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Metabolomic Profiling of Biolayers on the Surface of Marble in Nature and Urban Environment. Case Study of Karelia and St. Petersburg

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Abstract: The formation of biolayers of various taxonomic and biochemical composition occurs on the rock surfaces under various environmental conditions. The composition of metabolites in various types of biolayers on the marble surface in natural outcrops and urban environment was studied. Metabolome profiling was fulfilled by GC-MS. It was found that communities in urban environment are much less biochemically diverse than in a quarry. The seasonal differences in metabolite network between samples dominate over taxonomic ones in biolayers with predomination of algae and cyanobacteria and in biolayers with predomination of fungi. The biolayers of different stage of soil formation are less susceptible to seasonal variability.

Keywords: metabolomics community; metabolomic profiling; biolayers; microbial community; microbial metabolism

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1. Introduction

The interactions and interconversions of small biomolecules that form the metabolic network play an important role in the development of living organisms. System changes of metabolomic network determine the biological response of the organism to various influences, as well as the transition from one stage to the next ontogenesis [1–3]. In the natural habitat, in addition to the time factor and external abiogenic influences, the organisms are affected by biotic incorporation, i.e., they do not exist in isolation, but as part of complex biological communities. Until now, metabolomic studies were mainly concerned with biological systems represented by one single organism, colonies, or cell culture. In any case, it was one species of organism. Relatively recently, the term "metabolomics community" was proposed for use the metabolomic approach in the study of communities of organisms [4–6]. Over the last few years, the number of such researches has been increasing [7–9]. Nevertheless, the share of these studies in the total number of metabolic works is negligible.

Multispecies communities of organisms are a valuable resource for new substances with biological activity. Their research allows "in one fell swoop" to obtain information about valuable types of producers. Since many species of bacteria, fungi, and algae cannot be cultured in the laboratory, these organisms are largely unexplored. The new area of the metabolomics community is about to change this scenario. Just as metagenomics refers to

the analysis of all DNA from a given sample, the community of metabolomics considers a variety of naturally occurring metabolites from a metapopulation of a sample of a given environment, such as soil or water, and possibly even air [7].

Strikingly, the mathematical models developed to date in the context of mixed communities focus only on metagenomic datasets, bypassing metatranscriptomics, metaproteomics, and metabolomics. However, these omics techniques provide valuable information about the functioning of the ecosystem and, therefore, are necessary for accurate prediction of the emergent properties of the ecosystem. In general, the success of metabolomics in the context of mixed communities is limited in comparison with other -omic technologies, and, importantly, the identification of metabolites is not particularly informative in terms of microbial interactions. Non-targeted experimental strategies are usually limited to a small percentage of the total metabolites identified [10].

Thus, despite the limited possibilities of data interpretation, the metabolomics of communities is a promising area for the search of new biotechnologically significant producer species, as well as providing information on the systemic organization of small organic molecules at the supraorganism level. In our opinion, lithobiontic systems represent a convenient model for studying the metabolic network of organisms at the community level.

First, lithobiontic communities are not only multi-species; they include organisms of several large taxa of fungi, algae, bacteria, mosses, and lichens. Secondly, they are very diverse and allow observing different types of communities in a relatively small area and tracing their successions. Third, these communities are interesting for their geochemical activity since they are involved in such significant processes as the weathering of rocks and primary soil formation. Lithobiontic organisms are indeed the main driving forces of biogeochemical cycles and provide the recycling of basic organic elements such as carbon and nitrogen [11]. The study of the geochemical activity of the microbial community and its biochemical properties has raised important problems associated with the preservation of cultural heritage. Cultural heritage monuments made of stone, as well as stone in nature, are colonized by organisms. Monuments may be degraded by growth and activity of living organisms. Interactions between these organisms and stone can enhance or retard the overall rate of degradation. The nature of the environmental conditions influences the extent of biofilm colonization and the biodeterioration processes [12].

We will use the term biolayer, meaning the complex of biological objects and products of their vital activity on a solid substrate. Biolayers include subaerial biofilm (SAB) [13], mosses, as well as primary soils under mosses together with organic and mineral components from the outdoor environment and the underlying substrate.

The aim of this work is to describe the composition of metabolites and reveal their distribution (taking into account the spatial and temporal factors) in various types of biolayers on the marble surface in natural outcrops and urban environment.

2. Materials and Methods

2.1. Sampling

Biolayers were collected from the surface of Karelian marble in the Ruskeala quarry, as well as from the surface of the monuments of the Historical necropolises in Saint Petersburg (Museum Necropolis of 18th century), made of the same marble. Samples were collected from horizontal, vertical, and inclined rock surfaces. The Ruskeala quarry (Figure 1a,c) is located in the Northern Ladoga area (Sortavalsky district of the Republic of Karelia) (Figure 2a). Marble was mined here for facing buildings in St. Petersburg. Currently, no work is being carried out here, and the quarry is a monument to mining. It is ideal for studying marble biofouling under conditions of low anthropogenic load. Samples of the same types from the same places were taken in the spring–summer period (the stage of active vegetation) and in the autumn (at the end of the growing season). Historical necropolises (Figure 1b,d) are located in a small area in the central part of Saint Petersburg

(Figure 2b) and are suitable for studying biofouling processes under high anthropogenic load. Samples were taken here only at the active vegetation period.

(**a**) (**b**) (**c**) (**d**)

Figure 1. Sampling sites: Ruskeala quarry (**a**); Alexander Nevskay Lavra (**b**); Biolayers on marble in the Ruskeala quarry (**c**) and on the surface of museum necropolises in the Alexander Nevskay Lavra (**d**).

(**a**) (**b**)

Figure 2. Location of sampling sites: (**a**) Ruskeala quarry; (**b**) Alexander Nevskay Lavra.

The study areas are located in the same climatic zone. However, they differ in average annual indicators. St. Petersburg is more humid and warmer compared to Ruskeala (Table 1).

Table 1. Climatic features of sampling sites.

Study Area	Average Annual Temperature	Precipitation	Relative Humidity	Winds Main Direction
Ruskeala	3 °C	605 mm	74%	south
Central part of Saint Petersburg	5.6 °C	653 mm	80%	western and southwest

The surface of different rocks is covered by various types of biolayers, formed by fungi, algae, lichens, and mosses. On open rock surfaces in the Ruskeala quarry, the formation of mucous biofilms is usually observed, the color of which can vary (green, olive and reddish-brown). Close to such biofilms, as well as separately, one can observe denser dark cortical layers, tightly adhering to the surface of the stone. On inclined surfaces and closer to the soil surface, soft layers that are easily separated from the stone surface are formed, in which mosses and lichens are locally present (the initial stage of the formation of the primary soil). In cracks and near to the soil, a developed mosses cover can be observed, under which a layer of primary soil is located.

On the surface of the necropolis's monuments in the urban environment, there are usually no mucous pigmented biofilms and cortical dark biolayers. A significant part of the open surfaces of the monuments are covered with a film formed by algae or fungi. These films have the appearance of a dry coating of green, olive, brown, or black. In some areas, these layers also include lichens. Sometimes lichens form a continuous carpet and cover most of the monument. Like the biolayers in the Ruskeala quarry, near the monuments of the necropolises on horizontal surfaces and in damp places, one can observe the development of moss and various stages of the formation of the primary soil. Quite often, layers of dirt and abundant leaf litter can be observed on the surface stone.

