

Article

Bioglea as a Source of Bioactive Ingredients: Chemical and Biological Evaluation

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Abstract: This study focused on bioglea in thermal material sampled at Saturnia spa (Tuscany, Italy). Bioglea is the term used to define the thermal plankton consisting of biogenic substances that have been investigated little from the chemical and biological points of view. Bioglea is mainly formed of cyanobacteria, particularly from the Oscillatoriales subsection, and it seems to have an important role in the maturation of thermal mud for the development of organic matter. This cyanobacteria-dominated community develops in a large outdoor pool at the spa, where the spring water is collected, over the sediments, with matter floating at the surface. Throughout the year, the cyanobacterial species of bioglea were the same, but their relative abundance changed significantly. For chemical characterization an extractive method and several analytical techniques (HPLC, GC-MS, SPME) were used. We also studied the radical scavenging activity using in vitro tests (DPPH, ORAC, ABTS). We found various groups of compounds: saturated and unsaturated fatty acids, hydroxyl acids, alcohols, phenols, amino acids. Many of the compounds have already been identified in the mud, particularly the lipid component. SPME indicated several hydrocarbons (C₁₁–C₁₇) and long-chain alcohols (C₁₂–C₁₆). The qualitative composition of volatile substances identified in bioglea was very similar to that of the mud previously analysed. These results contribute to our knowledge on thermal photosynthetic community and its possible exploitation.

Keywords: bioglea; cyanobacteria; Saturnia spa; antioxidant activity; SPME analysis

1. Introduction

The communities of microorganisms growing at high temperature in the presence of sulphur in volcanic environments, are broadly known as bioglea [1–3]. They are believed to contribute to the particular characteristics of those volcanic muds [4–11] that are sometimes employed in the treatment of psoriasis [3] or in other topical treatments in dermatology and in skin care [12–16].

This study examined bioglea collected at Saturnia spa (Tuscany, Italy) that is mainly composed of cyanobacteria, blue-green algae that form a widely distributed morphologically different group of photosynthetic prokaryotes which present oxygenic photosynthesis analogous to plants [17].

They are found in very different habitats, such as hot springs, Antarctic ice shelves, or deserts. Cyanobacteria are abundant in marine and freshwater environments and contribute significantly to worldwide photosynthetic biomass production [18]. They are mainly found in habitats exposed to high solar irradiance [17].

Some cyanobacteria are also able to fulfil the anoxygenic photosynthesis using hydrogen sulphide instead of water as an electron donor and producing elemental sulphur instead of oxygen [19]. This occurs in environments where the sulphide is abundant such as sediments of coastal areas and the sulphurous hot springs [20]. In the last case, the high concentration of sulphide is related to the high temperatures.

In recent decades the study of microorganisms that grow in extreme environments has undergone a considerable increase. This is because adaptation to extreme conditions may have led to the evolution of peculiar characteristics that can be exploited in biotechnology (i.e., heat-resistant enzymes, new bioactive substances for application in the pharmaceutical field and spa) [21–23].

Cyanobacteria are an important source of biologically active molecules with antiviral, antibacterial, antifungal and anticancer properties [24–26]. Cyanobacteria are already employed in agriculture, wastewater treatment, as fertilizer, food and as a source of alternative energy [21,22,27].

Saturnia spa boasts various sulphur pools which, in agreement with geologists, are an expression of the geothermal activity of the Monti Volsini. Water gushes at a constant temperature of 37.0 °C into a small lake in a natural “crater”. Bioglea develops above the sediments and floating at the water surface.

The study was designed for the characterization of this thermal benthos from a chemical and biological point of view.

The main aims were: (a) to establish the composition of the bioglea species and analyse the variability of the microbial photosynthetic community along the year; (b) to determine the organic component using an extractive method and several analytical techniques: high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), solid phase microextraction (SPME); (c) to investigate the radical scavenging activity, using several *in vitro* tests: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, oxygen radicals absorbance capacity (ORAC) assay, 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid (ABTS) assay; (d) to confirm its role in the maturation of the mud by comparing the components identified in the two matrices.

2. Materials and Methods

2.1. Materials

Samples were collected in the natural pool of the Saturnia spa (from surface and bottom) where bioglea grows spontaneously. Each sample was stored in a glass container with screw cap for SPME analysis, and in a high-density polyethylene (PH-HD) container for transport before freeze-drying. All the organic solvents were of analytical grade (Aldrich, Milan, Italy). Further, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) and 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid (ABTS^{•+}) were purchased from Sigma-Aldrich. The ORAC reagents 2,2'-azobis-(2-aminopropane)dihydrochloride (AAPH) and R-phycoerythrin (R-PE) were purchased from Molecular Probes (Rome, Italy).

2.2. Analysis of the Microbial Assemblage Composition

The samples for the determination of cyanobacteria species composition were examined by direct light and phase contrast microscopy (Nikon Eclipse E200, Nikon Instruments S.p.A., Campi Bisenzio, Firenze, Italy). Cyanobacteria were classified on the basis of the morphological features [28]. The frequency of each cyanobacterium was calculated as a percentage of the total number of cyanobacterial units. For unicellular cyanobacteria each cell was counted, and for filamentous cyanobacteria, each trichome was considered as one organism.

2.3. Preparation of Samples

The bioglea samples were first treated to remove hydrogen sulphide. This treatment is based on neutralization with a solution of sodium hydroxide 1M. To promote the release of the gas the

sample was placed under agitation and heated to a temperature below 37 °C. The hydrogen sulphide developed is bubbled into the sodium hydroxide solution.

