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Characterization of cyanobacterial mats from an artificial hot spring in Uniejów (Poland) and the potential use of their biomass

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ABSTRACT

Artificial geothermal water systems are an efficient and low-cost alternative to natural ecosystems for phototrophic microorganism biomass production on an industrial scale. Our study focused on the investigation of mat-forming cyanobacteria produced in an artificial pool with a geothermal water source from a hot spring in Uniejów, Poland. The microorganisms inhabiting this ecosystem tolerate the high salinity (approximately 50 ‰) and temperature (45-55.2 °C) of the water. The structure, composition, and growth rates of the cyanobacterial mats were investigated under natural and laboratory conditions. We tested whether cyanobacteria harvested from this habitat represent a safe source of vital biomolecules for industrial applications. We found that the layered mats consisted of simple filamentous cyanobacteria, mainly of the genera *Leptolyngbya, Thermoleptolyngbya,* and *Anagnostidinema.* In the isolated cyanobacteria, we did not detect commonly studied cyanotoxins (i.e., ATX-a, BMAA, CYN, MC, and SXT) that could pose a direct risk to human health and lead to indirect risks through the contamination of bioproducts. The extracts and filtrates of the strains did not reduce the survival of *Daphnia*. In addition, we found that temperatures of 40-50 °C and pH values of 7.2-7.7 were optimal for mat formation and the growth of the dominant cyanobacteria. In the case of the *Desertifilum dzianense* strain, the highest biomass yield was noted at 26 °C. In summary, our study indicates that mat-forming cyanobacteria inhabiting ecosystems powered by geothermal waters from the Uniejów hot spring have strong potential as bioresources for different industrial applications.

1. Introduction

Cyanobacteria are among a limited number of photosynthetic organisms that can form masses under extreme conditions. These photosynthetic prokaryotes played a key role in the evolution of life and in early Earth [1]. At present, cyanobacteria are found in all types of ecosystems and play important roles. In aquatic environments, they are responsible for primary production together with other components of the phytoplankton community. Due to numerous adaptations that have resulted in a wide range of ecological tolerances to environmental factors, aquatic cyanobacteria are strong competitors that are frequently able to dominate communities, reduce water clarity and form massive water blooms, which are harmful, especially in eutrophic and degraded

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Abbreviations

Aps	anabaenopeptins
ATX-a	anatoxin-a
BMAA	β-methylamino-L-alanine
CYN	cylindrospermopsin
ELISA	Enzyme-Linked Immunosorbent Assay
FAs	fatty acids
EPSs	exopolysaccharides
HD	high density
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
MCs	microcystins
MC-LR	microcystin-LR
MC-RR	microcystin-RR
MC-YR	microcystin-YR
NOD	nodularin
OA	oleic acid
PA	palmitic acid
PCR	polymerase chain reaction
PUFAs	polyunsaturated fatty acids
PVC	polyvinyl chloride
STXs	saxitoxins
TPA	*trans*-palmitoleic acid
WC	Wright's cryptophyte
UFAs	unsaturated fatty acids

waters [2]. In terrestrial environments, they are a part of biological soil crusts and act as stabilizers of soil particles; they store carbon and nutrients; and they regulate water infiltration and runoff water cycles. Moreover, they can interact with the organs of growing plants and supply them with nutrients and consequently enhance their growth [3,4]. In turn, nontoxic strains of terrestrial cyanobacteria are considered promising organisms for the restoration of degraded land areas [5].

