MEP 19

Mining and Environmental Protection

7th INTERNATIONAL SYMPOSIUM
MINING AND ENVIRONMENTAL PROTECTION

VRDNIK, SERBIA, 25 – 28 SEPTEMBER 2019

27 September 2019

Microbial Diversity in Unique World Mine Allchar

-Reservoir of Minerals and Microbes-

Vaso Taleski[1], Ivica Dimkić[2],
Blazo Boev[1], Ivan Boev[1], Sanja Živković[3],
Slaviša Stankovic[2] and Milka Zdravkovska[1]

[1] University “Goce Delchev” Shtip, North Macedonia
[2] University of Belgrade, Faculty of Biology, Serbia
[3] University of Belgrade, Institute of Nuclear Sciences Vinča, Serbia

Allchar is low-temperature /hydrothermal gold-arsenic-antimony -thallium deposit in the mount Kozuf.

Allchar history:
A myth or thruth:
Some activities were conducted in ancient times

THE SECRET POWER OF ALEXANDER THE GREAT?

Christopher MacDonald

Published on June 29, 2015, **LONDON**

Alexander believed in the power of stones, crystals and talismans to bring success and good fortune. He carried with him gave his generals and favoured soldiers Lorandite, possibly because it was **believed the crystal** could help concentration and bring success through harnessing the **power of the sun.**

“The Alexander phalanx in the battles which he always fought exactly at noon and had his troops move from west to east covered their shields with lorandite, and thereby produced a strong reflection from their shields that blinded the opposing army.”

Allchar history:

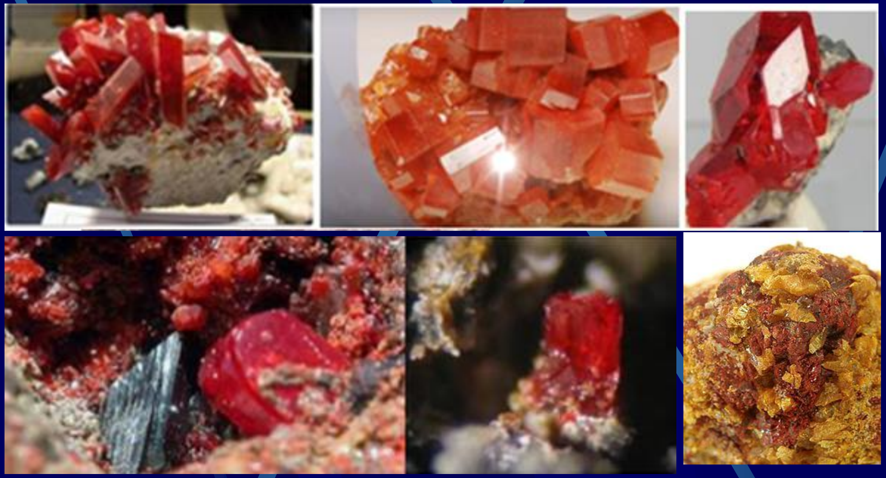
Others estimates that mine was known from 12 or 13 century.

In documents of Ottoman's Empery mine is mentioned from 1481 – Sale of **arsenic** made great income for Turkish sultan.

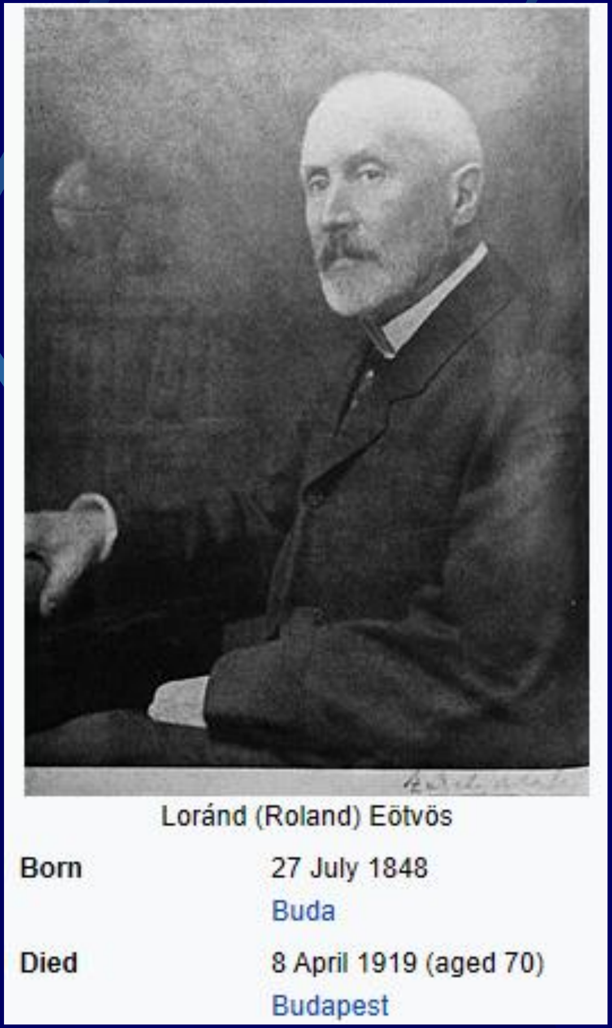
Deposit was mined extensively between 1881 and 1908 for: **Antimony, Arsenic and Thallium,** when British-French company got a concession from Ottoman’s Empire.

From 47 worldwide registered **minerals** containing **thallium (Tl), 13 exists in Allchar**:

- Bernardite,
- Fangite,
- Jankovicite,
- **Lorandite,**
- Parapierrotite,
- Pictopaulite,
- Raguinite,
- Rebulite,
- Simonite,
- Thalliumpharmacosiderite,
- Weissbergite,
- Vrbaite,
- Dollcharite.



Lorándite was first discovered at the Allchar deposit, in **1894** by **Krener**, and named after **Loránd Eötvös**, a prominent Hungarian physicist.

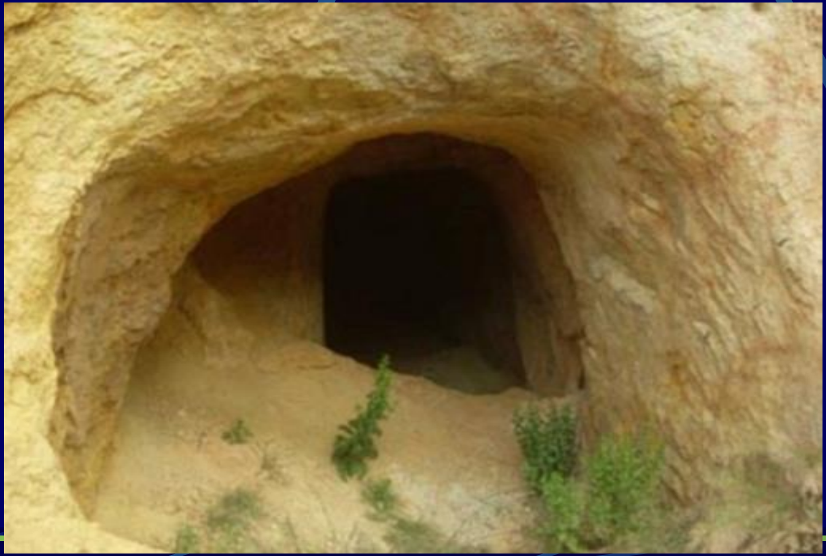


Loránd (Roland) Eötvös

Born	27 July 1848 Buda
Died	8 April 1919 (aged 70) Budapest

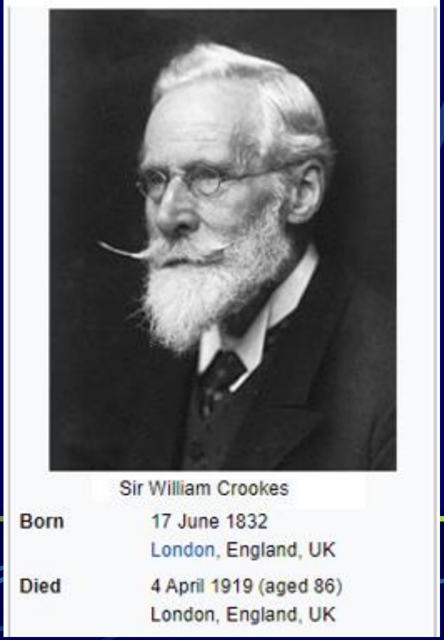
Allchar is as a unique mine in the world, richest of purest deposits of lorandite ($TlAsS_2$ - thallium arsenic sulfosalt), (contains only traces of K, Cr, Fe, Cu, Pb and Zn).