Lipophilic extract. Lyophilized were mixed with 60 mL/g (dry weight) of chloroform. The mixture was stirred overnight at room temperature (23 °C). The extract was filtered through a 0.45-µm filter and evaporated in a vacuum evaporator.

Hydrophilic extract. To the sample of bioglea, already extracted as described above, 60 mL/g (dry weight) of a solution of ethanol/water (7:3 v/v) were added. The mixture was stirred overnight at room temperature (23 °C). The extract was filtered through a 0.45 µm filter and evaporated in a vacuum evaporator. The dry residue was taken up with water (1 mL/g dry weight of bioglea).

Soxhlet extraction. Samples of bioglea were extracted six time using solvents with increasing polarity (petroleum ether, chloroform, chloroform/methanol 9:1, methanol, ethanol/water 2:1, and ethanol/water 1:1) to extract any substances with different polarity.

Starting with non-polar solvent, 500 mg of lyophilized bioglea were placed in a thimble and 150 mL of solvent were added. The extracts were dried by vacuum evaporation.

If necessary, the extracts were further fractionated by preparative chromatography to obtain products with high purity that were easier to investigate. Separation was done on a column of silica gel using a gradient elution with different eluents: petroleum ether; petroleum ether:ethyl ether (9:1 and 1:1); ethyl ether; chloroform; chloroform:methanol (9.9:0.1, 9:1, 3:1, 1:1) and methanol.

2.4. HPLC Analysis

Samples were dissolved in acetonitrile (10 mg/mL), filtered through a 0.45 µm filter and analysed using a Shimadzu Chromatograph LC-10 ADVP equipped with a Shimadzu SPD-M 10 AVP photodiode array detector (Shimadzu, Milan, Italy). For chromatographic determination, we used a Supelcosil LC-18 column (250 mm × 4.6 mm, 5 µm particle size) from Supelco (Milan, Italy). The solutions were injected with a manual injector (Rheodyne Model 7725) and a 20 µL sample loop. The mobile phase was an isocratic mixture of acetonitrile:water (95:5, v/v) with a flow rate of 1 mL/min. The detector wavelength was set at 254 nm. Responses were recorded and integrated using Shimadzu CLASS VP Chromatography Data System 4.2 version.

The most significant peaks from the chromatograms were selected and their UV spectra were recorded, since the instrument is equipped with a UV photodiode array detector.

2.5. UV Analysis

UV-VIS spectra were recorded on a Varian Cary 1E ver.3.03 spectrophotometer connected to a pc with software Varian Cary 13 ver.3.03 (Varian, Turin, Italy). Quartz cells with a path length of 1 cm have been used. Spectra were recorded between 200 and 800 nm.

All the cold extracts, lipophilic and hydrophilic, of the bottom and surface bioglea were determined. Dry lipophilic and hydrophilic extracts (5 or 10 mg each), exactly weighed, were diluted to 1 mg/mL (stock solution) with water/ethanol (7:3) and methanol respectively for hydrophilic and lipophilic extracts. It was sometimes necessary to make dilutions of 0.2–0.05 mg/mL (especially for chloroform extracts). Stock solutions of hydrophilic extracts were analysed directly by visible determination and diluted 1:5 for UV analysis.

2.6. GC-MS Analysis

GC-MS analysis was done on a Gas Chromatograph 3800 and Saturn Mass Spectrometer 2000 Varian (Turin, Italy). Sample derivatization and chromatographic determination were performed as previously reported [29].

2.7. SPME Analysis

For SPME analysis we used a Gas Chromatograph HP-5890 Series II equipped with a double capillary column (HP-WAX and HP-5, 30 m × 0.25 mm; 0.25 µm thick), FID detector and 1:30 split ratio.

We used a 100- μm thick polydimethylsilane (PDMS) fiber (Supelco). After 20-min conditioning the filter was exposed to the sample inside the container for 5, 10, and 30 min at room temperature. Adsorption on the fiber was maximum with 10-min exposure. After this sample check, the fiber was placed in the GC and GC-MS injector. GC analysis was carried out in the conditions previously reported [29]. Constituents were identified by comparing their retention times (Rt) with those of two internal standards (butyl cellosolve and cellosolve acetate). GC-MS analysis was done in the conditions previously described [29]. Constituents were identified by comparing the retention times and mass spectra with those of reference compounds from an experimental home-made MS library built up using pure substances and known oils. Computer matching against two commercial data bases (NIST 98) was also done. Spectra with at least 85–90% similarity were selected.

2.8. DPPH Assay

DPPH assay was employed on all surface bioglea extracts. They were analysed as previously described [30]. Reaction kinetics was plotted for each extract and the percentage of DPPH remaining at steady state was determined. Radical scavenging activity was expressed as IC_{50} ($\mu\text{g}/\text{mL}$).

2.9. ORAC Assay

The ORAC assay was run on all surface bioglea samples. The final concentration tested was 200 $\mu\text{g}/\text{mL}$. All samples were dissolved in acetate buffer then added to the R-PE used as a target for the free radical attack. The assay was carried out as previously reported [30]. Final results were calculated as described by Cao et al. [31] and expressed as Trolox equivalent (μM).

2.10. TEAC Analysis (ABTS assay)

ABTS assay was run on all samples (surface and bottom bioglea extracts). The antioxidant activities of the extracts were assayed by spectrophotometrically measuring the quenching of the stable cation radical $\text{ABTS}^{\bullet+}$ (acid 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic)) as reported by Re et al. [32].