Numerous species of cyanobacteria are extremophiles that are able to endure harsh conditions in which most microorganisms are unable to thrive due to the inhospitable nature of such habitats. They can be found as pioneers of bioengineering both in cold glacial ecosystems [6,7] and in extremely hot environments such as hot springs, volcanic soils or reservoirs [8,9]. Extremophiles are a "black box" for scientists because their taxonomic identity, diversity and mechanisms leading to high ecological tolerance to environmental extrema are still mostly unknown. The diversity of meso/thermophilic cyanobacteria with temperature preferences ranging from ~36 to ~68 °C can be quite high, as observed in hot springs in Bulgaria [10], Iran [11] and other ecoregions [12]. Thermophilic cyanobacteria can thrive at extremely high temperatures due to (i) morphological adaptations, such as the ability of some cyanobacteria to form sheaths [13,14]; (ii) their ability to synthesize and exude exopolysaccharides (EPSs) from cells to prevent desiccation [15]; (iii) the presence of thermostable enzymes [16]; and (iv) a variety of molecular adaptations that enhance photosynthesis, metabolism and transduction of stress signals [17]. The unique properties of cyanobacteria that have evolved at high temperatures make these organisms extremely valuable for basic and applied sciences. They can be promising, nature-oriented tools for many biotechnological applications, including the production of bioactive compounds, biofuels, pigments and bioremediation. [18,19]. Thermophilic cyanobacteria that are usable for biotechnological applications are usually thermophiles that occur in unique and often difficult-to-reach sites, e.g., springs in protected areas or volcanic lakes. However, artificial outlets of geothermal water on the ground surface made by geothermal power plants might constitute a cost-effective and accessible route for the acquisition and indoor/outdoor cultivation of cyanothermophiles for biotechnological purposes. This can be achieved in numerous countries worldwide where geothermal resources are widely accessible [20,21].

In this study, we demonstrate the results of phycological and ecotoxicological exploration of thermotolerant cyanobacterial communities (cyanobacterial bioconsortia) that form unique dense mats on the bottom of an artificial outdoor pool built in Uniejów, Poland. Specifically, we measured the physicochemical parameters of the released geothermal water, characterized the structure and morphometry of the microbial mat in the pool, assessed the factors facilitating mat formation in the field, and tracked the mat formation process at different thermal regimes in a mat-forming cyanobacterial strain isolated from the pool. The microbial mat samples from the field and cyanobacteria isolates were screened for the presence of several common toxic cyanometabolites (i.e., anabaenopeptins, anatoxin-a, β-methylamino-L-alanine, cylindrospermopsin, microcystins, nodularin, and saxitoxins) and genes responsible for the synthesis of anatoxin-a, cylindrospermopsin and microcystins (*ana*F, *cyr*J, and *mcy*E, respectively). We also studied the survival of *Daphnia* in the presence of filtered extracts and filtrates of cyanobacterial strains. Our data provide extensive insight into the ecology and biotechnological potential of the mat-forming cyanobacteria isolated from the studied geothermal pool.

2. Materials and methods

2.1. Sampling site

The mineral-rich geothermal hot spring is situated in Uniejów (Poland, Łódzkie Province), approximately 170 km to the west of Warsaw. In this area, the deposits of thermal waters are located mainly at depths of 1.7-2.2 km in Lower Cretaceous sandstones [22]. Geothermal water (with temperature ranging from 67 to 70 °C and a mineral level of 8 g/L) from depths above 2.031 m rises to the surface through borehole PIG/AGH-2, with a capacity of 120 m^3/h [23–25]. Interestingly, old geological layers rich in salts, which strongly mineralize the groundwater, are also preserved at this depth. They are the remains of the Zechstein Sea that existed 250 million years ago [26,27]. Therefore, high chloride concentrations ($Cl^- > 3.6$ g/L) and sodium concentrations ($Na^+ > 2.3$ g/L) have been recorded in the geothermal waters of Uniejów [24]. The thermal water is discharged into an artificial system on the surface that constitutes the research site (Fig. 1). This water system is occupied by cyanobacteria, which are the main focus of this research. The research site consists of an artificial pool (cooling tank) with a depth of approximately 1.6 m and an area of 1340 m^2. The walls and bottom of the tank are lined with thick black PVC foil. Currently, this pool allows the geothermal water to cool before it is discharged into the Warta River (Fig. 1).

2.2. Field research and samples

When macroscopic microbial mats formed at the research site (Fig. 1), they were sampled along with the water samples. At the examined site, the mats persisted for a substantial period of time and were sampled repeatedly from April to September (during the period from 2017 to 2019). During transport to the laboratory, the cyanobacterial mats (placed in PVC bags) were stored in a heated container.