Allchar has the largest lorandite deposit in the world. The “Crven Dol” holds estimated amount of 500 t of thallium.

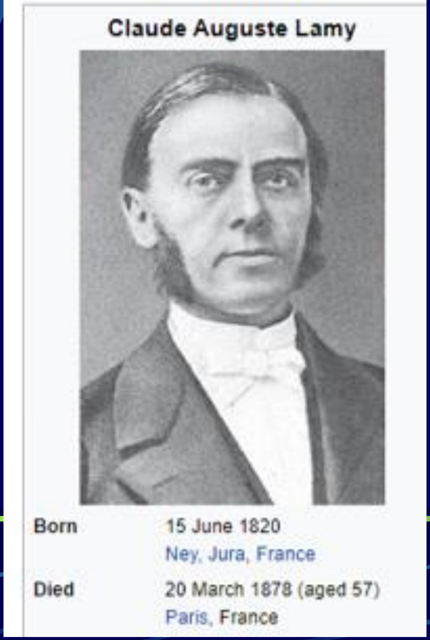


Thallium is a chemical element with atomic number 81.
A gray, soft, highly electrically conducting metal with a low melting point of 304 °C, that is **not found free in nature**.
When isolated,resembles tin, but discolors when exposed to air,

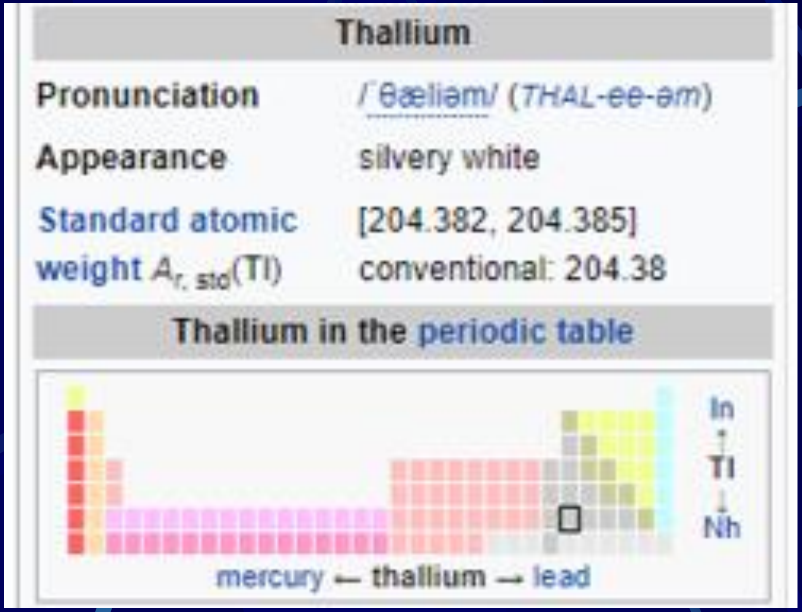
Chemists William Crookes and Claude-Auguste Lamy discovered thallium independently in 1861.


Sir William Crookes

Born	17 June 1832 London, England, UK
Died	4 April 1919 (aged 86) London, England, UK


Claude Auguste Lamy

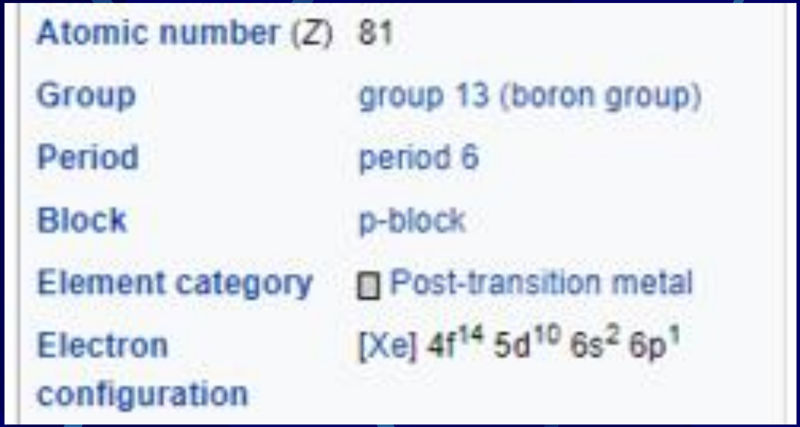
Born	15 June 1820 Ney, Jura, France
Died	20 March 1878 (aged 57) Paris, France



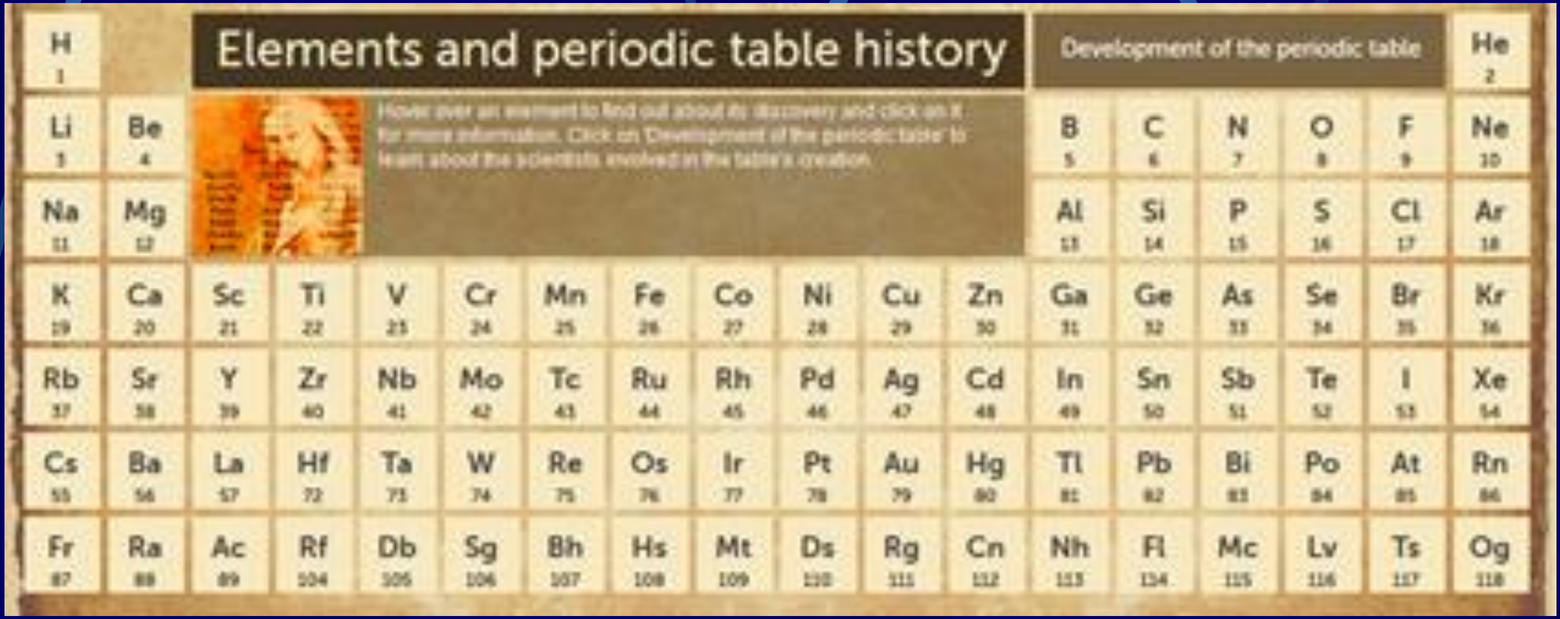
Thallium	
Pronunciation	/ˈθæliəm/ (*THAL-ee-əm*)
Appearance	silvery white
Standard atomic weight $A_{r,\,\mathrm{std}}$(Tl)	[204.382, 204.385] conventional: 204.38

Thallium in the periodic table

mercury ← thallium → lead



Atomic number (*Z*)	81
Group	group 13 (boron group)
Period	period 6
Block	p-block
Element category	☐ Post-transition metal
Electron configuration	$[\mathrm{Xe}]\,4f^{14}\,5d^{10}\,6s^{2}\,6p^{1}$



Elements and periodic table history

Development of the periodic table

Hover over an element to find out about its discovery and click on it for more information. Click on 'Development of the periodic table' to learn about the scientists involved in the table's creation.