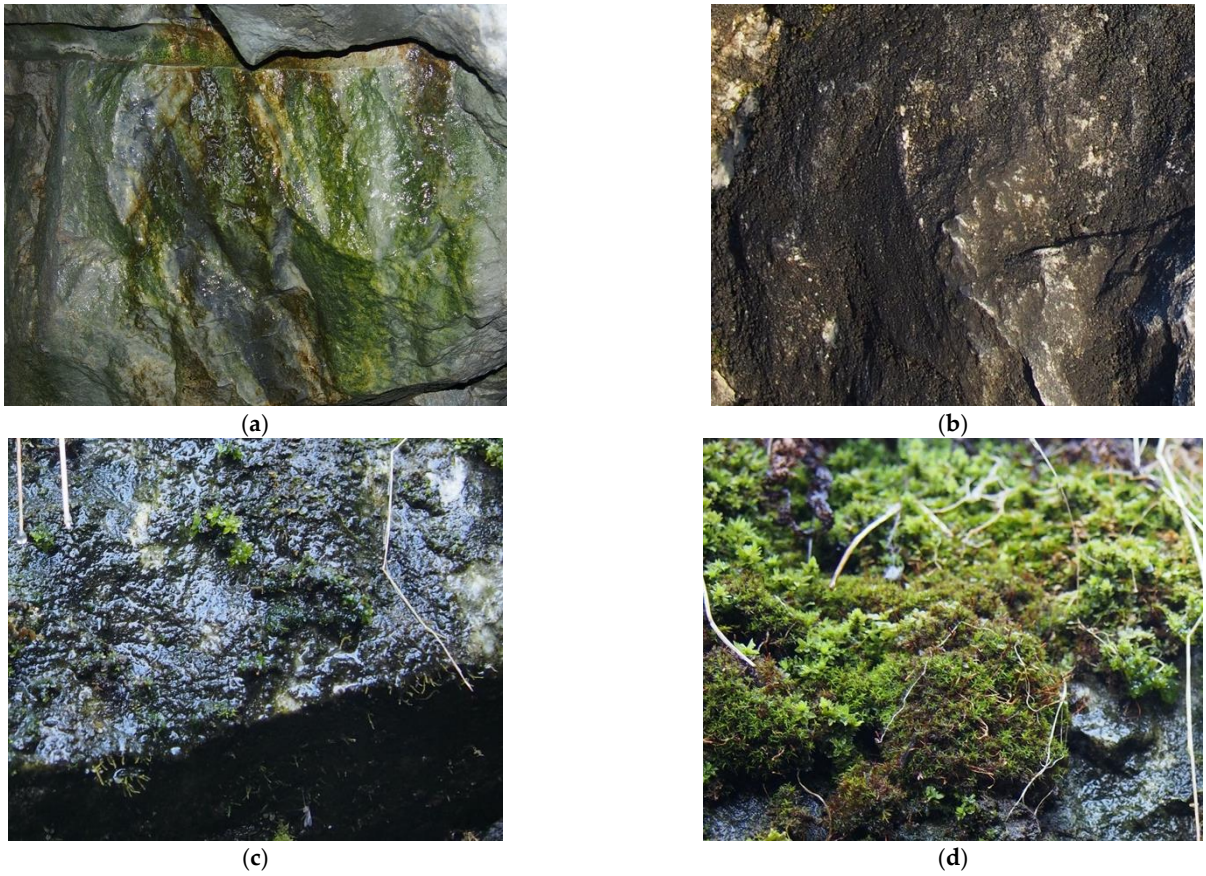
For this study, four types of communities classified according to the dominant groups of organisms according our previous study [14,15] were selected.

Type I—Biolayers dominated by algae and cyanobacteria. In the Ruskeala quarry—a slimy, pigmented film that has grown together with a stone, mainly of olive color, can acquire a reddish tint (Figure 3a). In an urban environment, they look like a dry green bloom on the surface of a stone (Figure 4a).

Type II—Biolayers with a dominance of fungi and a high abundance of algae and cyanobacteria. In the Ruskeala quarry, there are dark, crust-like layers, tightly adhering to the surface of the stone (Figure 3b). At the monuments of the necropolises, they are similar to type I biolayers, but have a dark color (Figure 4b). They are often observed in places of stone chipping.

Type III—Biolayers including individual tussocks of mosses and lichen thalli, which do not form a continuous cover (Figures 3c and 4c). It is characterized by a great variety of species and an abundance of fungi.

Type IV—Primary soil with a moss cover (Figures 3d and 4d). It is characterized by a high abundance of fungi. Primary soil formation is usually observed on horizontal surface.



(**a**) (**b**)

(**c**) (**d**)

Figure 3. The main types of biolayers on the surface of stone in Ruskeala quarry: (**a**) biolayers with a predominance of microscopic algae and cyanobacteria; (**b**) biolayers with a predominance of fungi; (**c**) biolayers including individual tussocks of mosses and lichen thalli; (**d**) moss and primary soil under the moss cover.

(**a**) (**b**)

Figure 4. *Cont*.

(**c**) (**d**)

Figure 4. The main types of biolayers on the surface of stone monuments of the historical necropolises: (**a**) biolayers with a predominance of microscopic algae; (**b**) biolayers with a predominance of fungi; (**c**) biolayers, including individual tussocks of mosses and lichen thalli; (**d**) moss and primary soil under the moss cover.

2.2. Study of Biodiversity

2.2.1. Mycological Analysis

All samples of biolayers were characterized by their appearance and dominant species of organisms. The identification of microscopic fungi was carried out by isolating them into a pure culture. For primary isolation and identified of micromycetes Czapek-Dox culture medium (HiMedia, Maharashtra, India) was used. Small fragments of biolayers were placed on the surface of the nutrient medium in Petri dishes. In addition, washings from the substrate surface were used for inoculation. The resulting cultures were incubated in a thermostat for 2–4 weeks at a temperature of 25 °C until sporulation appeared, after which microscopy and identification by morphological characteristics were carried out in accordance with guidebooks and monographs [16–19]. The species were verified in accordance with modern nomenclature using the Index Fungorum electronic database [20]. In this case, the number of colony-forming units (CFU) was determined per 1 g of substrate [21].

2.2.2. Algological Analysis

The identification of algae was carried out by morphological characteristics. For this purpose, we performed direct microscopy of samples (by means of Leica DM1000 microscope, Wetzlar, Germany) after settling in distilled water for a week. To determine the species composition, identifiers and monographs [22–24], as well as the electronic database AlgaeBase [25], were used.

2.3. Metabolomics Analysis

Extracts of biolayers were analyzed for the metabolomic study.

2.3.1. Quenching and Extraction

Quenching of cellular metabolism in biolayers was performed by 50% of cold methanol (−30 °C).

After quenching, samples of biolayers were extracted with 15 mL of cold methanol (−25 °C) and centrifuged (10 min, 400× *g*) at room temperature. The supernatant was transferred to a new vial and the samples were re-extracted with 10 mL of cold methanol. All extracts were combined and dried by a rotary evaporator at 40 °C.

The content of low molecular weight organic compounds in biofouling was determined by gas chromatography—mass spectrometry (GC-MS).