The data are presented as Trolox equivalent antioxidant capacity (TEAC) expressed as $\text{mgTrolox}/\text{mg}$ of extract. The results were obtained by comparison with a calibration curve plotted with Trolox at final concentrations of 0–15 mM.

2.11. Statistical Analysis

Data are reported as means with standard deviation (mean \pm SD). The experiments were done in triplicate. Statistical significance ($p < 0.05$) was determined using one-way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Composition of the Thermal Photosynthetic Community

At Saturnia spa, the environmental conditions are quite stable (constant temperature, pH and water composition) except for light intensity and quality. The species that form the photosynthetic community appear not to have significantly changed in the last four decades, as Bazzichelli et al. [33] reported a composition similar to that described in this work. The constancy of the environment is reflected in the composition of the population. The bottom of the pond is patchily covered with a brownish mat mainly composed of a phycoerythrin-containing *Oscillatoria* sp. (Figure 1a) and a green film dominated by *Spirulina* cf. *labyrinthiformis* and a *Leptolyngbya* sp. (Figure 1b). In addition to cyanobacteria the mat includes photosynthetic bacteria (mainly green sulphur bacteria) and non-photosynthetic sulphur bacteria such as *Beggiatoa*. The species forming the mats are the same in different periods of the year, but their relative abundance can change. In spring, early summer and autumn, the floating mat was dominated (42–92%) by a dark brown *Oscillatoria*, while the bottom biofilm was dominated by *Spirulina* cf. *labyrinthiformis* (64–90%). In August *S.* cf. *labyrinthiformis* was the main organism in

the floating mat which, however, showed evident signs of degradation and the previous presence of phycoerythrin-containing organisms, probably the dark brown *Oscillatoria*. At the same time, the bottom of the pool was amply covered by a thick mat of dark brown *Oscillatoria*. During the central hours of the day the *Oscillatoria* sp. mat starts to float, probably because gas bubbles remaining trapped inside of it. The gases, besides oxygen, are very likely those associated with the spring water, since inside the *Oscillatoria* sp. filaments sulphur granules are often visible, indicating probable anoxygenic photosynthesis.

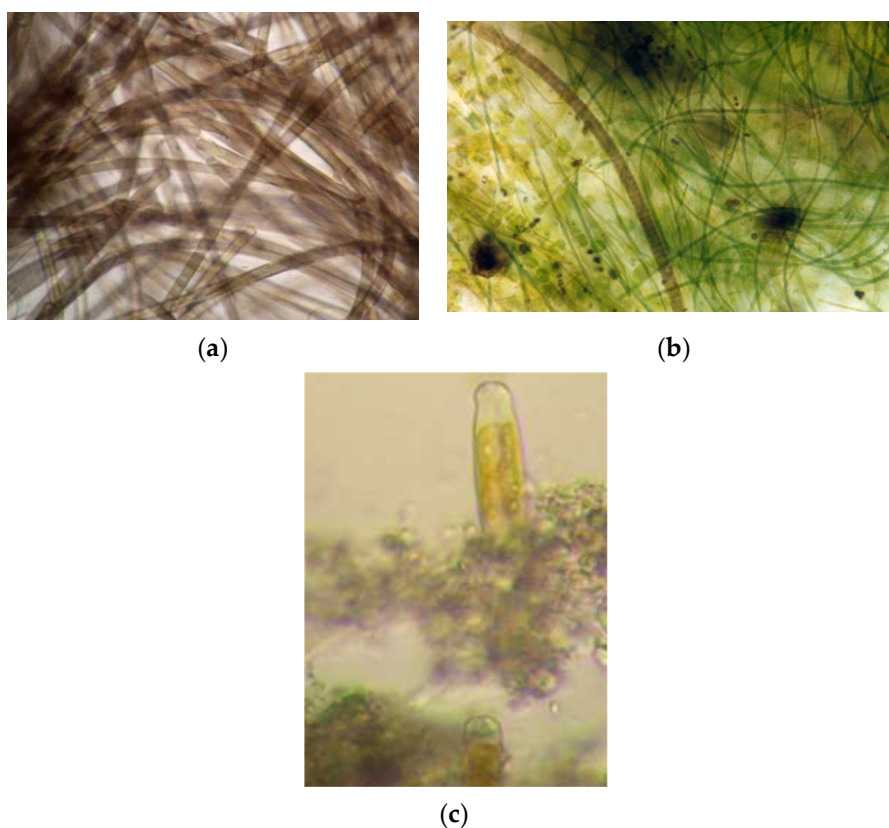


Figure 1. Cyanobacteria species composition. (a) *Oscillatoria* sp. from a floating mat; (b) green film dominated by *Spirulina* cf. *labyrinthiformis* and *Leptolyngbya* sp. a brown filament of *Oscillatoria* sp. is also visible; (c) Diatoms (*Pinnularia* sp.) growing on the surface of the mud in maturation.

In this ecosystem cyanobacteria appear to adopt two different strategies to protect cells from the high irradiances in the summer. In the mat dominated by *Oscillatoria* filaments alternately migrate within the mat and shelter in shaded zones and tend to pack together. This behaviour was previously observed in *Oscillatoria terebriformis* [34]. In the biofilm dominated by *S. cf. labyrinthiformis* and *Leptolyngbya*-like cyanobacterium filaments have less capacity for movement and evade the excess of irradiance mainly by altering their pigment ratio. However, when the *Oscillatoria* mat floats to the surface, the high irradiance and the increased temperature at the water-air interface can cause degradation of the trichomes during the summer months.