H 1																	He 2
Li 3	Be 4											B 5	C 6	N 7	O 8	F 9	Ne 10
Na 11	Mg 12											Al 13	Si 14	P 15	S 16	Cl 17	Ar 18
K 19	Ca 20	Sc 21	Ti 22	V 23	Cr 24	Mn 25	Fe 26	Co 27	Ni 28	Cu 29	Zn 30	Ga 31	Ge 32	As 33	Se 34	Br 35	Kr 36
Rb 37	Sr 38	Y 39	Zr 40	Nb 41	Mo 42	Tc 43	Ru 44	Rh 45	Pd 46	Ag 47	Cd 48	In 49	Sn 50	Sb 51	Te 52	I 53	Xe 54
Cs 55	Ba 56	La 57	Hf 72	Ta 73	W 74	Re 75	Os 76	Ir 77	Pt 78	Au 79	Hg 80	Tl 81	Pb 82	Bi 83	Po 84	At 85	Rn 86
Fr 87	Ra 88	Ac 89	Rf 104	Db 105	Sg 106	Bh 107	Hs 108	Mt 109	Ds 110	Rg 111	Cn 112	Nh 113	Fl 114	Mc 115	Lv 116	Ts 117	Og 118

Neighboring elements mercury and lead

Thallium and its compounds are **extremely toxic**.
There are numerous recorded cases of fatal thallium poisoning.

Thallium has 25 isotopes which have atomic masses that range from 184 to 210.

^{203}Tl and ^{205}Tl are the only stable isotopes and make up nearly all of natural thallium.

Electron neutrinos are produced in the Sun as a product of nuclear fusion.

Neutrinos travel from the center of the sun about **2.3 seconds** to reach the surface, and additional **8 minutes and 20 seconds to reach the Earth.**

They come straight through the earth at nearly the speed of light, in enormous numbers.

They are impossible to see and incredibly difficult to detect, because they are neutral, has no electrical charge, has a very small mass, which might even be zero and have very little interaction with matter.

The number of neutrinos can be predicted by the Standard Solar Model. The detected number of electron neutrinos was only 1/3 of the predicted number, and this was known as the solar neutrino problem.

Freedman proposed that one of the thallium isotopes (^{205}Tl) in the mineral lorandite, which during the process of interaction with neutrinos transforms into the lead isotope ^{205}Pb, could be used as a geochemical neutrino detector.

His idea was to analyse thallium-containing lorandite from Allchar and examines the quantity of lead in it as a basis for calculation of the number of neutrinos which over the millennia have passed through the lorandite, enabling the calculation of the Sun's age.

LOREX (LORandite EXperiment) is a project (running between 2008 and 2010) using lorandite from the ore deposit Allchar, to determine the solar proton–proton neutrino flux.

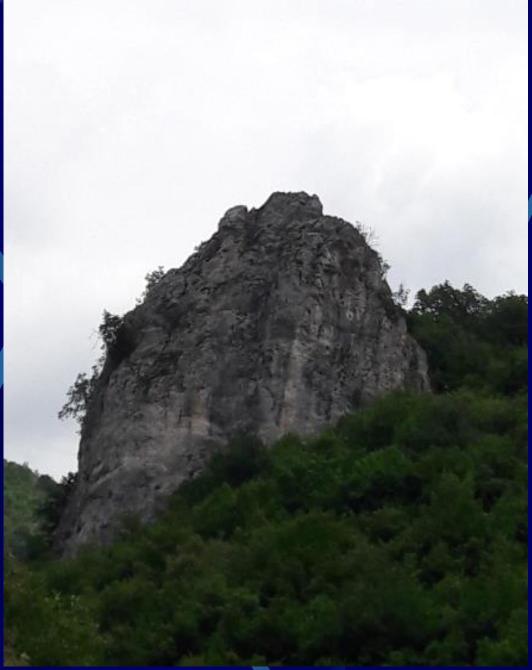
Lorandite is thought to have the potential to help to understand the work and power of the Sun.

Some say it holds the secrete of the Universe.

Science-fiction or reality?

Several studies showed high biological diversity around Allchar locality:

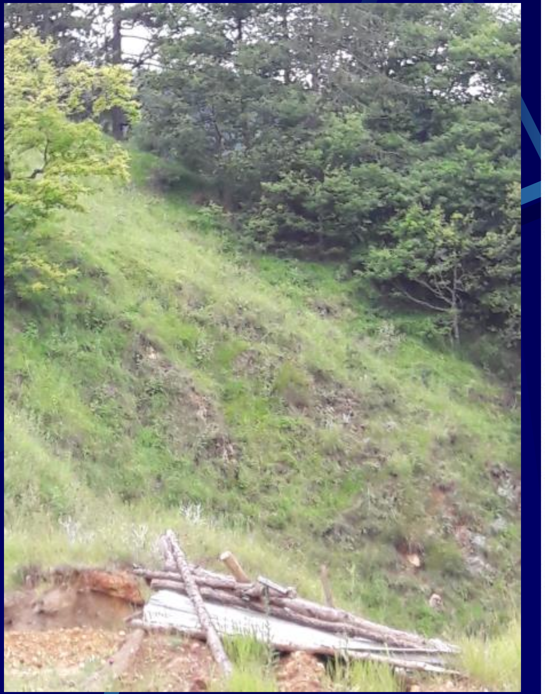
- Fungi
- Flora and
- Fauna

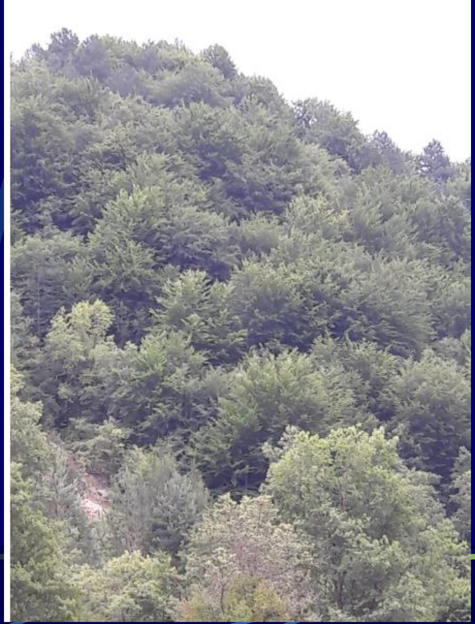


Allchar – Kozuf

- ❖ 100 km from Skopje
- ❖ 1 h driving from Kavadarci
- ❖ 1 h walking to get Allchar

June 23rd 2018




Beautiful and…

Beautiful and…

Dangerous and…

Very exciting

Entrance– Crven Dol Allchar

Entrance– Crven Dol Allchar

New flora and fauna???

MATERIAL

Samples were collected on June 23rd 2018 at the location “Crven Dol”, of mine Allchar.

Sampling sites were divided on three areas :

1. Floor (sample name VT1)
2. Beams (sample name VT2) and
3. The side walls of the mine (sample name VT3).

Samples were collected from all areas in three replicas and DNA extraction was performed separately from each of them.

Tunnel at CRVEN DOL (Allchar)

Tunnel at CRVEN DOL (Allchar)

Tunnel walls – Crven Dol Allchar

Sampling

Sampling

Sampling

Supporting beams

Microbial diversity in various mine sites are connected with the environment conditions and **usually remain undervalued**.

The huge bio diversity and uncultivable nature of certain m.o. make it immensely difficult for accurate representation of microbial communities in a particular ecological niche.

Metagenomics is a revolutionary concept in the aspect of studying microbial bio diversity, their adaptation to the ecological niches and their evolution.

Metagenomic data are obtained by high-throughput **sequencing** of environmental samples provides an aggregation **of all the genetic materials** of the studied environment.

This strategy **overcomes** the problems associated with conventional molecular methods of retrieving genetic information for a particular environment.

High throughput **bioinformatic analysis enables the accurate exploration of a gene of interest**.

The geological and geobiochemical environment, metagenomic approaches have enabled straight forward **investigation of the microbiome in deep mining deposits** (Turnbaugh, *et al.*, *2007,* Brazelton *et al*., 2012).

Some studies have also provided **novel genes, metabolic processes, the evolutionary history of the dwelling microorganisms, the mechanism of their metal tolerance and solubilation abilities** (Delmont *et al*., 2011, Li *et al*. 2015).

This study presents the **first metagenomic study** of the microbial diversity of the mine Allchar.