2.3.2. Gas Chromatography–Mass Spectrometry

The dried extracts were soluble in pyridine (30 µL) and BSTFA (N,O-bis—3-methylsilyl-3-F-acetamide) (30 µL), incubated at 100 °C for 15 min. The derivatized samples were analyzed by gas chromatography–mass spectrometry (GC-MS) on a Maestro instrument (Interlab, Russia) with an Agilent 5975 mass-selective detector (Santa Clara, CA, USA). Column HP-5MS, 30 m × 0.25 mm × 0.25 µm. Chromatography was performed with linear temperature programming from 70 °C to 320 °C for 6 °C/min in the mode of constant carrier gas flow through the column (1 mL/min). The carrier gas is helium. Mass spectra were scanned in the range of 50–750 m/z with a frequency of 1.6 scans/sec. Chromatograms of the samples were recorded using the total ion current. Mass spectrometric information was processed and interpreted using AMDIS program (2.65 version) [26], the standard NIST2005 library, and the library of standard compounds of BIN RAS. Quantitative interpretation of chromatograms by internal standard method was also performed using tridecane in the UniChrom program (5.0.19.1180 version) [27].

2.4. Statistical Analysis

Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLSDA), as well as heatmaps, were performed using MetaboAnalyst software (5.0 version) [28].

3. Results

3.1. Biodiversity of Biolayers on Marble Surface

The taxonomic composition of fungi, algae, and cyanobacteria was determined for each selected type of biolayer (Table 2). Comparative analysis of mycobiota showed that in the urban environment the number of micromycetes (CFU number, Figure 5) in all types of biolayers is 1.5–2 times higher than in the Ruskeala quarry. At the same time, the species diversity in museum necropolises is higher only in types III and IV of biolayers, and in types I and II, on the contrary, it is lower than in the quarry. The most common species both at monuments and in quarries were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladposporioides*, *Cladosporium herbarum*, and *Fusarium oxysporum*. In general, the presence of dark-colored micromycetes (*Hormonema dematioides*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Alternaria alternata*, *Coniosporium* sp.) is more typical for marble in the urban environment.

In general, an increase in the dark-colored fungi species in II type of bio-layers and in primary soils can be noted. In the primary soil, the proportion of sterile light mycelium is rather high. Primary soil is also characterized by a high occurrence of species from genus *Penicillium*, which is typical for soils in the northern regions.

Biofilms of type I are represented by the greatest diversity of cyanobacteria. The species *Calothrix parietina* and *Leptolyngbya* sp., Which form characteristic mucous deposits on the surface of the stone (trichomes of *C. parietina* are dressed in sheaths and collected in tufts, which are abundantly covered with mucus), were identified as dominants of the I type of biofilms on marble. *Gloecapsopsis magma* is more abundant in dark biolayers (Types II and III) than in the I type. A greater diversity of cyanobacteria from the genus *Gloeocapsa* is noted in biolayers II and III.

The dominant species are likely to determine the appearance of biolayers. Type I are green cyanobacteria with mucous sheaths; types II and III are pigmented species with staining biofilms in tones from reddish brown to almost black.

Of the common species for the quarry and the city, only green algae and cyanobacteria of the genus *Nostoc* and genus *Leptolyngbya* are noted.

Table 2. Diversity of phototrophic and heterotrophic microorganisms in samples of bio-layering on marble from the Ruskeala quarry (Karelia) and from the monuments of the museum necropolises of St. Petersburg.

Fungi	Type of Biolayers		Algae and Cianobacteria	Type of Biolayers	
	Quarry	St. Petersburg		Quarry	St. Petersburg
Alternaria alternata	I, II, III, IV	I, II, III, IV	*Aphanocapsa* sp.	I, II, III	-
Cladosporium cladosporioides	I, II, III, IV	I, II, III, IV	*Gloeocapsa minor*	I, II, III	-
Cladosporium herbarum	I, II, III, IV	I, II, III, IV	*Gloeocapsa punctata*	I, II, III	-
Coniopsorium sp.	I, II, III, IV	I, II, III, IV	*Leptolyngbya sp.*	I, II	I
Light coloredmyceliasterilia	I, II, III, IV	I, II, III, IV	*Nostoc sp.*	IV	I, II
Dark colored mycelia sterilia	I, II, III, IV	I, II, III, IV	*Calothrix parietina*	I, II	-
Fusarium oxysporum	I, II, III, IV	I, III, IV	*Chroococcus cohaerens*	I, II	-
Penicillium decumbens	I, II, III, IV	II	*Chroococcus spelaeus*	I, II	-
Penicillium waksmanii	I, II, III, IV	-	*Gloeocapsa violascea*	I, II	-
Scytalidium lignicola	I, II, III, IV	-	*Gloeocapsopsis magma*	I, II	-
Trichoderma viride	I, II, III, IV	-	*Gloeothece rupestris*	I, II	-
Aureobasidium pullulans	-	I, II, III, IV	*Leptolyngbya foveolarum*	I, II	-
Penicillium brevicompactum	II, III,	III, IV	*Leptolyngbya sieminskae*	I, II	-
Penicillium oxalicum	III, IV	IV	*Nostoc commune*	I, II	-
Epicoccum nigrum	II	II, III	*Synechocystis aquatilis*	I, IV	-
Mortierella lignicola	I, III, IV	-	*Euglena sp.*	II, III	-
Metarhizium marquandii	II, III, IV	-	*Gloeothece palea*	II, III	-
Hormonemadematioides	-	I, II, III	*Bacillariophyta*	III, IV	-
Mucor hiemalis	IV	IV	*Klebsormidium sp.*	-	I, II
Penicillium citrinum	I	I	*Gloeocapsopsis dvorakii*	-	I, II
Penicillium herqueri	I	IV	*Gloeocapsopsis* sp. 1	-	I, II
Penicillium roqueforti	I, IV	-	*Netrium sp.*	I	-
Paecilomyces variotii	II, IV	-	*Chroococcus minutus*	I	-
Sarocladium strictum	III, IV	-	*Chroococcus turgidus*	I	-
Trichoderma koningii	III, IV	-	*Chalicogloea* sp.	I	-
Alternaria chartarum	-	I, III	*Chroococcus* sp. 1	I	-
Arthrinium phaeospermum	I	-	*Cyanothece aeruginosa*	I	-
Doratomyces stemonitis	I	-	*Eucapsis* sp.	I	-
Trichocladium asperum	I	-	*Gloeocapsa atrata*	I	-
Aspergillus niger	II	-	*Gloeocapsopsis* sp. 2	I	-
Polyscytalum fecundissimum	II	-	*Gloeothece* sp.	I	-
Acremonium potronii	III	-	*Leptolyngbya gracillima*	I	-
Fusarium incarnatum	III	-	*Leptolyngbya sp.* 1	I	-
Fusarium solani	III	-	*Leptolyngbya sp.* 2	I	-
Pseudogymnoascus pannorum	III	-	*Lyngbya putealis*	I	-
Penicillium purpurogenum	-	III	*Hassalia byssoidea*	-	I
Phoma herbarum	-	III	*Phormidesmis sp.*	-	I
Scopulariopsis acremonium	IV	-	*Anabaena* sp.	II	-
			Gloeocapsa alpina	II	-
			Gloeocapsa compacta	II	-
			Gloeocapsa kuetzingiana	II	-
			Gloeocapsa sp.	II	-
			Petalonema incrustans	II	-
			Phormidium tergestinum	II	-
			Cyanosarcina sp.	-	II
			Aphanocapsa cf. *fusco-lutea*	-	II
			Aphanocapsa parietina	-	II
			Chroococcus sp. 2	III	-
			Phormidium papyraceum	III	-
			Vaucheria sp.	III	-