Water samples immediately above the cyanobacterial mats were collected using 1.0-l sterile plastic bottles (Roth, Karlsruhe, Germany). In the field, the water samples were filtered through a plastic sieve to eliminate organic contamination (e.g., leaves of vascular plants). Subsequently, the 1.0-L water samples were placed two sterile 0.5-L high-density polyethylene plastic containers (Roth, Karlsruhe, Germany). The first subsample (0.5 L) was preserved using 0.5 mL of chloroform

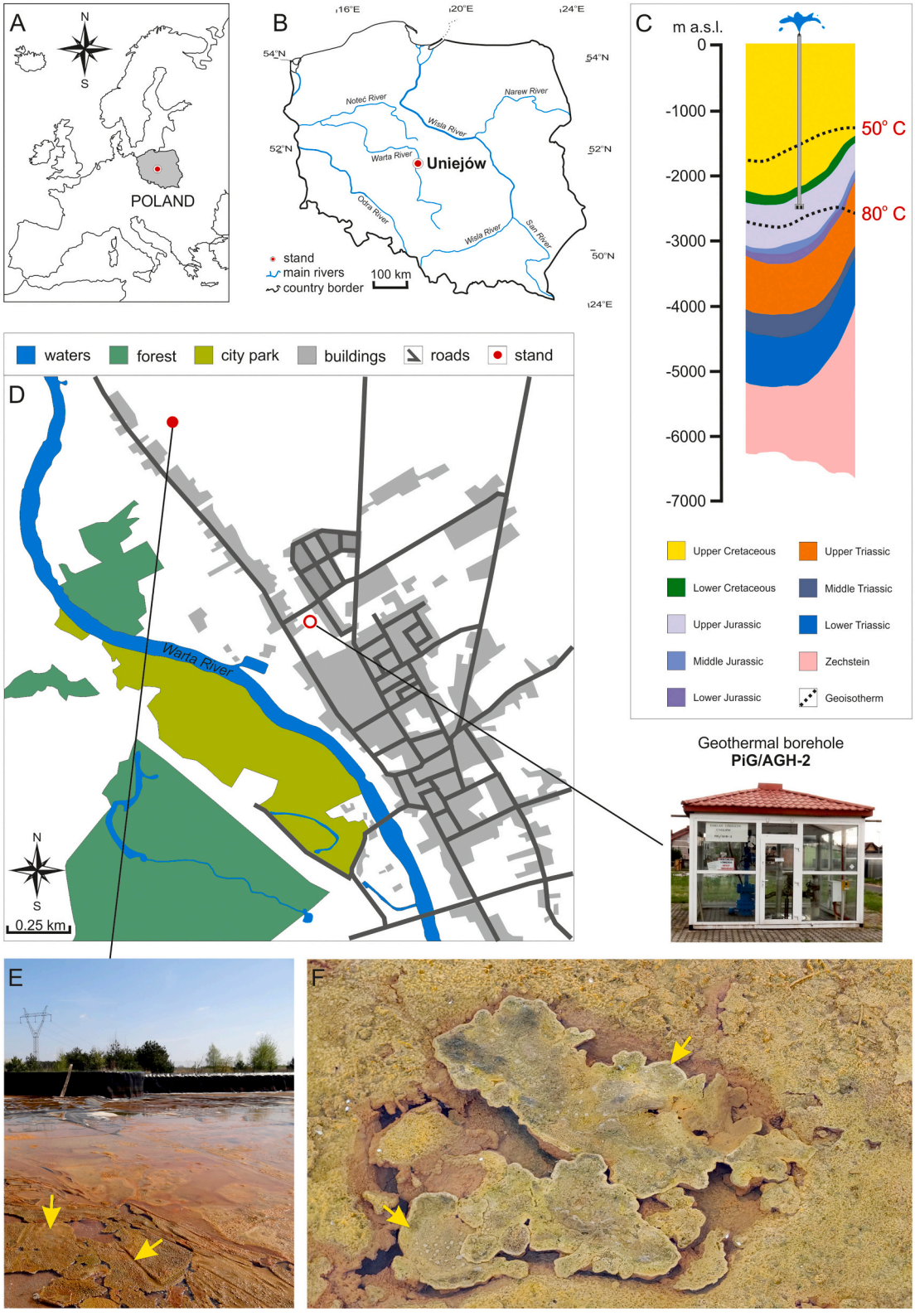

Fig. 1. Hot spring in Uniejów, location on the map of Europe (A) and Poland (B); C – geological cross-section through the geothermal borehole (PiG/AGH-2), D – sampling location, filled red dot – studied pool, unfilled red dot – geothermal borehole; E and F – shallow pool with geothermal water covered by dense cyanobacterial mats of various shapes and sizes (yellow arrows). Photo credit: A.S. Rybak. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Sigma–Aldrich, Seelze, Germany) and cooled to 4 °C, and the second subsample of water (0.5 L) meant for the chemical analyses was preserved by adding 5 mL of ultrapure 15 % HCl (Sigma–Aldrich, Seelze, Germany). At the laboratory, all preserved samples were passed through a microbiological filter with a pore size of 0.45 μm (Sartorius, Germany) and stored in a freezer at -20 °C for further chemical analyses.

2.3. Physicochemical properties of the water

In the field, water temperature, pH, and conductivity were measured using a Professional Plus multiparameter instrument (YSI, Yellow Springs, OH, USA). Water depth was measured using a plastic staff gauge. Water velocity was measured at the surface layer (above the cyanobacterial mats) using a hydrometric universal current meter and at the point of discharge and outflow of geothermal water into the pool. In the laboratory, the chloride (Cl^-) content in samples of geothermal water from the research site where cyanobacteria formed mats was determined by a titration (argentometric) technique based on ISO 9297:1989 using silver nitrate with chromate as the indicator (commonly known as Mohr's method) [28]. Sulfates (SO_4^{2-}) were determined by a gravimetric method involving the precipitation of sulfate (VI) ions with barium chloride according to the ISO 9280:2002 guidelines. The concentrations of nutrients such as total phosphorus (TP), nitrate nitrogen ($N\text{-}NO_3^-$) and ammonium nitrogen ($N\text{-}NH_4^+$) were determined using a UV-1601 spectrophotometer (Shimadzu, Japan) in accordance with standard methods (ISO 6878:2006, PN-82/C-04576/08 and PN-76/C-04576/01). During standard solution preparation, high sodium chloride concentrations were maintained, which were recorded in the tested field samples. In turn, the concentrations of metals in water samples were determined using an ICP–OES optical emission spectrometry method on an Agilent 5100 ICP–OES spectrometer (Agilent, USA).

2.4. Assessment of cyanobacterial mat growth at the sampling site

Analysis of cyanobacterial mats formed along a temperature gradient was performed *in situ*. The layout of the research site, i.e., a shallow large pool with a flat bottom made of thick PVC foil and a shallow depth of water, allowed us to observe the formation of large mats under a constant supply of geothermal water (Fig. A.4). The samples of the mats were taken from 30 subsites, from the point of direct discharge of water where the highest temperatures were recorded (approx. 55 °C) to the points where the thermal water gradually cooled (the lowest measured water temperature was approximately 26 °C). Water parameters such as depth, temperature, pH, salinity and conductivity were measured in all subsites using a Professional Plus multiparameter instrument (YSI, Yellow Springs, OH, USA). A microbial mat of varying thickness and surface area of 100 cm^2 was cut at each subsite using a plastic spatula. The thickness of the mats was also measured in the field using a digital caliper, model 31C628 (Topex). Samples of cyanobacterial mats ($n = 30$) collected from the pool were placed in separate HD PVC bags and then in a polystyrene container. The samples were then transported to the laboratory. The biometric parameters of the mats, such as the fresh and dry weights, percentage of water content, and amount of organic and inorganic matter, were measured. Following the PN-88/B-04481 procedure, each cyanobacterial mat sample was dried in glass Petri dishes at 105 °C for 24 h using a drying oven with air circulation (model SLW 15 ECO, POL-EKO) until a constant weight was reached. The dried samples were crushed with an agate mortar and pestle. Then, they were ground using a laboratory mill (model SM-450 L, MRC). All samples were stored in a desiccator prior to further analysis. The content of organic substances in each sample was determined using the mass loss on ignition method at 440 °C for 4 h [29]. Thermal decomposition of organic substances was carried out using a L9/11/B510 muffle furnace (Nabertherm).