S. cf. labyrinthiformis is at least partially able to switch to anoxygenic photosynthesis, excreting granules of elemental sulphur outside the cells [35].

A different species composition can be found in the film covering the mud during its maturation [36].

The first photosynthetic organisms to develop on the mud surface are diatoms (Figure 1c). After about one month, cyanobacteria too start to develop mainly *S. cf. labyrinthiformis*, *Leptolyngbya* sp. and a high abundance of unicellular forms of the sub section Chroococcales such as *Synechococcus* sp. [36].

3.2. Extracts

Variouly coloured extracts (orange, brown, green) were obtained by extraction of the bioglea collected at the surface or the bottom of the natural pool of the Saturnia spa (Table 1). The colour of the extracts indicates the presence of substances such as carotenoids, chlorophyll, and other pigments known for cyanobacteria [22].

Table 1. Amount (mg/g bioglea) and colour of the various extract.

Type of Extract	Type of Sample			
	Surface Bioglea		Bottom Bioglea	
<i>Hot extraction</i>	<i>mg/g bioglea</i>	<i>Colour</i>	<i>mg/g bioglea</i>	<i>Colour</i>
PE	19.25 ± 0.95	Orange	3.70 ± 0.66	Orange
C	17.19 ± 1.02	Brown	26.23 ± 0.61	Brown
C/M (9:1)	13.23 ± 1.04	Reddish brown	6.23 ± 0.59	Reddish brown
M	31.35 ± 1.00	Green	6.30 ± 0.63	Brown
E/W (2:1)	11.52 ± 0.97	Light green	4.30 ± 0.53	Green
E/W (1:1)	14.13 ± 0.84	Light green	2.89 ± 0.43	Dark green
<i>Cold extraction</i>	<i>mg/g bioglea</i>	<i>Colour</i>	<i>mg/g bioglea</i>	<i>Colour</i>
C	24.43 ± 1.02	Reddish brown	6.72 ± 0.65	Brown
W/E (7:3)	63.09 ± 0.83	Dark green	16.72 ± 0.69	Green

PE: petroleum ether; C: chloroform; M: methanol; E: ethanol; W: water.

The largest amounts of extracts were obtained from the surface bioglea with the exception of the hot chloroform extract where the highest was from the bottom bioglea. The largest amount was obtained for the cold water/ethanol (7:3) extract of the surface bioglea (63.09 mg/g bioglea). The smallest for the hot ethanol/water (1:1) extract of the bottom bioglea (2.89 mg/g bioglea).

We focused mainly on the surface bioglea that the Saturnia spa uses for treatments and for the preparation of cosmetic products.

3.3. HPLC and UV Analyses

Bioglea and its cold extracts were first examined by HPLC and UV spectrophotometry. HPLC analysis was done under the same conditions as for the thermal mud [29] with the aim of comparing their chromatographic profiles, not for the identification of their composition. Figure 2 shows the chromatogram for the bioglea *in toto*.

There were peaks very similar to those of the mature mud (six months) in the area with a Rt between 2 and 6 min. The spectra show the presence of substances that absorb at 400–450 nm and 560–660 nm, which can be attributed to carotenoids, biliproteins, and chlorophyll. The chromatogram also gave peaks of photoprotective substances that absorb at 300 nm. This is in agreement with the ability of cyanobacteria to process radical scavenging and UV absorbing compounds [37–41]. These data were confirmed by spectrophotometry.

The products highlighted by spectrophotometry were mainly concentrated in the UV (200–400 nm) and visible (400–700 nm) ranges. The spectra of the hydrophilic extracts show an absorption band at about 662 nm characteristic of chlorophyll *a* which is the only active chlorophyll in blue-green algae. The peak of chlorophyll (chl) was more evident in the surface bioglea extracts than in the bottom extracts (Figure 3).