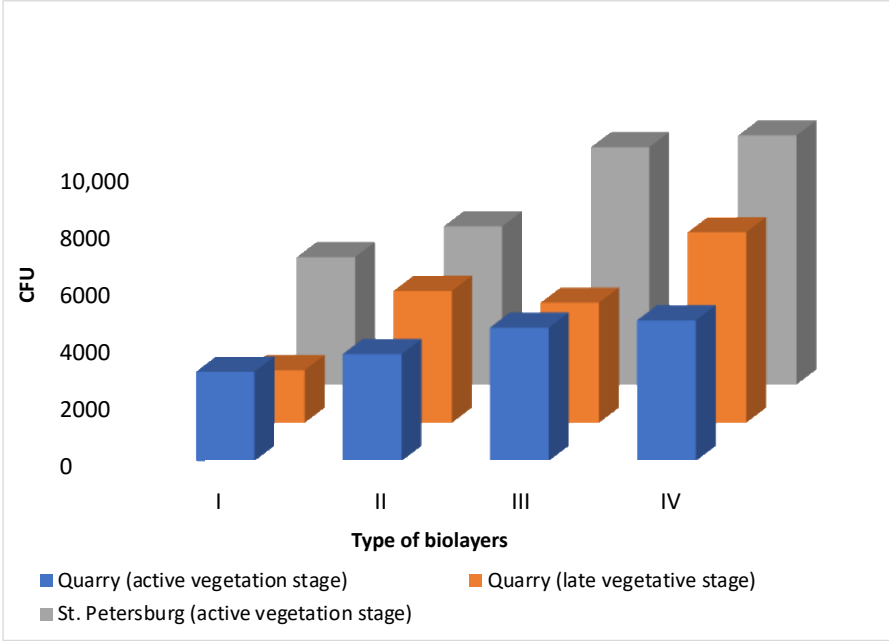


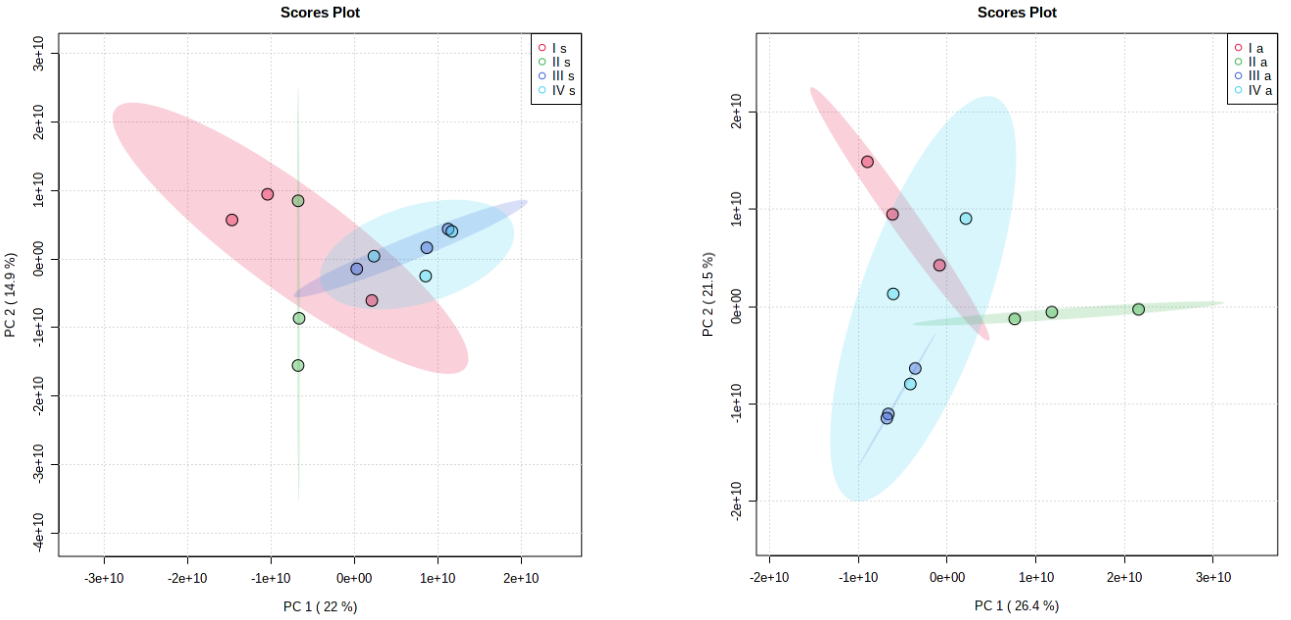
Figure 5. CFU number of micromycetes in different types of biolayers.

3.2. Metabolome Analysis of Biolayers in Ruskeala Quarry at Different Seasons

More than 200 different compounds belonging to the following main classes: mono-, di-, and trisaccharides; carboxylic acids; fatty acids; amino acids; phenolic compounds; terpenodes; and sterols were found in the samples.

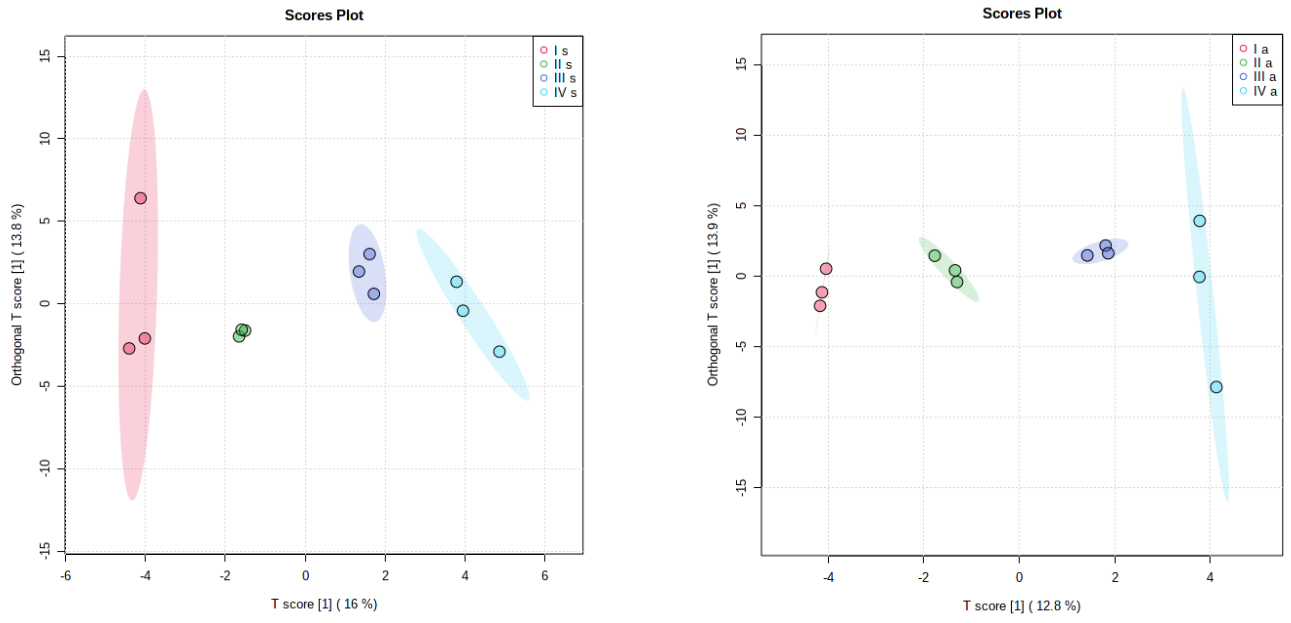
The PCA analysis of the samples from Ruskeala quarry grouped the data by the types of biolayers with overlapping clusters characterizing the metabolomes of the biolayers types (Figure 6). In the case of active vegetation stage (spring–summer samples), PCA does not visualize isolated positions of types 3 and 4 and also shows partial overlap of clusters of types 1 and 2. For late vegetative stages (autumn samples), PCA visualizes clustering of types 1, 2 and 3, but does not show isolated position of type 4 relative to 3 and partially 1. Clear clustering of metabolic data was visualized only when using OPLSDA (Figure 7).