2.5. Morphological assessment

The morphology of the microbial mats and cyanobacterial composition were assessed within two days after sampling in the field. Macroscopic observations were performed using Stemi 305 and Stemi DV4 stereomicroscopes (Zeiss, Oberkochen, Germany). The species composition was analyzed using an Axioplan 2 light microscope (Zeiss, Oberkochen, Germany). Photographs of fresh and preserved samples were taken with a Jenoptic Gryphax AVIOR digital microscope camera (Jena, Germany).

2.6. Morphological taxonomic identification, nomenclature and eco-classification

The initial identification of phototrophic organisms from the Uniejów artificial hot spring was based on the examination of morphological features and literature reports [12,30–41]. The nomenclature of cyanobacterial and algal taxa listed here was verified based on AlgaeBase [42]. Cyanobacteria were classified as non-thermophilic (with a thermal optimum <35 °C), mesophilic (between 35 and 45 °C), or thermophilic (> 45 °C) according to their Castenholz ranking [43–45].

2.7. Molecular taxonomic identification

Biological material for DNA isolation was obtained from a cyanobacterial mat collected from a research stand. Genetic material was extracted according to Giovannoni et al. [46] with some modifications, which improved the quality and quantity of the extracted DNA, as described by Mankiewicz-Boczek et al. [47]. For centrifugation, a rate of 13,000 ×g instead of 10,000 ×g was used. For the enzymatic lysis step, a final proteinase K (Fermentas, Lithuania) concentration of 275 μg/mL instead of 160 μg/mL was used. During the phenol/chloroform step, a volume of chloroform/isoamyl alcohol (24:1) equal to the volume of the supernatant was used.

The analysis was performed using a cyanobacterial gene fragment of 16S rRNA, with the primer pair Cyano 16S F (forward): CGGACGGGTGAGTAACGCGTG, and Cyano 16S R (reverse): CCCATTGCGGAAAATTCCCC. The primer set amplified a PCR product of 258 bp [48]. The amplification reaction was carried out in a 20 μL mixture containing approximately 100 ng of DNA template, 1× PCR buffer, 3 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 μM of each primer, 0.1 mg/L BSA and 1.25 U of Pfu Taq polymerase (Thermo Scientific). The PCR program, performed in an Eppendorf gradient mastercycler, started with an initial denaturation at 95 °C for 10 min. Subsequently, 26 cycles of denaturation at 94 °C for 10 s, primer annealing at 58 °C for 30 s, and extension at 70 °C for 1 min were performed. The reaction was terminated by a final extension at 72 °C for 10 min. Prior to sequence analysis, the PCR products were separated and excised in a 1.5 % agarose gel incubated in ethidium bromide and purified with a QIAEX II® Gel Extraction Kit according to the manufacturer's instructions. The purified products were sent to and sequenced by Genomed® laboratories in Warsaw, Poland [49]. The assembled DNA sequence was edited and aligned using the software CLC Main Workbench 8.0.1 (Qiagen). A basic local alignment search tool (BLAST) was used to verify gene homology in the NCBI database [50]. The 16S rRNA sequence was deposited in GenBank under accession number OR044717.