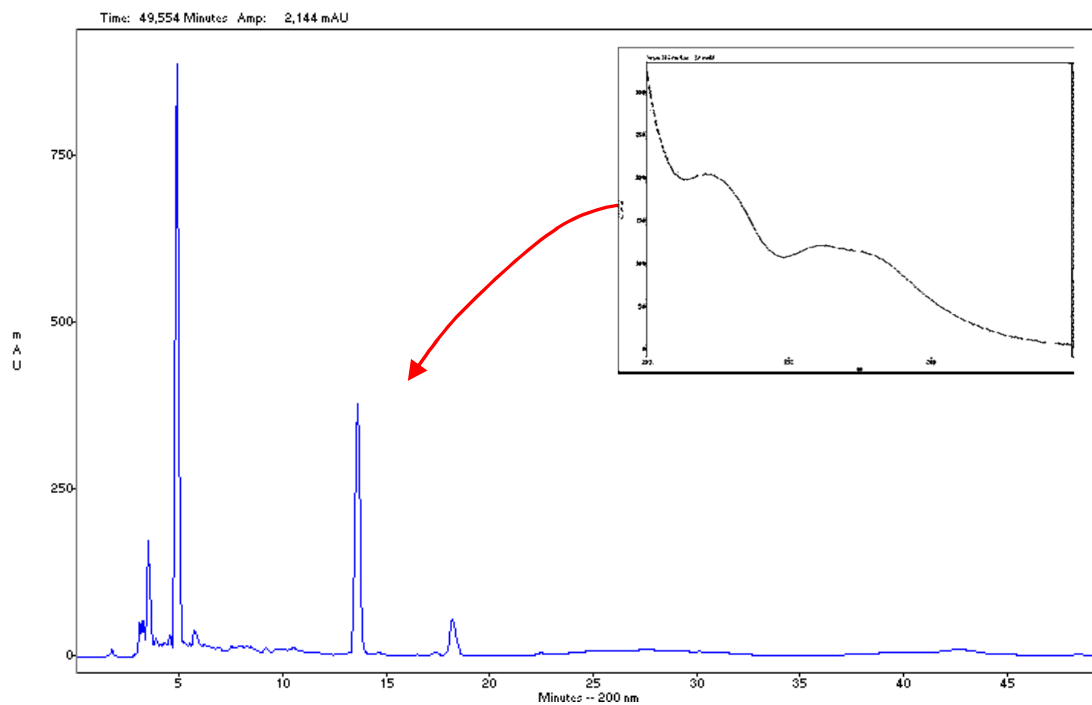


Figure 2. HPLC chromatogram of bioglea *in toto*. The peak with interesting UV absorption at 300 nm was highlighted.

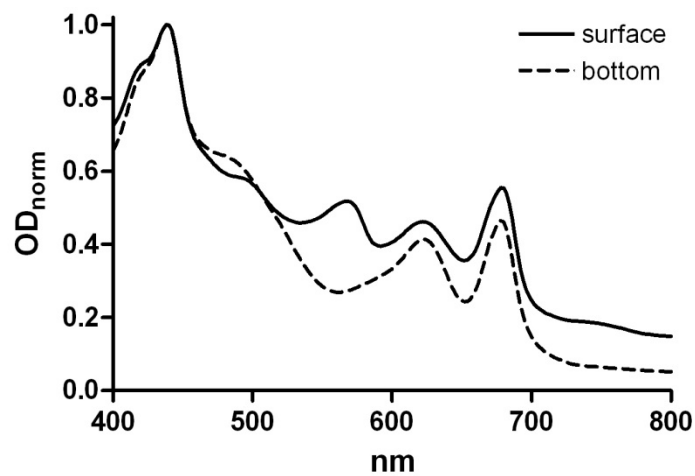


Figure 3. Absorption UV spectra of hydrophilic extracts of surface and bottom bioglea.

In both samples there was a peak at 411 nm probably due to carotenoids (400–450 nm) [23]. Bioglea extract of the surface material had two absorption bands at 564 and 611 nm. These can be assigned to the biliproteins such as red phycoerythrin (545–570 nm) [40] and blue phycocyanine (about 620 nm) [42,43].

Lipophilic extracts, like hydrophilic extracts, showed chlorophyll *a* with absorption bands at 661 and 665 nm respectively for the bottom and the surface bioglea. Carotenoids are also present in all extracts with more intense absorption bands than those of the hydrophilic extracts. There were also peaks between 605 and 614 nm typical of phycocyanine.

The production of chlorophyll, phycobiliproteins and carotenoids is an adaptation mechanism for acclimatization. Their composition and amount are influenced by the light intensity [44]. The carotenoid/chl ratio in cyanobacteria was higher after UV irradiation [17].

3.4. GC-MS Analysis

All extracts and the fraction separated by chromatography were analysed by GC-MS to identify some components. By comparing the spectra with those of substances present in the library we could identify different classes of compounds: saturated and unsaturated fatty acids, hydroxyl acids, dicarboxylic acids, alcohols, phenols, aromatic compounds, sterols, amino acids, and compounds of various kinds (Tables 2–4). Lipids were the main compounds.

Table 2. Fatty acids and sterol profiles (% weight) identified in the bioglea extracts.

Class of Compounds	Rt	C _(c) Extract	W/E _(c) (7:3) Extract	PE Extract	C _(h) Extract	C/M (9:1) Extract	M Extract	E/W (2:1) Extract	E/W (1:1) Extract
<i>Saturated fatty acids</i>									
(C ₉) Nonanoic acid	5.920	0.2	0.1	0.6	0.5	0.5	0.3	0.1	0.1
(C ₁₀) Decanoic acid	6.564	–	–	0.2	0.1	–	0.1	–	–
(C ₁₂) Dodecanoic acid	7.723	0.3	–	–	0.8	–	0.2	0.1	0.1
(C ₁₄) Tetradecanoic acid	8.783	3.8	0.8	12.5	6.5	–	2.8	1.5	1.2
(C ₁₅) Pentadecanoic acid	9.273	5.4	1.0	16.3	9.7	–	4.2	2.0	1.5
(C ₁₆) Palmitic acid	9.774	0.3	–	0.8	0.6	2.5	–	–	–
(C ₁₇) Heptadecanoic acid	10.235	2.5	–	9.2	7.5	–	3.5	1.8	1.5
(C ₁₈) Stearic acid	10.650	0.5	–	–	0.9	1.7	0.1	–	–
(C ₂₀) Eicosanoic acid	11.692	–	0.5	–	0.3	–	0.1	0.2	0.1
(C ₂₂) Docosanoic acid	13.128	–	–	–	0.2	–	0.1	–	–
<i>Unsaturated fatty acids</i>									
(C ₁₆) Palmitelaidic acid	9.673	3.2	0.7	10.5	7.8	–	0.8	0.7	0.3
(C ₁₈) Oleic acid	10.599	2.4	0.5	6.8	5.7	1.8	1.5	1.2	0.8
(C ₁₈) Linoleic acid	10.548	–	–	0.8	0.3	–	0.2	0.1	0.1
<i>Sterols</i>									
Cholesterol	15.730	–	–	1.5	1.3	0.7	0.4	0.2	0.1
β-sitosterol	17.817	–	–	1.2	0.8	0.5	0.2	0.3	0.1
Lanost-8-en-3-beta-ol	20.529	–	–	–	0.5	–	–	–	–
Stigmasterol	21.864	–	–	–	–	–	–	0.2	–
Desmosterol	22.797	–	–	–	–	0.3	–	–	–

C: Chloroform; E: Ethanol; M: Methanol; PE: Petroleum ether; W: Water; (c): cold extract; (h): hot extract.