The variable importance in projection (VIP) metabolites included 22 compounds for spring–summer samples (fructose, galactose, glyceric acid, gluconic acid, valeric acid (C 5:0), stearic acid (C 18:0), linoleic acid (C 18:2), behenic acid (C 22:0), cerotic acid (C 26:0) azelaic acid, abietic acid, sitosterol, camposterol, methylglucoside, trehalose, hydroxyquinone, 1,2-hydroxypropionic acid, hydroxypropionate, lupeol, unidentified compound (RI 1188), unidentified compound (RI 1207), unidentified compound (RI 1232)) and 19 compounds for autumn samples (glucose, serine, valine, proline, oxoproline, myo-inositol, chiro-inositol, palmitic acid (C 16:0), stearic acid (C 18:0), linoleic acid (C 18:2), arachidic acid (C 20:0), behenic acid (C 22:0), sitosterol, quinic acid, dulcitol, glycerol, orcinol, fructose, and trehalose).



(a) **(b)**

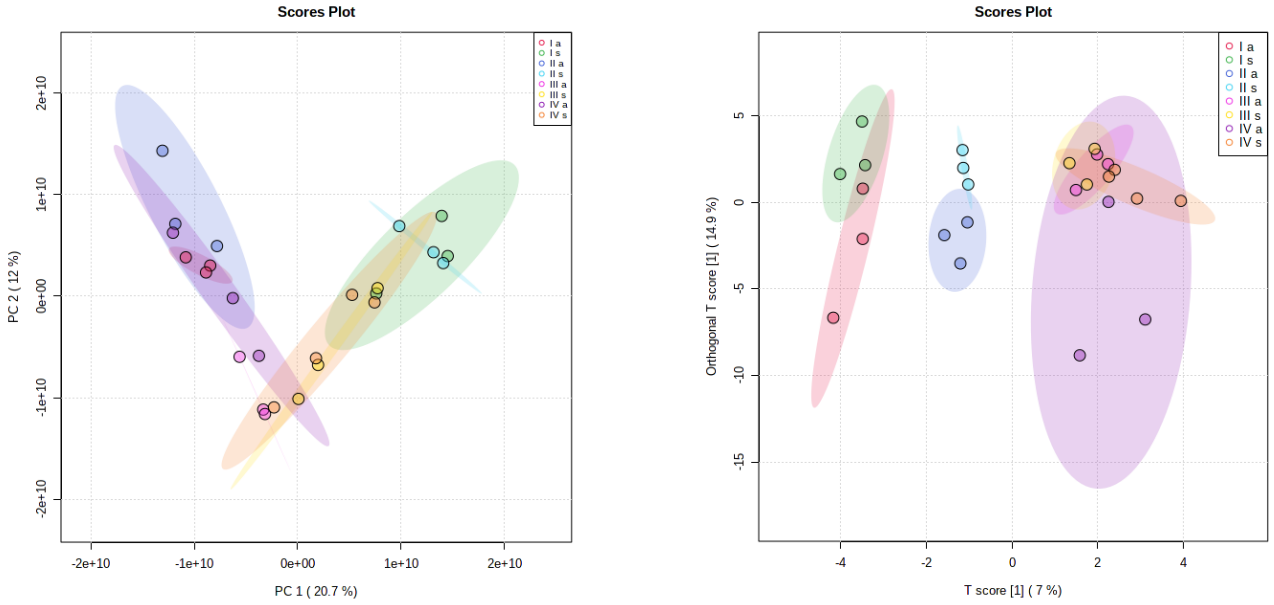
Figure 6. PCA analysis of metabolite network of biolayers from Ruskeala quarry: (**a**) active vegetation stage; (**b**) late vegetative stage.



(**a**) (**b**)

Figure 7. OPLSDA analysis of metabolite network of biolayers from Ruskeala quarry: (**a**) active vegetation stage; (**b**) late vegetative stage.

Joint PCA analysis of all samples from the Ruskeala quarry resulted in a V-shaped model, in which samples of types 1 and 2 are clearly divided into two groups depending on the season, and types 3 and 4 are grouped together (Figure 8a). Analysis by the OPLSDA method allows visualizing the differences, both by the types of communities and by seasons; types I and II, and III and IV are grouped together (Figure 8b).



(**a**) (**b**)

Figure 8. PCA (**a**) and OPLSDA (**b**) analysis of metabolite network of biolayers from Ruskeala quarry (united for active vegetation stage and late vegetative stage).

For this model, the number of VIP metabolites included 23 compounds: valeric acid (C 5:0), stearic acid (C 18:0), linolenic acid (C 18:1), linoleic acid (C 18:2), behenic acid (C 22:0), unidentified monosaccharide (furanose, RI 1807), glucose, arabinose, galactose, mannose, glycine, dulcitol, tregalose, sucrose, arabitol, methylglucofuranoside, phytol, gluconic acid, azelaic acid, abietic acid, glyceric acid, 2-hydroxypropionic, and acid sitosterol.

The heat map (Figure 9) illustrates the differences between the metabolomes of the samples of different types (the averaged data for the group are shown) taken both at the active vegetation stage and the late vegetative stage.

A significant part of the differences between groups of samples taken at active vegetation stage and late vegetative stage associated with the same metabolite. Some compounds tend to accumulate at the beginning of the growing season, while others in autumn, regardless of the taxonomic composition of the community.

As the general patterns of differences between the types of samples, both taken at active vegetation stage and late vegetative stage, can note the greater quantitative content of most metabolites in types I and II compared to III and IV. The samples of type I were characterized by a high content of linoleic acid (C 18:2), behenic acid (C 22:0), lignoceric acid (C 24:0), sitosterol, camposterol, glycerol, dulcitol, arabinose, and fructose. Comparison of the spring–summer and autumn samples of this type showed the samples taken at active vegetation stage contained more tocopherol, sitosterol, sugars (mannose and galactose), sugar acids (threonic, gluconic, and keto-gluconic acids), fumaric, succinic, malic, glyceronic, maleic acids, polyols (erythritol, mannitol), fatty acids (valeric acid (C 5:0), myristic acid (C 14:0), arachidic acid (C 20:0)), abietic acid, lupeol, hydroquinone, glycerol-3-P, and hydroxybenzaldehyde. The autumn samples, in comparison with the spring–summer ones, were richer in the content of amino acids (valine, leucine, alanine, proline, oxoproline, glycine, ethanolamine), pelargonic acid (C 9:0), lauric acid (C 12:0), linolenic acid (C 18:1), orcinol, mandelic acid, quinic acid, phytol, and polyols (arabitol, myo-inositol).