METHODS

1. DNA extraction, library preparation and NGS sequencing

The extraction of ultra-pure DNA from three mine samples area (approximately 200 mg of each) was done using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) in three replicas, following manufacturer's protocol. **Total DNA from each replica was pooled into one sample for each sampling site.**

The DNA yield of analyzed samples was measured using Qubit Fluorometric Quantitation (Qubit 4 Fluorometer, Invitrogen™, USA). The amplicons were amplified following the target gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A).

The gene-specific sequences used in **this protocol target the 16S rRNA gene V3 and V4 region**, with the defined primers for 16S forward (5′-CCTACGGGNGGCWGCAG-3′) and reverse sequences (5′-GACTACHVGGGTATCTAATCC-3′).

2. Sequence data process and taxonomy annotation (Valencia, Spain)

Quality assessment was performed by the use of ***prinseq-lite* program.**

Taxonomic annotation tables are summarised using **Krona** tool as an **interactive viewer** for taxa distributions by samples.

3. Bioinformatic analysis

Have been carried out within **R statistics environment** with reported packages.

Bacterial differences at the taxonomic level including phylum, family and genus, were compared, and species level in the case for fungal communities for the alpha diversity.

4. Sample preparation for ICP-OES analysis

According to the EPA Method 3051, using Thermo Scientific™ iCAP™ 7400 ICP-OES analyser.

June 27th 2019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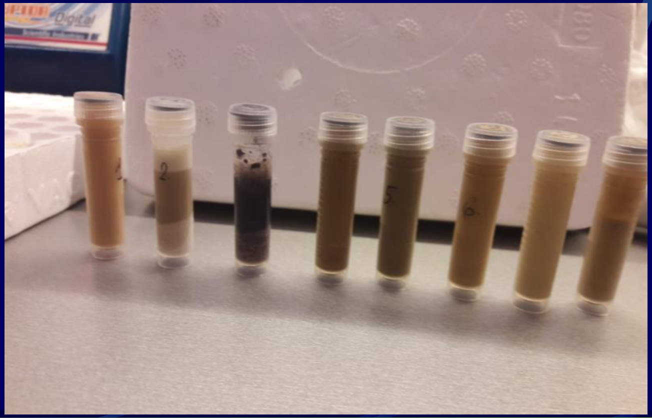
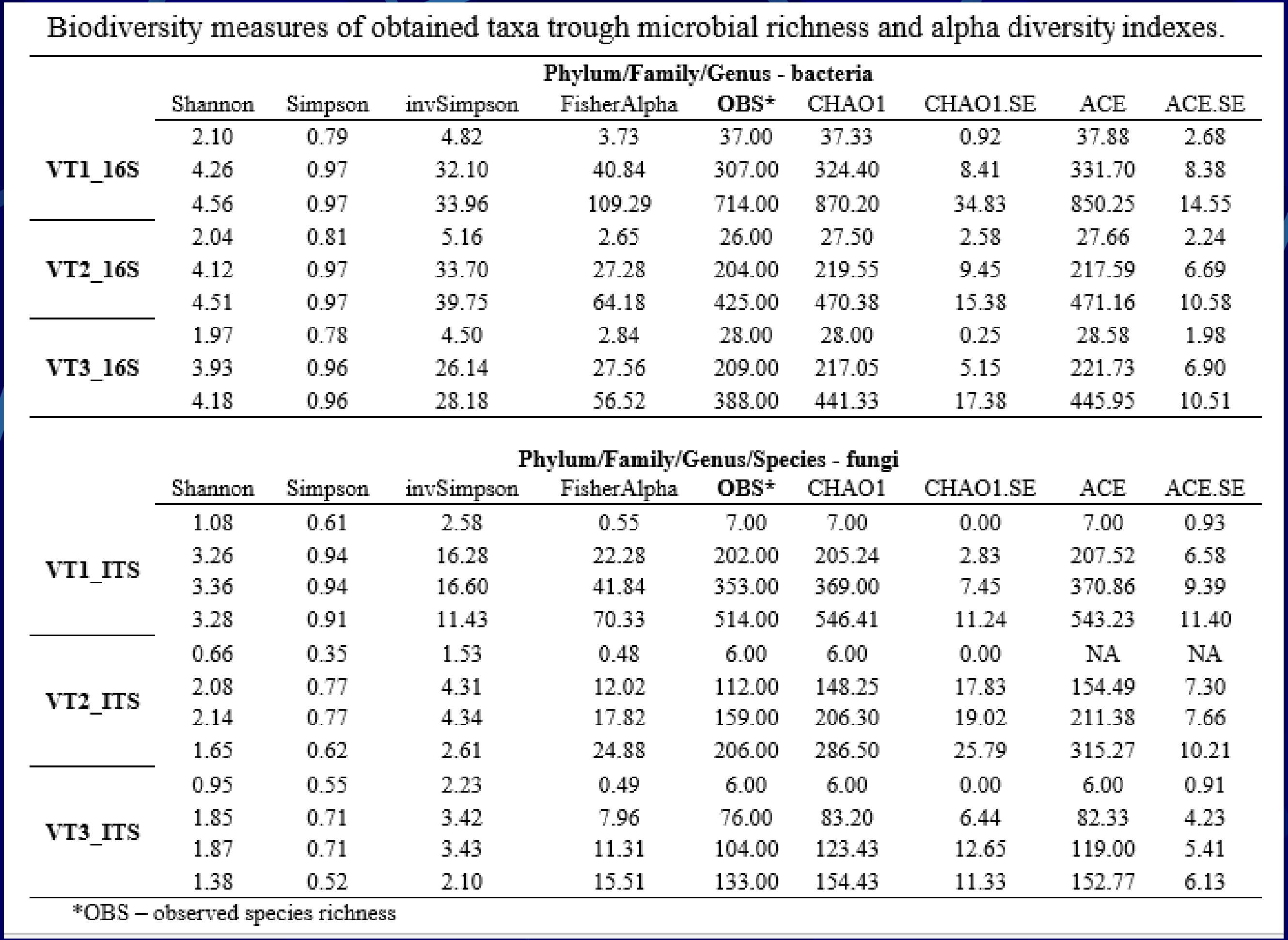

1
2
5
6

Results and discussion

1. Diversity of microbial communities



Biodiversity measures of obtained taxa trough microbial richness and alpha diversity indexes.

	Phylum/Family/Genus - bacteria								
	Shannon	Simpson	invSimpson	FisherAlpha	**OBS***	CHAO1	CHAO1.SE	ACE	ACE.SE
VT1_16S	2.10	0.79	4.82	3.73	37.00	37.33	0.92	37.88	2.68
	4.26	0.97	32.10	40.84	307.00	324.40	8.41	331.70	8.38
	4.56	0.97	33.96	109.29	714.00	870.20	34.83	850.25	14.55
VT2_16S	2.04	0.81	5.16	2.65	26.00	27.50	2.58	27.66	2.24
	4.12	0.97	33.70	27.28	204.00	219.55	9.45	217.59	6.69
	4.51	0.97	39.75	64.18	425.00	470.38	15.38	471.16	10.58
VT3_16S	1.97	0.78	4.50	2.84	28.00	28.00	0.25	28.58	1.98
	3.93	0.96	26.14	27.56	209.00	217.05	5.15	221.73	6.90
	4.18	0.96	28.18	56.52	388.00	441.33	17.38	445.95	10.51

	Phylum/Family/Genus/Species - fungi								
	Shannon	Simpson	invSimpson	FisherAlpha	**OBS***	CHAO1	CHAO1.SE	ACE	ACE.SE
VT1_ITS	1.08	0.61	2.58	0.55	7.00	7.00	0.00	7.00	0.93
	3.26	0.94	16.28	22.28	202.00	205.24	2.83	207.52	6.58
	3.36	0.94	16.60	41.84	353.00	369.00	7.45	370.86	9.39
	3.28	0.91	11.43	70.33	514.00	546.41	11.24	543.23	11.40
VT2_ITS	0.66	0.35	1.53	0.48	6.00	6.00	0.00	NA	NA
	2.08	0.77	4.31	12.02	112.00	148.25	17.83	154.49	7.30
	2.14	0.77	4.34	17.82	159.00	206.30	19.02	211.38	7.66
	1.65	0.62	2.61	24.88	206.00	286.50	25.79	315.27	10.21
VT3_ITS	0.95	0.55	2.23	0.49	6.00	6.00	0.00	6.00	0.91
	1.85	0.71	3.42	7.96	76.00	83.20	6.44	82.33	4.23
	1.87	0.71	3.43	11.31	104.00	123.43	12.65	119.00	5.41
	1.38	0.52	2.10	15.51	133.00	154.43	11.33	152.77	6.13

*OBS – observed species richness

Alpha diversity at all observed taxonomic levels indicated **high bacterial diversity in all samples,** with slightly higher in VT1 and VT2 than in VT3, especially at the genus level.