Table 3. Amino acids composition (% weight) identified in the bioglea extracts.

Class of Compounds	Rt	C _(c) Extract	W/E _(c) (7:3) Extract	PE Extract	C _(h) Extract	C/M (9:1) Extract	M Extract	E/W (2:1) Extract	E/W (1:1) Extract
L-alanine	4.914	–	–	–	–	–	–	5.8	5.1
L-isoleucine	5.487	–	0.5	–	–	–	0.5	4.2	4.1
L-valine	6.033	–	–	–	–	–	–	6.0	5.4
L-leucine	6.065	–	–	–	–	0.8	–	6.1	5.6
Glycine	6.545	–	–	–	–	–	–	4.3	3.9
L-proline	6.823	–	–	–	–	–	–	2.7	2.2
L-proline-5-oxo	7.066	–	0.1	0.1	0.1	–	–	0.2	0.1
N-(2-methyl-1-oxopropyl) glycine	7.325	–	–	–	–	–	0.1	–	–
L-serine	7.366	–	–	–	–	–	–	2.5	2.3
N-(2-methyl-1-oxobutyl) glycine	7.446	–	–	–	–	–	0.1	–	–
Glutamine	7.556	–	0.5	–	–	–	–	2.8	3.1
L-threonine	7.615	–	–	–	–	–	–	3.3	3.0
L-aspartic acid	8.369	–	–	–	–	–	–	0.3	2.3
L-methionine	8.747	–	–	–	–	–	–	1.7	1.6
L-phenylalanine	9.638	–	–	–	–	–	0.7	3.1	2.9
L-tyrosine	11.868	–	–	–	–	–	–	2.9	3.0
N-acetyl tyrosine	13.977	–	–	–	–	–	–	0.4	0.5

C: Chloroform; E: Ethanol; M: Methanol; PE: Petroleum ether; W: Water; (c): cold extract; (h): hot extract.

Table 4. The main class of organic compounds identified in the extracts of bioglea.

Class of Compounds	The Predominant Individual Compound	Rt	Class of Compounds	The Predominant Individual Compound	Rt
<i>Carboxylic acids</i>	2-butendioic acid	5.580	<i>Carbohydrates</i>	D-ribonic acid gamma lactone	10.118
	Butanedioic acid	5.600		D-ribofuranose	10.786
	2-pentendioic acid	6.715		Methyl- α -D-mannofuranose	10.900
	4-methoxy benzoic acid	8.753		D-fructose	10.992
	3-methoxy cinnamic acid	9.294		D-altrose	11.228
	Abietic acid	14.633		Methyl- α -D-glucofuranose	11.288
	Dehydroabietic acid	14.655		α -D-galactopyranose phosphate	11.608
<i>Hydroxy acids</i>	2-hydroxypropanoic acid	4.472	<i>Alcohols</i>	2-acetylamino-2-deoxy- α -D-mannopyranose	12.914
	2-hydroxybutanoic acid	5.151		Dodecanol	7.221
	3-hydroxybutanoic acid	5.434		Hexadecanol	9.328
	2-hydroxy-3-methyl butanoic acid	5.500		Phytol	10.376
	4-hydroxyvaleric acid	5.837		Octadecanol	10.428
	3-hydroxyvaleric acid	6.172		Olean-13(18)-ene	13.269
	4-hydroxybutanoic acid	6.173		Squalene	16.953
<i>Esters</i>	3-methoxy-4-hydroxy cinnamic acid	12.758	<i>Others</i>	Lycopersene	16.960
	2,3-dihydroxy benzoic acid methyl ester	11.258		2,4-bis-hydroxy pyrimidine	7.145
				5-methyl-2,4-bis-hydroxy pyrimidine	7.725

Table 2 shows the distribution profiles of fatty acids, which were the main components in the various extracts of the bioglea. The relative proportions of the various compounds were attributed on the basis of the peaks areas. Palmitic acid had the largest amounts in all the extracts. In fact, this compound seems to be the main fatty acid developed by cyanobacteria [45]. Oleic acid was found in all the extracts.

Other interesting compounds were sterols (Table 2) and amino acids (Table 3). Sterols are present only in the hot extracts and the amino acids concentrated in the hot hydrophilic extracts.

Table 4 shows other compounds detected at very low concentration (<0.1%) or in traces. We also identified synthetic compounds (not reported in the Tables), present as contaminants, used in cosmetic products such as antioxidants (BHT), preservatives (sorbic acid, 4-hydroxy benzoic acid, phenoxyethanol), sunscreens (Ethylhexyl methoxycinnamate, Methylbenzylidene camphor), active ingredients (urea, azelaic acid), and various perfumes. These probably come from the cosmetics used by bathers. The results obtained for each extract are illustrated below.

Cold extracts. The chloroform extract was mainly characterized by the presence of saturated and unsaturated fatty acids (C₉–C₂₀). Some fatty acids are also identified in the water/ethanol (7:3) extract where there were certain amino acids as well. The compounds identified are shown in Tables 2 and 3.