2.8. Fatty acid extraction and analysis

The fatty acid composition was estimated in samples of cyanobacterial mats colonizing an artificial pool containing hot mineral water (Fig. 1). In this pool, filamentous cyanobacteria had the greatest surface coverage and reached considerable biomass. Such biomass production was made possible by the stable environmental conditions prevalent, including the constant geothermal water supply at an average temperature of 38.7 °C and salinity of 50 ‰ (Table 1). After collection, the mats

Table 1
Physicochemical profile of water habitat colonized by cyanobacteria. Data are means ($n = 4$).

Parameter	Units	
Temperature	°C	38.70
pH	–	7.09
Conductivity	mS/cm	11.55
Salinity	‰	50.82
Depth of water	cm	4-5
Cl^-	mg/l	3020
SO_4^{2-}	mg/l	66.9
Na^+	mg/l	1670
K^+	mg/l	29.8
Ba^{2+}	mg/l	0.044
Li^+	mg/l	0.193
Ca^{2+}	mg/l	98.9
Mg^{2+}	mg/l	26.0
Fe^{3+}	mg/l	0.080
Mn^{2+}	mg/l	0.167
Cd^{2+}	mg/l	<0.001
Co^{2+}	mg/l	0.006
Cr^{3+}	mg/l	<0.001
Cu^{2+}	mg/l	0.002
Ni^{2+}	mg/l	<0.001
Pb^{2+}	mg/l	0.039
Sb^{2+}	mg/l	0.007
Se^{2-}	mg/l	0.043
Sn^{4+}	mg/l	0.004
Sr^{2+}	mg/l	5.80
Zn^{2+}	mg/l	<0.001
Al^{3+}	mg/l	0.077
TP	mg/l	0.014
$N\text{-}NO_3$	mg/l	0.705
$N\text{-}NH_4^+$	mg/l	1.73

TP - total phosphorus.

were transported to the laboratory within 2 h, where they were further processed. During transport, the fresh material was stored in a thermal container with heating, and the temperature was maintained at 35 °C. Before fatty acid (FA) content analysis, the samples of cyanobacterial mats were freeze-dried in a LyoQuest 85 Arctic apparatus (Telstar, USA). The obtained material was ground in a mortar using an agate pestle. Approximately 0.5 g of the sample (± 0.1 mg) was placed in a round-bottomed flask, and 5 mL of Folsch solution (chloroform-methanol 2:1 v/v) was added [51]. The mixture was sonicated for 30 min and then heated to reflux for 2 h. The solid components of the mixture were filtered and washed with Folsch solution (5 × 1 mL). The solution was evaporated on a rotary evaporator at 45 °C, and the residue was dried under vacuum. The weight of the obtained dry extract was approximately 3 mg. Then, 0.5 mL of tert-butyl methoxymethyl ether, 0.25 mL of trimethylsulfonium hydroxide solution (0.25 M in methanol) and 10 μL of internal standard (methyl undecanoate; 24.2 mg in 10 mL of tert-butyl methoxymethyl ether) were added to the material. The analyses were performed on a Varian 450-GC gas chromatograph (Bruker, USA). One microliter of the sample, prepared according to the procedure described below, was introduced into the injection chamber. The injection chamber temperature was 250 °C; helium was used as the carrier gas (with a flow of 1 mL/min^{-1}) in splitless mode (1:50); the column was HP-INNOWAX (30 m × 0.53 mm, film with a thickness of 1 μm) (Agilent J&W, USA); the temperature program was as follows: isothermal at 50 °C for 2 min, linear gradient of 10 °C/min^{-1} to 240 °C (20 min), and isothermal at 240 °C for 22 min. A flame ionization detector (FID) at a temperature of 250 °C was used. Chromatographic grade standards of FAs (Sigma, USA) were used for tentative identification based on a comparison of retention times. Each analysis was repeated three times. The detection limit of FAs in the sample was 0.2 mg/L.

2.9. Molecular analysis of cyanobacterial toxigenicity

The cyanobacterial mat samples formed in the geothermal water of the pool were also genetically analyzed for their cyanobacterial toxigenic potential to produce microcystins (*mcy*E gene), cylindrospermopsin (*cyr*J gene), or anatoxins (*ana*F gene). The method for DNA isolation is described in Section 2.7.