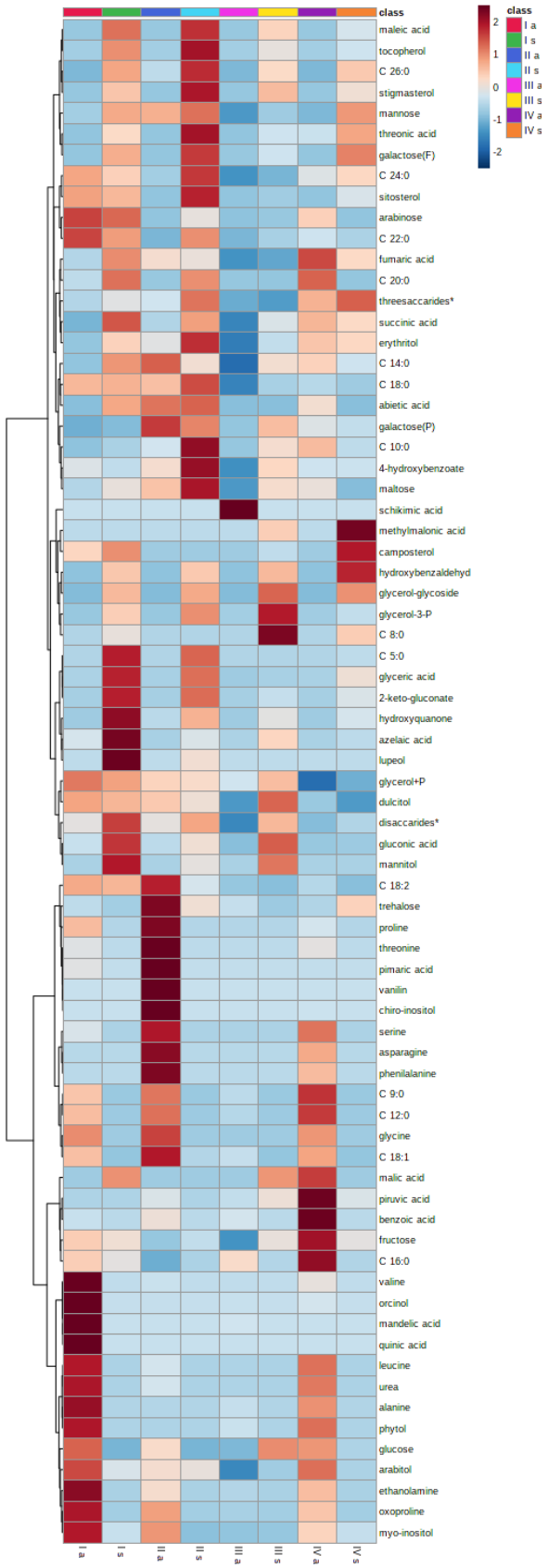


Figure 9. Heat maps of united spring-summer and autumn metabolome data of biolayers from Ruskeala quarry (group average shown). Notes: a—autumn samples, s—spring-summer samples, *—summarize concentration, P—pyranose, F—furanose.

The samples of type II contained a lot of myristic acid and stearic acid, abietic acid, and galactose. Spring–summer samples of group II differed from autumn ones by the dominance of almost the same metabolites as samples I, with the exception of azelaic acid, lupeol, gluconic acid, mannitol, and myristic acid. Compared to spring–summer samples, autumn samples contained more fatty acids (C 9:0; C 10: 0; C 12:0; C 18:2; C 18:1), sugars (maltose, glucose, trehalose), polyols (myo- inositol and chiro-inositol), amino acids (proline, threonine, ethanolamine, valine, serine, asparagine, phenylalanine, glycine), and pimaric acid.

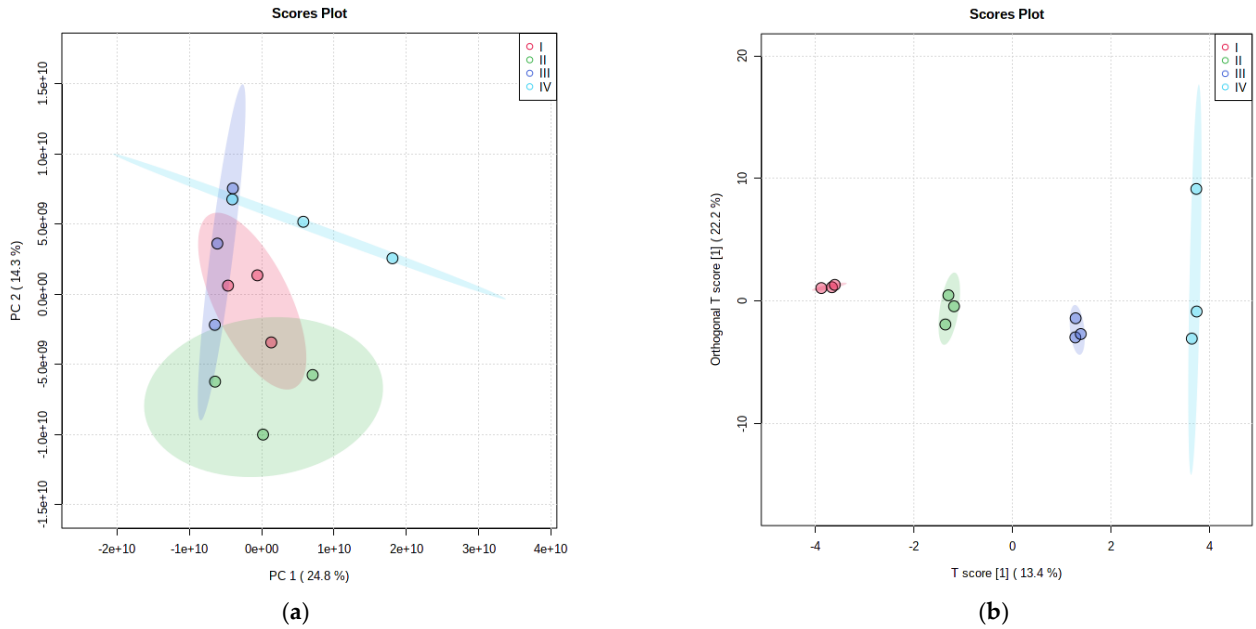
The samples of III type, and especially the autumn samples, were rather poor in the content of small organic molecules. Spring–summer samples contained a lot of disaccharides and some organic acids (malic, gluconic), mannitol, glycerol-3-P, glycerol-glycoside, and glycerol-benzaldehyde.

Type IV biolayers were distinguished by a high content of trisaccharides and some organic acids (fumaric and succinic). Spring–summer samples contained more methylmalonic acid, camposterol and stigmasterol, hydroxybenzaldegyl, glycerol glycoside, cerotic acid (C 24:0), mannose, galactose, threonic acid, and trisaccharides. Autumn samples compared to spring–summer samples, as well as samples I and II, contained higher concentrations of amino acids (leucine, alanine, oxoproline, ethanolamine, glycine, serine, asparagine, phenylalanine), fatty acids (C 9:0, C 12:0, C 16:0, C 18:1), glucose, fructose, myo-inositol, arabitol, malic acid, pyruvic acid, phytol, and urea.

3.3. Metabolome Analysis of Biolayers in Different Location

In samples from the urban environment (from the surface of museum necropolises), about 100 compounds were detected, which is two times less compared to samples from the Ruskeala quarry.

Metabolic analysis of samples from the urban environment with PCA data processing revealed clustering of metabolomes according to the putative types with partial overlapping of clusters (Figure 10a). OPLSDA clearly visualizes the clustering of samples (Figure 10b), as in the case of samples from the Ruskeala quarry.



(**a**) (**b**)

Figure 10. PCA (**a**) and OPLSDA (**b**) analysis of metabolite network of biolayers from museum necropolises.

The VIP metabolites included 13 compounds: mannitol, glucitol, myo-inositol, erythritol, succinic acid, glyceric acid, stigmasterol, galactose, fructose, tregalose, stearic acid, unidentified monosaccharide (F RI 1833), and unidentified monosaccharide (RI 2376).

