for their metabolism. These findings are in agreement with SMITH et al. (1978), where sufficient phosphate was released by the partial dissolution of apatite crystals at pH 7.8 to support the growth of bacteria. The fact that all inoculated bacteria survived and were spontaneously immobilized onto minerals confirmed that the investigated phosphorites were excellent substrates for the immobilization of selected bacteria.

Table 2: Number of bacteria per 1.0 g of dry mineral after 3 min and after 4 days of contact with water. * significantly different at 4 days when compared to 3 min; [A] significantly different to Ervenik_white; [B] significantly different to Ervenik_mix.

Parameter	Ervenik_white	Ervenik_mix	Ervenik_pink
		3 min	
Saprophytic bacteria (10^3 CFU/g)	5.17±1.04	6.70±0.95	2.37±1.12[A,B]
Bacterial spores (CFU/g)	55±15	72±12	45±15
Faecal streptococci (CFU/g)	0	0	0
Final pH	7.74±0.02	7.76±0.02	7.81±0.02[A,B]
		4 days	
Saprophytic bacteria (10^3 CFU/g)	42.33±9.45*	52.33±9.07*	2.43±0.87[A,B]
Bacterial spores (CFU/g)	61±7	78±9[A]	43±8[A,B]
Faecal streptococci (CFU/g)	0	0	0
Final pH	7.47±0.02*	7.47±0.02*	7.66±0.02*[A,B]
Multiplication of saprophytes (c_{4days}/c_{3min})	8.2±1.0	7.8±0.3	1.0±0.1[A,B]

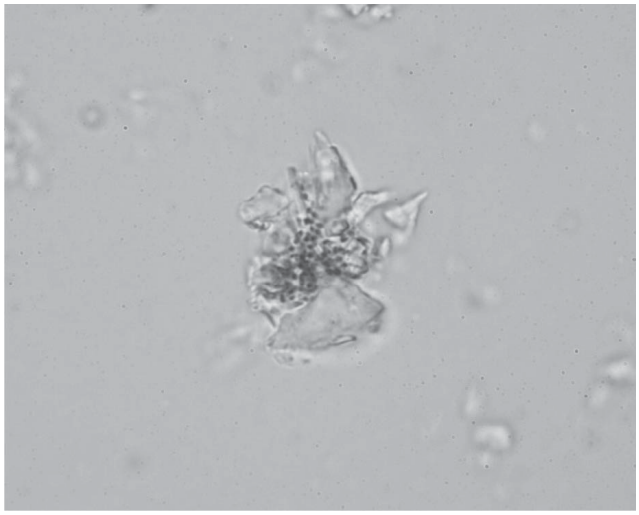

Figure 7: Cells of *A. junii* immobilized onto mineral Ervenik_white.

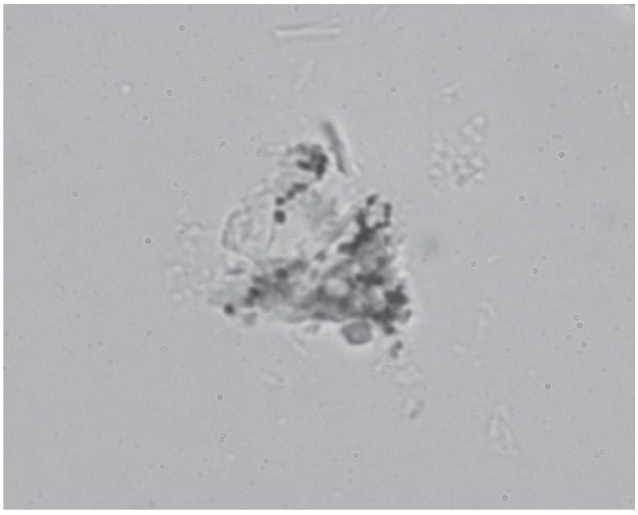

Figure 8: Granules of polyphosphate inside the cells of *A. junii* immobilized onto mineral Ervenik_white.

After the contact of the sample Ervenik_white with *A. junii*, the solution was filtered through a Sartorius nitrocellulose filter with a pore diameter of 0.2 μm and the water was analysed by ICP-MS to compare it with a blank solution of 0.05M NaCl, in order to detect the bacteriological activity on the phosphate minerals (Table 3). A set of 22 trace elements were measured: Al, As, Ba, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Mo, Ni, Pb, Rb, Sb, Sn, Sr, Tl, U, V, and Zn, (most of these elements are common constituents of apatite group minerals), as well as the macroelements Ca, K, Mg and Na which were expected at higher concentrations.