The extracted DNA was used as the template for qualitative polymerase chain reaction (PCR) analyses of *mcy*E (405 bp), a gene fragment universal in microcystin-producing cyanobacteria; *cyr*J (578 bp), a gene fragment universal in cylindrospermopsin-producing cyanobacteria; and *ana*F (467 bp), a gene fragment universal in anatoxin-producing cyanobacteria. The characteristics of these genes and the primers used for their amplification are listed in Table 2. The conditions for PCR amplification of the present toxigenicity genes were described in several publications [48,52–54].

2.10. Cyanotoxin analysis

2.10.1. Detection of cyanotoxins in microbial mats from the Uniejów geothermal pool

For the determination of cyanotoxins (microcystins MCs, cylindrospermopsin CYN and anatoxin-a ATX-a), 1.5 mL of fresh cyanobacterial scum was extracted by sonication for 15 s in a Misonix (Farmingdale, NY, USA) ultrasonicator with an ultrasonic liquid processor XL. The extract was then centrifuged at 11000 ×*g* for 10 min at 4 °C in an Eppendorf 5804 centrifuge (Germany). A volume of 400 μL of the supernatant was collected for the MCs and for CYN and ATX-a and was evaporated to dryness in an SC110A Speedvac® Plus (ThermoSavant, Holbrook, NY, USA). The samples were redissolved in 400 μL of 75 % aqueous methanol for MC analysis and in 400 μL of distilled water for CYN and ATX-a analysis and filtered through a Gelman GHP Acrodisc 13 mm syringe filter with a 0.45 μm GHP membrane and minispike outlet (East Hills, NY, USA).

Chromatographic separation was performed using an Agilent (Waldbronn, Germany) 1100 series HPLC system consisting of a degasser, a quaternary pump, a column compartment thermostat set to 40 °C, and a diode array detector operated at 200-300 nm on a Merck (Darmstadt, Germany) Purospher STAR RP-18e column (55 mm × 4 mm I.D. with 3 μm particles) protected by a 4 mm × 4 mm guard column.

The mobile phase for the CYN and ATX-a analysis consisted of water (solvent A) and acetonitrile (solvent B), both of which contained 0.05 % trifluoroacetic acid. The flow rate was 1.0 mL/min with the following linear gradient program: 0 min, 1 % B; 5 min, 7 % B; 5.1 min, 70 % B; 7 min, 70 % B; 7.1 min, 1 % B; and stop time, 12 min. The injection volume was 20 μL. Cyanotoxins in the samples were identified by comparing the retention time and UV spectrum (200-300 nm), with an absorption maximum at 262 nm for CYN and 227 nm for ATX-a.

The mobile phase for MC analyses consisted of water (solvent A) and acetonitrile (solvent B), both of which contained 0.05 % trifluoroacetic acid. The flow rate was 1 mL/min with the following linear gradient program: 25 % B for 0 min, 70 % B for 5 min, 70 % B for 6 min, and 25 % B for 6.10 min; and the stop time was 9 min. The injection volume was 20 μL. The contents of microcystin-LR (MC-LR), microcystin-YR (MC-YR) and microcystin-RR (MC-RR) in the samples were analyzed by comparing the retention time and UV spectrum (200-300 nm with an absorption maximum at 238 nm).

2.10.2. Analysis of cyanotoxins in cyanobacterial cultures obtained from the Uniejów geothermal pool

The biological material for isolating cyanobacterial cultures was collected from the geothermal pool in 2017 and processed at the Department of Hydrobiology at Adam Mickiewicz in Poznań (Poland) by L. Wejnerowski and M. K. Dziuba. The isolation process involved picking an entangled mass of filaments from the mat and separating them into single filaments using microcapillaries and a serial dilution technique. The isolated cyanobacterial filaments were incubated in 2 mL of WC media at 36 °C. A few reisolations of trichomes from positively growing samples were performed until monoclonal strain cultures were obtained.