2. Microbial community diversity

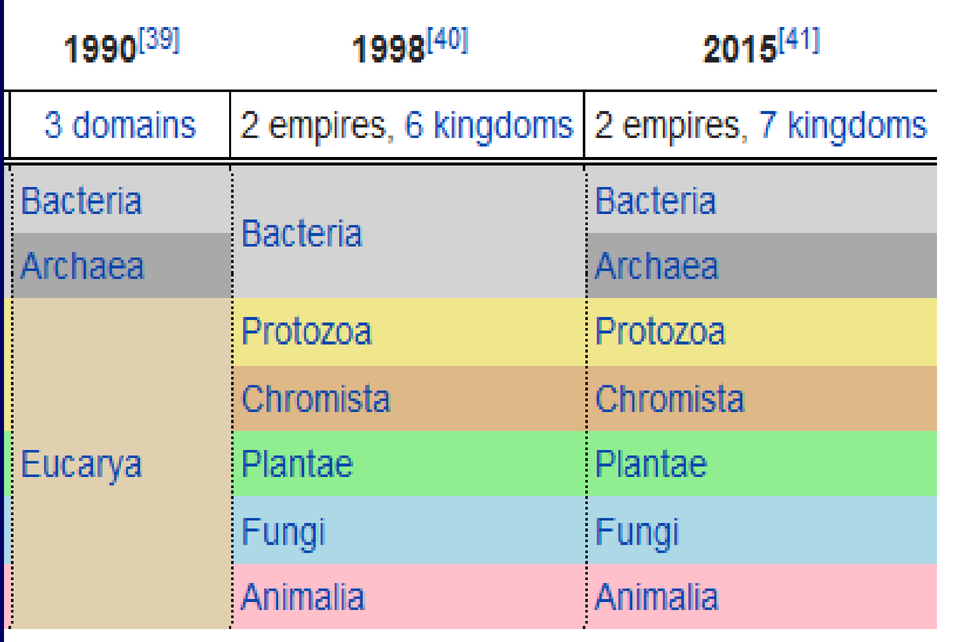
Bacterial and fungal taxa were determined by 16S rRNA and ITS gene sequences, classified according to alignment from the RDP reference database.

Phylum level - for bacteria:

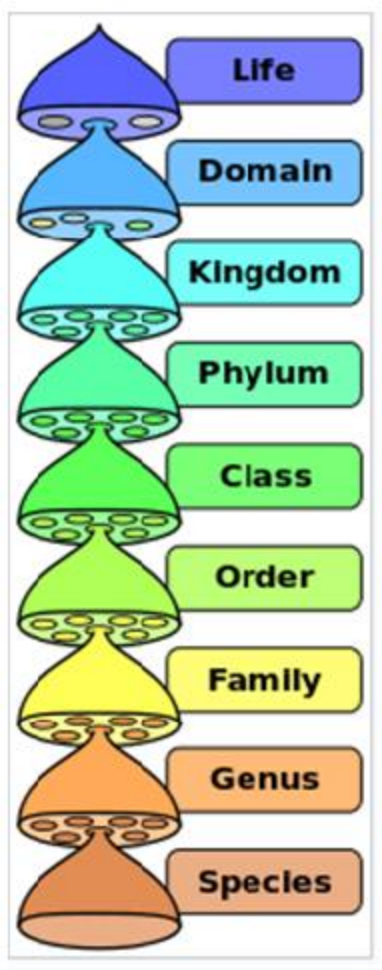
- **Proteobacteria** the most dominant for all three samples (36 to 41%).

- **Bacteroidetes, Verrucomicrobia, Planctomycetes** and **Nitrospirae** more abundant in sample **VT2**
- **Acidobacteria Candidatus Saccharibacteria** and
- **Gemmatimonadetes** in higher proportion for VT3.

- **Chlamydiae** was present at same proportion in all samples.

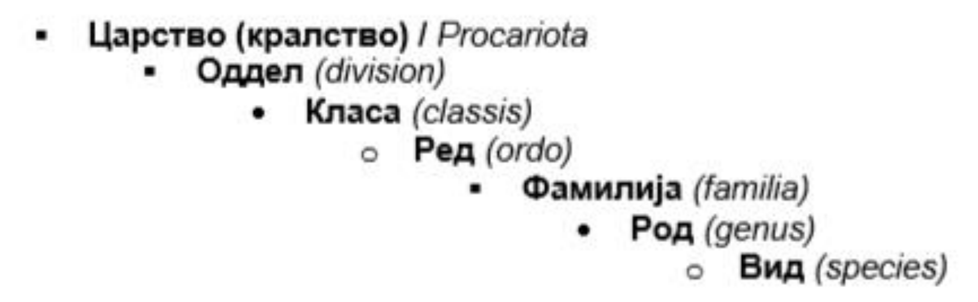


Woese et al. 1990[39]	Cavalier-Smith 1998[40]	Cavalier-Smith 2015[41]
3 domains	2 empires, 6 kingdoms	2 empires, 7 kingdoms
Bacteria	Bacteria	Bacteria
Archaea		Archaea
Eucarya	Protozoa	Protozoa
	Chromista	Chromista
	Plantae	Plantae
	Fungi	Fungi
	Animalia	Animalia



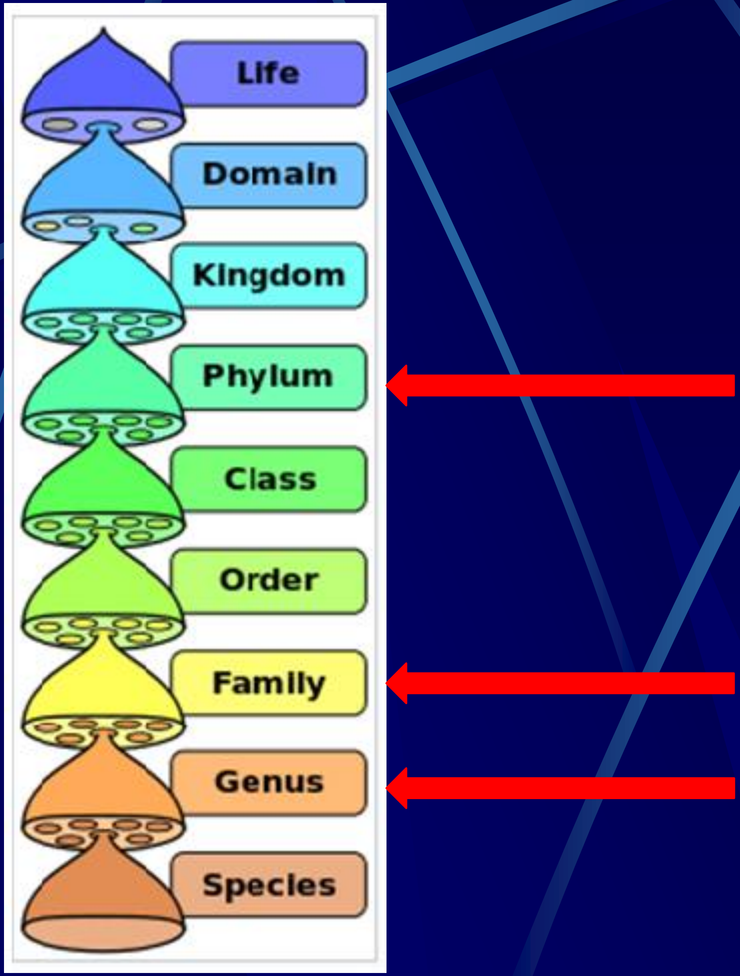
The hierarchy of biological classification's eight major taxonomic ranks.

A kingdom contains one or more phyla.