The heat map (Figure 11) illustrates the differences between types of biolayers from museum necropolises, as well as the differences between the metabolite composition of biolayers in a quarry and in an urban environment (taken during the active growing season). Samples of type I were distinguished by the accumulation of some sugars (mainly ribose and fructose), chiro-inositol, glyceric, and succinic acids. The samples of type II were characterized by the accumulation of erythritol, stearic acid, glycosides, disaccharides, and some monosaccharides. Samples of type III, in comparison with other types, accumulated more monosaccharides and polyols (mannitol, arabitol, and erythritol). Samples of type IV were distinguished by the accumulation of sterols (camposterol, sitosterol, and stigmasterol), tocopherol, and a number of unidentified compounds.

As noted above, the diversity and concentration of compounds in biolayers in urban environment was significantly lower than in the quarry. There were almost no amino acids, phenolic compounds, and terpenoids in the biolayers of the museum necropolises.

Some samples from the urban environment contained polyols (mainly arabitol, mannitol, erythritol) in very high concentrations. Furthermore, in these biolayers, there was more palmitic acid. The concentrations of all other compounds common to the museum necropolises and the Ruskeala quarry were significantly higher in the biolayers sampled in the quarry.

Comparative PCA and OPLSDA analysis of the biolayers from Ruskeala quarry and from urban environmental showed the samples from the museum necropolises form a single densely grouped cluster. The samples from the Ruskeala quarry are distributed by types and almost do not overlap with the samples from the urban environment (Figure 12).

Partial overlapping of clusters was observed only for the IV type of samples (primary soil). The VIP metabolites for this model include glyceric acid, erythritol, dulcitol, succinic acid, keto-1-gluconic acid, camposterol, abietic acid, fatty acids (C 5:0, C 16:0, C 18:0, C 18:2, C 22:0, C 26:0), glycerol glycoside, hydroxyquinone, fructose, glycoside, galactose, mannose, glucose, arabinose, and trisaccharide.

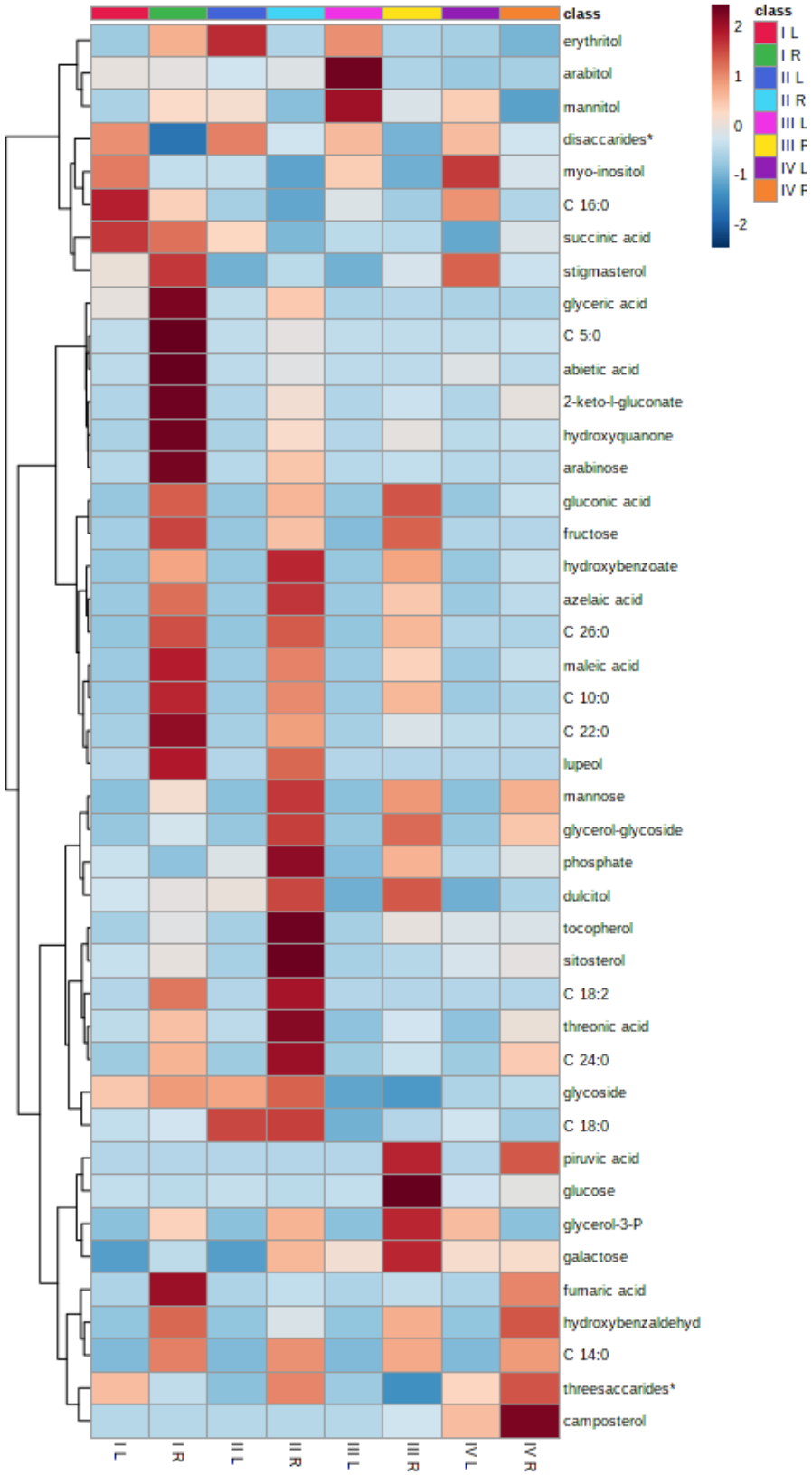
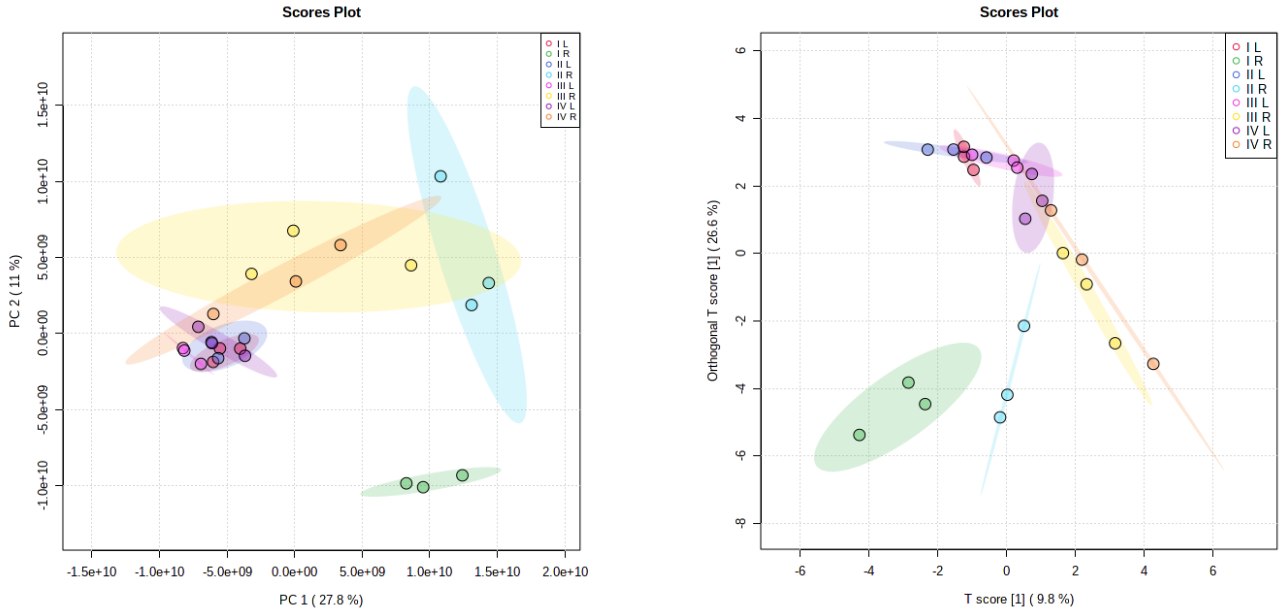


Figure 11. Heat map of united metabolome data of spring-summer samples of biolayers from Ruskeala quarry and museum necropolises (group average shown). Notes: R—Ruskeala quarry samples, L—museum necropolises samples, *—summarize concentration, P—pyranose, F—furanose.