Petroleum ether extract. Saturated and unsaturated fatty acids with between 9 and 18 carbon atoms were the major compounds (Table 2). Some fatty acids with odd number of carbon atoms were found, in particular the saturated acids C₉ and C₁₇. Other substances were also detected such as a carotenoid ψ,ψ -7,7',8,8',11,11',12,12',15,15'-decahydro-carotene, known as lycopersene (Table 4). It is an intermediate in the enzymatic synthesis of β -carotene and lycopene [46], two compounds with radical scavenging activity.

Terpenes such as olean-13(8)ene and sterols such as cholesterol and β -sitosterol were identified. Environmental pollutants such as abietic and dehydroabietic acids were released from the plants around the spa.

Chloroform extract. In this extract, too, fatty acids were the main components (Table 2). However, we also identified the C₁₂ and C₁₅ fatty acids and acids with longer chain (C₂₀ and C₂₂). Other compounds detected were sterols, such as cholesterol, β -sitosterol, and lanost-8-en-3- β -ol, and the carotenoid lycopersene and dehydroabietic acid as contaminant.

There were traces of benzoic and cinnamic acids derivatives (2,3-dihydroxy benzoic acid methyl ester, 4-methoxy benzoic acid, 3-methoxy cinnamic acid, and 3-methoxy-4-hydroxy cinnamic acid) (Table 4).

Chloroform/methanol (9:1) extract. This extract had a smaller lipid component: only nonanoic, palmitic, stearic and oleic acids were identified (Table 2). The hydrocarbon squalene, a compound not present in the previous extracts was detected. This is an intermediate in the biosynthesis of cholesterol, it stimulates the acetyl-CoA and has also an antioxidant activity similar to that of trans retinols [47].

There were some sterols: in addition to cholesterol and β -sitosterol already found, there was desmosterol (a precursor of cholesterol) [48]. Other compounds were phytol (also present in the previous extracts) and the amino acid L-leucine.

Methanol extract. The methanol extract had similar lipid profile the chloroform extract. This extract contained some substances not identified in the previous extracts, such as amino acids (Table 3) and carbohydrates (Table 4). Cyanobacteria are able to synthesize polysaccharides by different biosynthetic pathways. They may be complex heteropolymers represented by 2–14 different monosaccharides, methyl sugars, and/or amino sugars [49] like those identified in this extract.

We identified essential L-amino acids such as L-isoleucine and L-phenylalanine and glycine derivatives. Also noted were traces of pyrimidine bases such as 2,4-bis-hydroxy pyrimidine and 5-methyl-2,4-bis-hydroxy pyrimidine (Table 4), tautomeric forms of uracil and thymine respectively.

Ethanol/water (2:1) extract. This first hydrophilic extract still showed the presence of lipidic component with saturated and unsaturated fatty acids from C₉ to C₁₈. We identified compounds that were not present in the previous extracts such as stigmaterol (Table 2) and a group of hydroxyacids (see Table 4). The extract was characterized by the large number of amino acids, six of them essential.

Ethanol/water (1:1) extract. The composition of the ethanol/water (1:1) extract was very similar to the previous extract. It was rich in fatty acids and amino acids (Tables 2 and 3).

GC-MS analysis did not show any carotenoids (exception of lycopersene). This might be due to two factors: (i) the extracts had no added antioxidant, which is necessary for products that are so easily alterable; (ii) the compounds may be decomposed during the analysis.

All non-aqueous extracts showed sulfur (retention time 11.728).

Comparison of the results of GC-MS analysis and those obtained previously on a sample of mature mud [29] shows that some classes of compounds such as saturated and unsaturated fatty acids free and esterified with glycerol, alcohols, sterols, dicarboxylic acids, hydroxyl acids and other compounds were identified in both thermal matrices. This confirms our previous hypothesis concerning the direct involvement of the bioglea in the maturation of the mud. The identification of a lipid component is in agreement with the activity of cyanobacteria.

Lipid synthesis by cyanobacteria is known and various biological activities are attributed to them [25,50,51]. They play a fundamental role for the protection of cyanobacteria when they are exposed to environmental stresses (low temperature, high concentration of salt, desiccation, high irradiation) [52]. An interesting finding was the presence of fatty acids with odd numbers of carbon atoms such as pentadecanoic and heptadecanoic acids, that are also found in marine algae [52]. The composition is probably linked to the environmental conditions in which the bioglea develops. The characteristics of fatty acids as alkyl chain length and degree of unsaturation can both be influenced by temperature [53].

3.5. SPME Analysis

The SPME allows the quantification of volatile constituents released in the space around the tested material. It is one of the most widely applied techniques to examine different matrices without altering the sample and in a short time [54].

In the bioglea sample several hydrocarbon substances from C₁₁ to C₁₇ were found in more or less significant percentage (Table 5). The heptadecene was the only unsaturated hydrocarbon in the matrix. Cyanobacteria can naturally produce hydrocarbons from fatty acids. Cyanobacteria synthesize long-chain alkanes and alkenes using two different pathways, one of which involves a deformation of fatty aldehydes and the other decarboxylation of fatty acids [55].

Table 5. Percentage composition and retention time in the bioglea SPME analysis.

Compounds	Rt	Percentage
Dimethylsulfur	6.65	9.50
Limonene	8.51	2.66
<i>p</i> -cresole	10.15	5.76
Undecane	11.13	0.87
Decanal	15.76	0.85
Tridecane	19.90	1.59
Tetradecane	24.33	4.64
Branched Alcohol	26.37	20.42
Pentadecane	28.61	3.81
Hexadecane	32.70	6.19
Branched Alcohol	34.45	4.91
Heptadecene	36.58	16.13
Hepatadecane	36.60	15.27

Limonene (2.66%) and *p*-cresole (5.76%) were also identified. The main compound (20.42%) with R_t 26.37 was not been identified with certainty; by comparison with the data in the library, this could be a more or less branched long chain alcohol (C_{12} – C_{16}) which corresponds to a superior homologous with R_t 34.45 (4.91%) (Table 5).