Na was measured in both blanks and in sample waters in the same order of concentrations. The concentrations of Ca, Mg and K in the aqueous phase of the samples were higher after bacteriological activity. The highest increase in concentration was observed for Ca (124-fold) when compared to concentrations of Mg and K (5 and 14 times, respectively). This observation is in agreement whit the statement that Mg and K ions were essential elements for *A. junii* while Ca was not (HRENOVIĆ et al., 2010c). Additionally, Ca is the dominant cation in apatite and Mg and K are minor constituents. Among the trace elements, the bacterial activity has the highest influence on the concentrations of Sb, U, V and As, which increased 973, 105, 452 and 226 times, respectively. A significant increase in the concentrations was also established for Ni, Cu and Sr (24, 61 and 57 times, respectively). Li, Cd, Cr, Mn and Co increased their concentrations about 10 times, Rb, Mo, Tl, and Al increased about 5 times, Sn, Fe and Ba about 2 times, Pb concentration remained the same, while Cs concentration significantly decreased. It is possible that *A. junii* has the ability to accumulate Cs within its cells or on their surface, which has been observed for other bacteria (NAKAO et al., 2005), but this needs to be further examined. Increased concentrations of elements may be explained as a result of the dissolution of hydroxylapatite, which can contain these elements in its crystal lattice. These elements are not involved in bacterial metabolic processes.

Table 3: Concentrations of macro and trace elements in water after dissolution experiments in the presence (sample) and absence (blank) of *A. junii* bacteria.

Macroelements	Concentration (mg L^{-1})	
	Probe	Blank
Ca	62.00	0.50
K	14.10	1.00
Mg	0.90	0.20
Na	1294	1280
Trace elements	**Concentration (μg L^{-1})**	
	Probe	Blank
Al	8.40	1.87
As	2.26	0.01
Ba	17.3	11.2
Cd	0.16	0.01
Co	0.29	0.03
Cr	7.53	0.94
Cs	0.02	0.13
Cu	40.16	0.66
Fe	0.98	0.46
Li	1.97	0.17
Mn	3.34	0.20
Mo	0.65	0.14
Ni	3.14	0.13
Pb	0.09	0.11
Rb	1.93	0.34
Sb	9.73	<0.01
Sn	0.09	0.06
Sr	70.71	1.23
Tl	0.04	0.01
U	1.05	0.01
V	9.04	0.02
Zn	128.8	91.4

4. DISCUSSION AND CONCLUSION

From the results of the X-ray diffraction analysis it is possible to identify that the minerals investigated (according to their structure) belong to the apatite group of minerals (hexagonal apatites). Apatite group minerals consist of 15 different minerals. Most of them (11) have the same hexagonal structure and the space group $P6_3/m$ (PASERO et al., 2010). By calculating unit cell parameters, it is possible to conclude that these are apatite group minerals ($Ca_5(PO_4)_3X$, X=F, OH, Cl), having unit cell parameters comparable to fluorapatite and hydroxylapatite. Unit cell dimensions of apatite group minerals in the separated parts of spherulites significantly differ from each other, suggesting that there are two end members present in these nodules, partly making solid solutions. The most interesting members of the apatite group are hydroxylapatite and fluorapatite, as they can be affected by biological activity.

The plumbogummite subgroup of the alunite-jarosite group of minerals (BACK & MANDARINO, 2008) contains phosphates that are very important as weathering products, commonly present as constituents of soils. According to unit cell calculations and EDS measurements, this is crandallite $CaAl_3(PO_4)_2(OH, H_2O)_6$. This mineral phase is related to the presence of aluminium in bauxitic material surrounding the spherulites.

The identified phosphate minerals are assumed to be associated with biomineralization processes, these being a consequence of organic matter interaction with the carbonate phases of the host rock limestones (POSILOVIĆ et al., 2010).

Careful investigation of the collected samples prior to bacteriological/biological experiments, resulted in the interesting observation that fungi only grow on phosphate rich sections of the mineral substrate, and not on carbonate nor iron and aluminium rich oxide phases.

Due to the higher solubility of hydroxylapatite, with respect to fluorapatite, hydroxylapatite enriched zones were preferentially dissolved resulting in hollow apatite crystals.

Crandallite formation on the contact of phosphorite nodules with the surrounding bauxite matrix is the result of increased aluminium availability. Apatite and crandallite spherulites were formed during early diagenesis of the cave sediment; each single spherulite was precipitated in a partially closed microenvironment of the sediment voids and pores. Apatite occupying the central portions of the spherulite centre, formed prior to crandallite in the outer spherulite region. The calculated stability fields for apatite and crandallite may be used to explain the bi-phase precipitation in the spherulites. Higher phosphate and lower aluminium concentrations with higher pH will favour apatite precipitation; such conditions prevailed during precipitation of the spherulite core. As a consequence of apatite precipitation, phosphate concentration is decreased and aluminium is increased, the stability field of crandallite prevails and crandallite becomes the stable phase precipitating in the spherulite outer sectors (POSILOVIĆ et al., 2010).

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