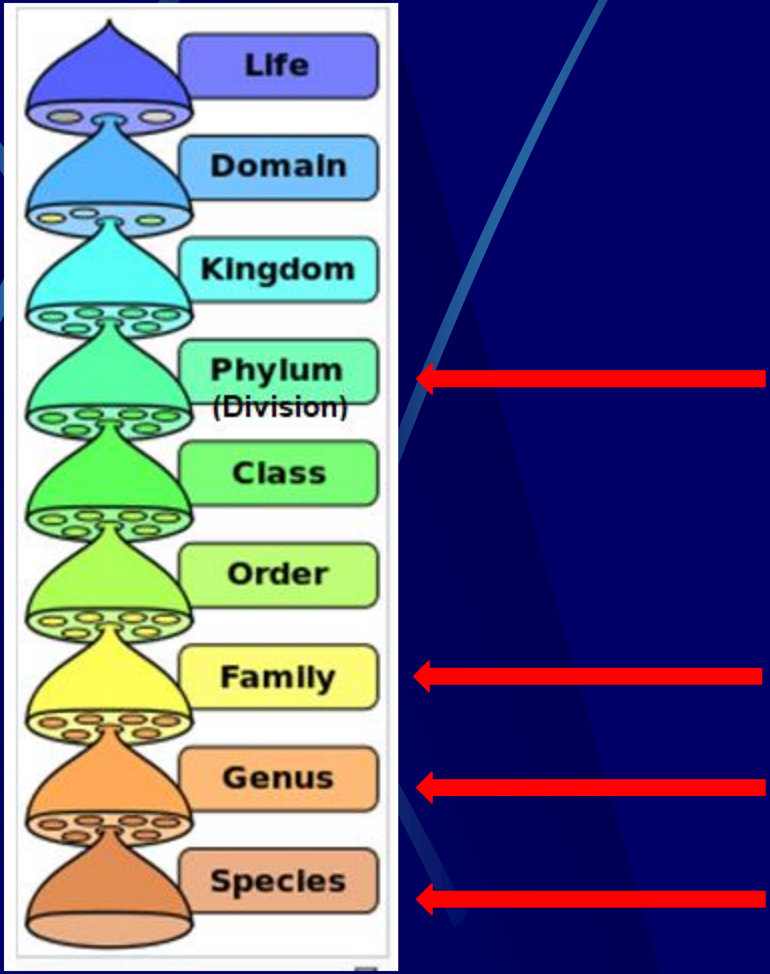


- **Царство (кралство)** / *Procariota*
  - **Оддел** *(division)*
    - **Класа** *(classis)*
      - **Ред** *(ordo)*
        - **Фамилија** *(familia)*
          - **Род** *(genus)*
            - **Вид** *(species)*

Metagenomics microbial taxonomy level - detection



Bacteria



Fungi

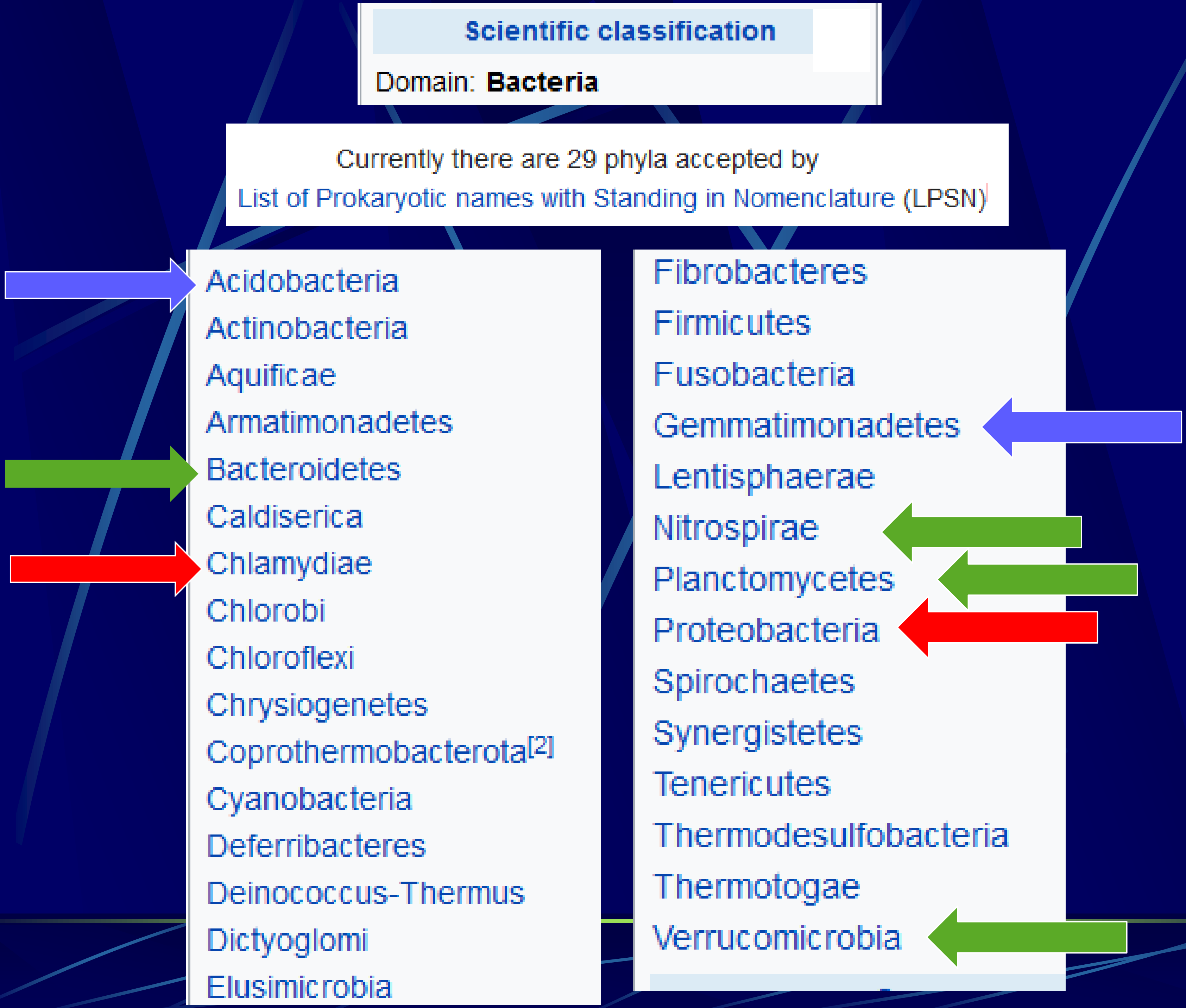
3. Microbial community composition

Bacterial and fungal taxa were determined by **16S rRNA and ITS gene sequences**, classified according to alignment from the RDP reference database.

Our results (Fig. 1) show that, on phylum level the most dominant were **Proteobacteria** for all three samples, with relative abundance between 36 and 41%.

Phyla such as **Bacteroidetes, Verrucomicrobia, Planctomycetes** and **Nitrospirae** were more abundant in sample **VT2**, while **Acidobacteria**, Candidatus Saccharibacteria and **Gemmatimonadetes** were related for VT3 in higher proportion ratio.

The phylum **Chlamydiae** was present at same proportion in all samples.



Scientific classification

Domain: **Bacteria**

Currently there are 29 phyla accepted by
List of Prokaryotic names with Standing in Nomenclature (LPSN)

- Acidobacteria
- Actinobacteria
- Aquificae
- Armatimonadetes
- Bacteroidetes
- Caldiserica
- Chlamydiae
- Chlorobi
- Chloroflexi
- Chrysiogenetes
- Coprothermobacterota[2]
- Cyanobacteria
- Deferribacteres
- Deinococcus-Thermus
- Dictyoglomi
- Elusimicrobia
- Fibrobacteres
- Firmicutes
- Fusobacteria
- Gemmatimonadetes
- Lentisphaerae
- Nitrospirae
- Planctomycetes
- Proteobacteria
- Spirochaetes
- Synergistetes
- Tenericutes
- Thermodesulfobacteria
- Thermotogae
- Verrucomicrobia