The qualitative composition of volatile substances in bioglea was very similar to that of the mud previously analysed [29].

3.6. Antioxidant Activity

As a preliminary step for studying the radical scavenging activity, we determined the quenching ability against the DPPH radical. The DPPH test is run in a homogeneous phase and is not very specific. It measures the H radical/electron-transferring capacity against the stable free radical DPPH, so it only provides an approximate information on the scavenging activity. The results are shown in Table 6.

Table 6. Antioxidant activity of bioglea extracts in vitro.

Sample	DPPH IC ₅₀ (µg/mL)	ORAC Trolox Equivalent(µM)	TEAC	
			mg TROLOX eq/mg extract	
			Surface	Bottom
Petroleum ether extract	270.50 ± 0.58	3.15 ± 0.15	3.2 ± 0.2	4.1 ± 0.2
Chloroform extract	203.84 ± 0.91	4.35 ± 0.12	4.5 ± 0.3	4.9 ± 0.7
Choloroform/methanol 9:1 extract	498.29 ± 0.87	4.25 ± 0.15	9.1 ± 0.5	11.6 ± 0.6
Methanol extract	351.27 ± 0.72	4.20 ± 0.75	12.8 ± 0.6	15.2 ± 0.8
Ethanol/water 2:1 extract	379.10 ± 0.77	5.58 ± 1.20	11.1 ± 0.6	32.0 ± 1.6
Ethanol/water 1:1 extract	359.95 ± 0.65	6.75 ± 0.85	22.1 ± 1.1	20.8 ± 1.0
Hydrophilic extract (RT)	868.41 ± 0.85	2.80 ± 0.70	4.9 ± 0.2	1.8 ± 0.1
Lipophilic extract room (RT)	974.91 ± 0.47	1.90 ± 0.25	5.9 ± 0.3	1.4 ± 0.1

RT: room temperature.

The chloroform extract had the highest activity (IC_{50} = 203.84 µg/mL) while both extracts obtained at room temperature, lipophilic and hydrophilic, had the lowest.

Oxygen radical absorbance capacity (ORAC) measures the quenching ability of peroxy radicals produced by thermal decomposition of the radical initiator (AAPH). It provides an indication of the protective capacity of each sample against peroxy radical, whose target is the phycoerythrin protein. It is more specific than the DPPH assay.

The analysis showed that the hydrophilic extracts were more active than the others extracts at the same concentration. The extract ethanol:water (1:1) was the most active (Table 6). In this case, too, the extracts obtained at room temperature had the lowest activity.

The radical scavenging activity on sample collected from the bottom and the surface of the natural pool was determined by TEAC analysis (ABTS assay). The results with this method are shown in Table 6.

The extracts obtained by hot extraction are more active than those obtained by cold extraction. In the first case the extracts of the bottom bioglea were more active than those of the surface bioglea and hydrophilic extracts had greater antioxidant power than the lipophilic extracts.

The extract ethanol/water (2:1) was the most active, followed by the ethanol/water (1:1) extract. Again, out of the extracts obtained by cold extraction, the water/ethanol (7:3) extract of the surface bioglea showed the most activity.

The highest activity of the hydrophilic extracts may be attributed to amino acids and polyphenols. These results are in agreement with previous results for the mature mud where the hydrophilic extract was more active than the lipophilic extract [29].

According to recent studies, the radical scavenging activity of cyanobacteria, the main constituents of bioglea, seems mainly due to the biliprotein phycocyanin [42,43,56]. This is a photosynthetic pigment whose antioxidant and anti-inflammatory activity is tested with different methods. It may be hold potential as a therapeutic agent for diseases induced by oxidative stress. Moreover, the protective effects of algae on the skin have been proved to be closely related to the antioxidant properties of natural carotenoids, which in turn raise the levels of beta-carotene in the skin and serum [57,58].

4. Conclusions

In the thermal pool of Saturnia spa, a sulphide-rich thermal spring, a fairly stable cyanobacteria community (bioglea) grows all the year round. This study contributes to our knowledge of the thermal photosynthetic community and its possible exploitation.

The analytical procedures employed here allowed the characterization, for the first time, of the organic composition of bioglea and an analysis of its biological properties. An important result is the identification of a significant lipidic component similar to that of the mature mud, which confirms the fundamental role of bioglea in the mud's maturation.

In spite of the wide popularity of spa treatments, few studies have assessed the biological activity of hot thermal spring cyanobacteria. The good antioxidant properties mean that bioglea from Saturnia spa are suitable for use in the preparation of cosmetic products

Judging from the results and the composition, we can consider bioglea a "multiactive ingredient" and, besides the activity we report for some extracts, it might also be worth investigating the moisturising, photoprotective, antiaging, etc. properties.

This work can therefore provide a useful outline for examining new natural raw materials for use in cosmetic products, and for topical treatments, exploiting their composition, and hence their activity. This is a highly topical subject in view of the growing demand for research and development of raw materials of natural origin. Their sustainability is to be welcomed in all areas, particularly cosmetics.

The bioglea is certainly an important source of ingredients with biological activity that could be used in the dermo-cosmetological field. Further research will be needed in order to make the best use of its potential.

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