(**a**) (**b**)

Figure 12. United PCA (**a**) and OPLSDA (**b**) analysis of metabolite network of biolayers from Ruskeala quarry and museum necropolises.

4. Discussion

Biological fouling and the formation of biolayers of various taxonomic and biochemical composition occur on the marble surface under various environmental conditions.

A clear clustering of metabolic data by the types of communities identified on the basis of dominant groups of organisms was visualized only by OPLSDA. This is to be expected because the uncontrolled PCA algorithm provides the means to achieve objective dimensionality reduction, and its application reveals group structure only when within-group variation is significantly less than between-group variation. OPLSDA is a method similar to PCA, but based on a combined decomposition of two matrices. The first matrix contains experimental measurements, and the second matrix includes a priori data, i.e., class belonging to a sample [29]. As a result, we get a matrix of scores, where objects are presented in a space of a lower dimension, but the differences between classes are maximum [30].

PCA demonstrated the grouping of samples into clusters with their partial overlapping. Of course, although samples from one type are similar, they are not identical in species composition, and the intragroup differences are quite large. Moreover, the biolayers of different types are partly formed by the same species. Thus, about 70% of the species of fungi were common for all types of communities in the quarry albeit with different frequency of occurrence. The differences for microalgae and cyanobacteria were more significant: two thirds of the total diversity of phototrophs identified in the quarry were found in only one type of biolayer. Furthermore, the species common to all four types of biolayers were not found at all. According to the metabolic data, the samples from types 3 and 4 turned out to be the closest. These communities really represent different stages of a process of primary soil formation.

The VIP for intergroup differences included only about 10% of the total number of metabolites in the samples. VIP indicates the significance of a variable for classification. If VIP > 1, the variable is considered to be significantly related to the difference in classes [29]. The main differences for both spring and autumn samples from Ruskeala quarry were related to sugars, polyols, organic acids of tricarboxylic acid (TCA) cycle, fatty acids, and sterols. Only five VIP compounds (fructose, stearic acid, behenic acid, sitosterol, and trehalose) were the same for the models of spring and autumn samples.

The heatmap shows that, at the trend level, the sample groups do differ. These differences are mainly related to the concentrations of metabolites. Likely these differences are not based on individual marker compounds, but rather on differences in the structures of metabolic networks of biolayers. It is interesting that some compounds tend to accumulate at the active vegetation stage, while others in autumn. Compared to autumn samples, spring–summer samples of all types of biolayers contained more TCA organic acids, sugar acids, some monosaccharides, and sterols. Autumn samples contained more amino acids, terpenoids, phenolic compounds, and polyols. It is very likely that these differences can be associated with the seasonal dynamics of the metabolome of individual organisms.

Joint PCA analysis of all samples from the Ruskeala quarry resulted in a V-shaped model, in which samples of types I and II are clearly divided into two groups depending on the season, and types III and IV are grouped together. Analysis by the OPLSDA method also allows visualizing the differences both by the types of communities and by seasons for types I and II, but types III and IV are grouped together (Figure 7b). These seasonal differences between samples dominate over taxonomic ones in types I and II. Types III and IV are less susceptible to seasonal variability.

In the statistical comparison of samples from museum necropolis in urban environmental and samples from the Ruskeala quarry, only partial overlapping of clusters was observed for samples of group IV, i.e., for primary soil. Thus, according to analyzing all the obtained statistical models, it is obvious that the primary soil is the most stable form of communities, with the minimum variance of data and the least susceptible to various changes. Communities of types I and II are more labile. They significantly change over time (when comparing samples from Ruskeala quarry taken in different seasons), but the points characterizing the metabolome of the system continue to group similarly, i.e., the trajectories of the attractor model remain unchanged.

In general, communities in the urban environment are much less biochemically diverse than in a quarry. The samples from the monuments contained very few amino acids, phenolic compounds, and terpenoids. However, some samples from the urban environment contained polyols (mainly arabitol, mannitol, erythritol) in very high concentrations. This is probably due to the higher proportion of fungi. In urban samples, fungi were more abundant (by CFU number). Our data on the biodiversity of fungi are only partially (for types III and IV of communities) consistent with the data of similar studies of the fungal diversity on rock in the original quarry located in a rural area (Zogelsdorf, Austria) and on the historical monuments in the city of Vienna [31]. However, the number of microfungi (CFU) was always higher in the city. It is known that polyols are synthesized and perform physiological functions in plants; however, to a greater extent, they are characteristic of fungi, partly replacing sugars [32]. The concentrations of all other compounds common to the urban environment and the quarry were significantly higher in the samples from the quarry. The species diversity of both fungi and algae in biolayers from museum necropolises was often inferior to the diversity in the quarry.

According to the classical concepts of stone colonization it begins with the colonization of the substrate by phototrophic organisms (algae and cyanobacteria). The growth of heterotrophic organisms (fungi and most bacteria) occurs later and is provided by metabolites of other (autotrophic) organisms or organic substances coming from the environment. Phototrophic organisms develop either on the surface of the stone, or penetrate a few millimeters into the depth of the substrate. The hyphae of fungi, as a rule, penetrate deeper, which is partly achieved due to the dissolution of minerals by metabolites secreted by the mycelium. As the community of microorganisms develops, its biomass increases, and the effect of fungal and bacterial metabolites on the stone surface is accompanied by weathering of rocks and the formation of primary soil [33,34].

We identified and characterized all the types of bio-layering described above, including communities dominated by phototrophs, communities dominated by fungi, the initial stage of primary soil formation, and primary soil with a developed moss cover. Whether these communities are temporary stages of succession and, accordingly, whether

the revealed patterns are temporary or association only the spatial specificity of the stone overgrowth is a discussion question and requires longer observations and next studies. Nevertheless, the revealed patterns of the organization of the metabolite networks of lithobiontic communities will undoubtedly be useful for further studies of the biochemical aspects of the processes of primary soil formation and biogeochemical processes.

5. Conclusions

According to metabolomic data, primary soil is the most stable form of communities, with the minimum variance of metabolomic data and the least susceptible to various changes. Communities of biolayers formed by algae, cyanobacteria, and fungi are more labile. The seasonal differences between samples dominate over taxonomic ones in types I (with predomination of algae and cyanobacteria) and II (with predomination of fungi). Types III and IV (different stages of soil formation) are less susceptible to seasonal variability.

Some compounds tend to accumulate at the active vegetation stage, while others in autumn, regardless of the taxonomic composition of the community. It is likely that the formed differences do not involve individual marker compounds, but rather differences in the structures of metabolite networks of different types of biolayers. In general, communities in urban environment are much less biochemically diverse than in a quarry.

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