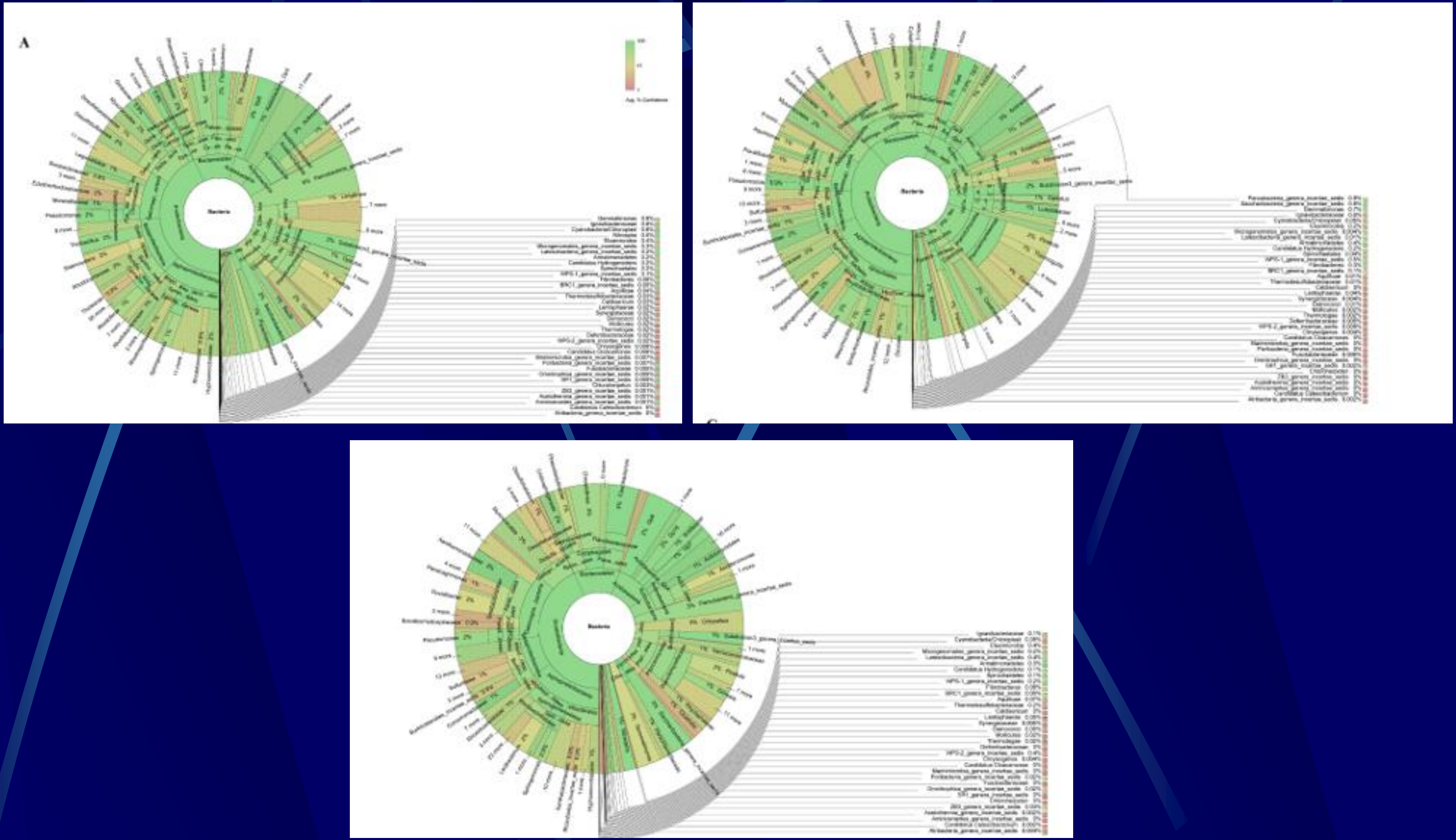


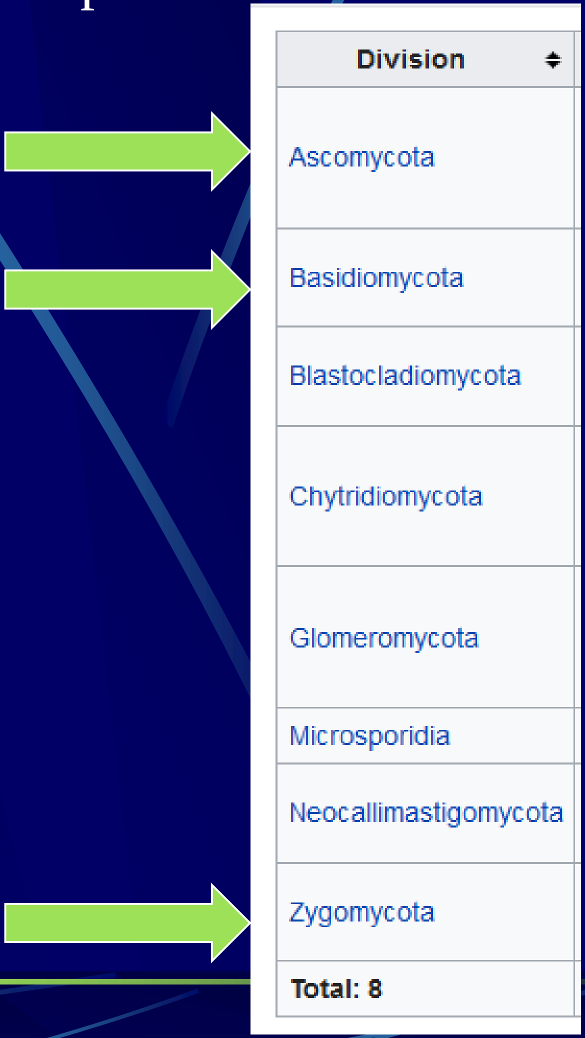
Figure 1. Relative abundance of bacterial taxa at the genus level, according to RDP classifier across VT1 (A), VT2 (B) and VT3 (C) samples, presented by **Krona interactive viewer**. Different colours indicate average % of confidence.

Results on Fungi diversity:

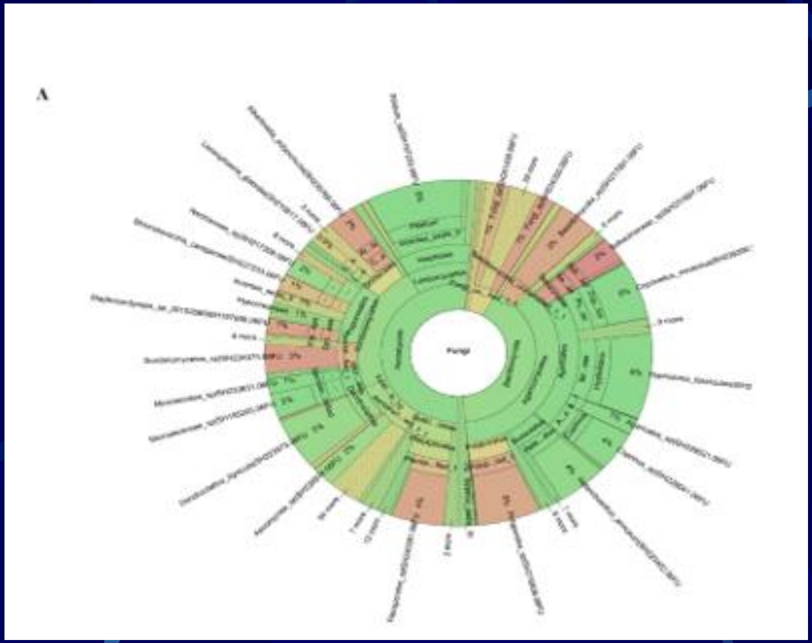
Phylum level:

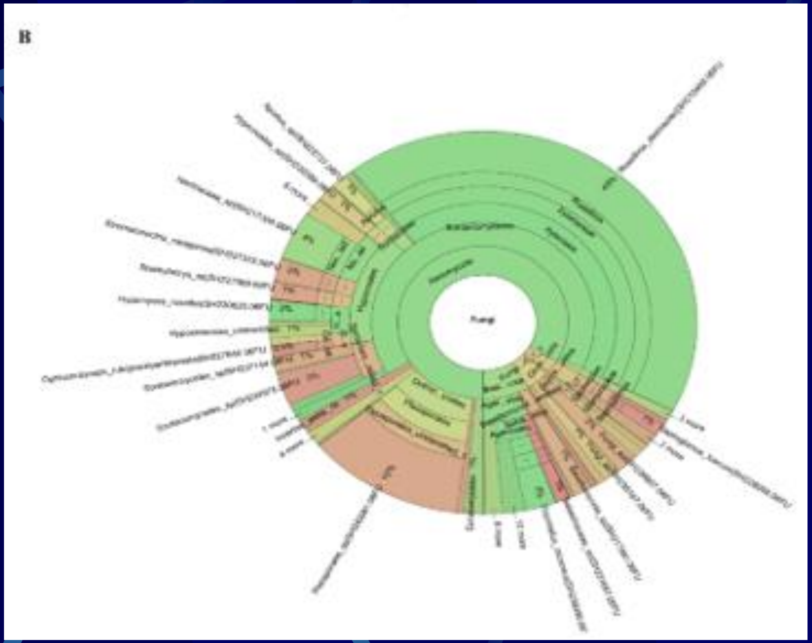
- ❖ ***Ascomycota*** the most dominant was for VT1 (49.9%) and VT2 (79.4%),
- ❖ ***Basidiomycota*** more than 55% related to VT3 sample.
- ❖ ***Zygomycota*** most abundant in VT3.

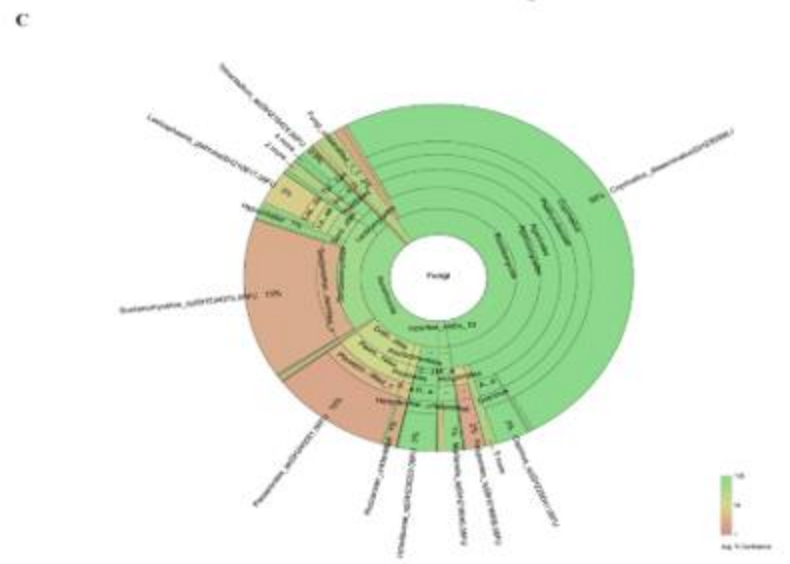
- ❖ Similar situation was noticed on family level.



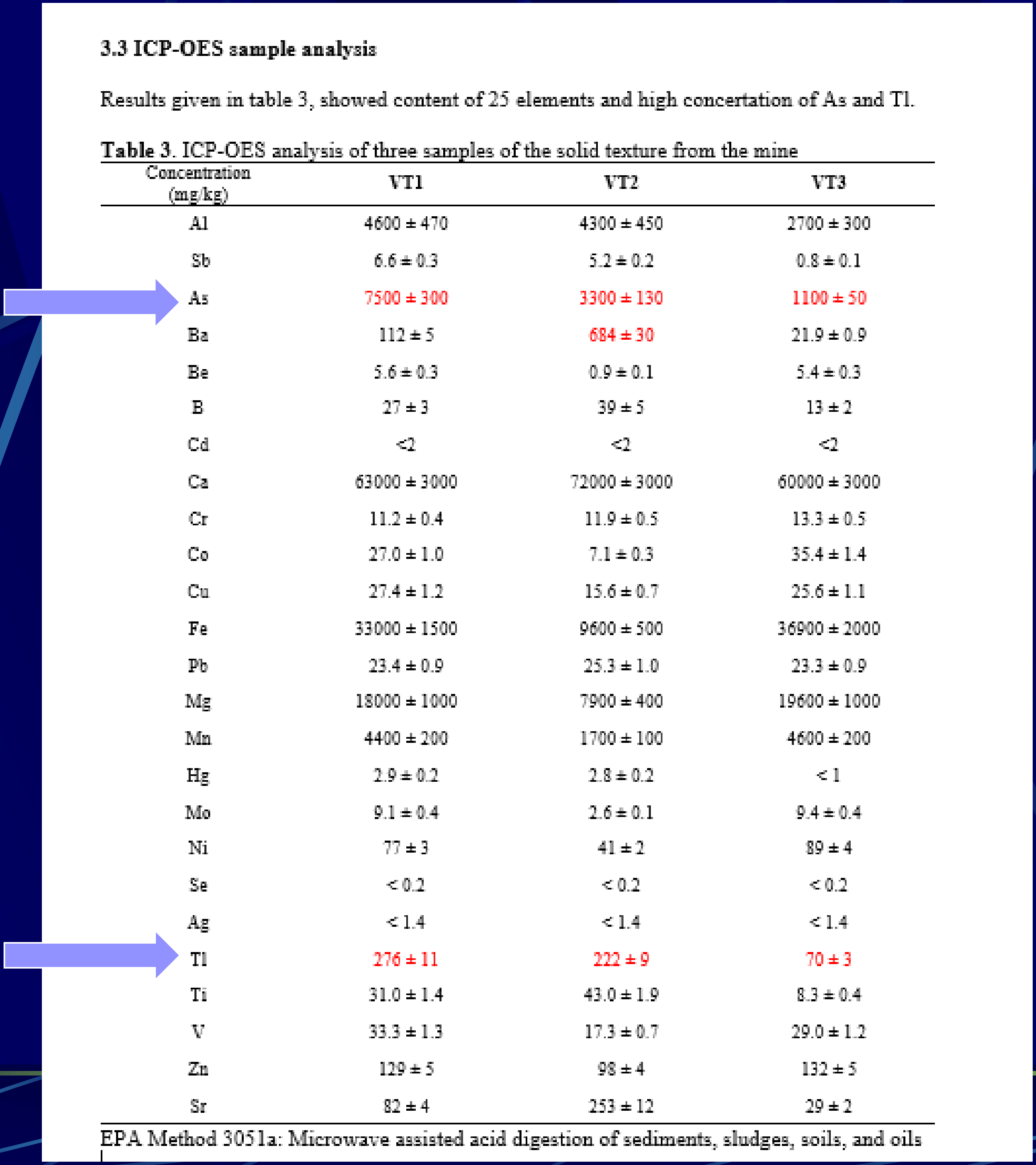
Division ⬍
Ascomycota
Basidiomycota
Blastocladiomycota
Chytridiomycota
Glomeromycota
Microsporidia
Neocallimastigomycota
Zygomycota
Total: 8







Fungal taxa at the species level, according **Krona interactive viewer.**



3.3 ICP-OES sample analysis

Results given in table 3, showed content of 25 elements and high concertation of As and Tl.

Table 3. ICP-OES analysis of three samples of the solid texture from the mine

Concentration (mg/kg)	VT1	VT2	VT3
Al	4600 ± 470	4300 ± 450	2700 ± 300
Sb	6.6 ± 0.3	5.2 ± 0.2	0.8 ± 0.1
As	7500 ± 300	3300 ± 130	1100 ± 50
Ba	112 ± 5	684 ± 30	21.9 ± 0.9
Be	5.6 ± 0.3	0.9 ± 0.1	5.4 ± 0.3
B	27 ± 3	39 ± 5	13 ± 2
Cd	<2	<2	<2
Ca	63000 ± 3000	72000 ± 3000	60000 ± 3000
Cr	11.2 ± 0.4	11.9 ± 0.5	13.3 ± 0.5
Co	27.0 ± 1.0	7.1 ± 0.3	35.4 ± 1.4
Cu	27.4 ± 1.2	15.6 ± 0.7	25.6 ± 1.1
Fe	33000 ± 1500	9600 ± 500	36900 ± 2000
Pb	23.4 ± 0.9	25.3 ± 1.0	23.3 ± 0.9
Mg	18000 ± 1000	7900 ± 400	19600 ± 1000
Mn	4400 ± 200	1700 ± 100	4600 ± 200
Hg	2.9 ± 0.2	2.8 ± 0.2	< 1
Mo	9.1 ± 0.4	2.6 ± 0.1	9.4 ± 0.4
Ni	77 ± 3	41 ± 2	89 ± 4
Se	< 0.2	< 0.2	< 0.2
Ag	< 1.4	< 1.4	< 1.4
Tl	276 ± 11	222 ± 9	70 ± 3
Ti	31.0 ± 1.4	43.0 ± 1.9	8.3 ± 0.4
V	33.3 ± 1.3	17.3 ± 0.7	29.0 ± 1.2
Zn	129 ± 5	98 ± 4	132 ± 5
Sr	82 ± 4	253 ± 12	29 ± 2

EPA Method 3051a: Microwave assisted acid digestion of sediments, sludges, soils, and oils

4. Conclusion

In this study we confirmed presence of:

- **25 different elements** and **high concentration of As and Tl**,
- **Huge microbial diversity, including bacteria and fun**gi.

Study will continue exploring:

- **microbial diversity of other part of Allchar, different areas where different composition and concentration of mineral deposits exists (Antimony),**
- Additional detection of **Archea.**

Funding: This study was funded by University “Goce Delchev” Shtip, Macedonia, Research project: Isolation and identification of bacteria from mines Allchar and Sasa, no. 0307-12/105, 14.05.2016.