Table 2
Molecular markers and sequences of primers used in the present study.

Targeting gene	Primers	Sequence (5' to 3')	Length (bp)	Reference	Annealing temperature (°C)
16S rRNA	Cyano 16SF	CGGACGGGTGAGTAACGCGTG	258	[48]	58
	Cyano 16SR	CCCATTGCGGAAAATTCCCC			
*cyr*J	cynsulfF	ACTTCTCTCCTTTCCCTATC	578	[52]	57
	cylnamR	GAGTGAAAATGCGTAGAACTTG			
*mcy*E	mcyE-R1	ATAGGATGTTTAGAGAGAATTTTTTCCC	405	[53]	59
	mcyE-S1	GGGACGAAAAGATAATCAAGTTAAGG			
*ana*F	atxoaf	TCGGAAGCGCGATCGCAAATCG	467	[54]	60
	atxar	GCTTCCTGAGAAGGTCCGCTAG			

Finally, two strains of filamentous cyanobacteria were obtained: UNIE 1 and UNIE 2. Stock cultures (culture volume ~ 50 mL) of UNIE 1 and UNIE 2 were maintained in 75 cm^3 Nunc cell culture flasks (Thermo Scientific, San Jose, California, USA) filled with 50 mL of WC culture medium [55]. The sources of chloride ions in the medium were $CaCl_2$ and $FeCl_3$. Stock cultures were grown in a phytotron Conviron (Winnipeg, Canada) under a set temperature (20 °C ± 0.5 °C), photoperiod (16:8 light-dark cycle), and light intensity (50 μmol photons/m/s). Manipulations with cultures (e.g., inoculating cultures, supplying fresh medium or collecting samples for observation) were performed in a laminar airflow cabinet CLEAN FLUX O/130 (FoLabo Instruments S.r.l., Buccinasco, Italy). Other researchers who received access to these strains sequenced them and described the UNIE 1 strain as a new species, *Thermoleptolyngbya hindakiae* (correct "*hindakii*", see AlgaeBase [42], and the UNIE 2 strain as a *Desertifilum dziansense* [12].

The concentrated biomass of 21-day-old cultures of each strain was screened for the presence of fourteen toxic cyanometabolites using enzyme-linked immunosorbent assay (ELISA; anabaenopeptins – APs, anatoxin-a – ATX-a, β-methylamino-L-alanine – BMAA, saxitoxins – STXs; Eurofins Abraxis, Warminster, PA, US; Product No. 520070, 520060, 520040, 52255B, respectively), high-performance liquid chromatography with diode array detection (HPLC-DAD; ATX-a, cylindrospermopsin – CYN, microcystins - dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-LY, MC-LW, MC-LF, nodularin – NOD), mass spectrometry (LC–MS; CYN, MCs except the dmMC-LR, and NOD), and a noncompetitive time resolved fluorescence immunoassay (TRFIA; MCs, and NOD).

Sample preparation for ELISA tests and chromatography and the analyses were performed at the Biochemistry, Faculty of Sciences and Engineering, Åbo Akademi University in the same manner as described in Fig. A.1 of Wejnerowski et al. [56]. Identification of cyanometabolites by chromatographic techniques was based on comparing the retention times and absorption spectra of particular compounds (HPLC-DAD) or their retention times and observed m/z values (LC–MS) to those of authentic standards. A noncompetitive time-resolved fluorescence immunoassay (TRFIA) was performed at the Biotechnology branch of the Department of Life Technologies, University of Turku, according to methods in Akter et al. [57] with slight modifications. The assay included a 100 μL reaction mixture per well in which the standard or sample (1:10) volume was 25 μL/well. MC-LR (National Research Council Canada) was used as a standard.

2.11. Assessment of the toxicity of the UNIE 1 and UNIE 2 strains

An approximately 5 mm^2 fragment of the cyanobacterial mat of the UNIE 1 and 2 strains was inoculated into separate 175 cm^2 cell culture flasks (Nunc™ EasYFlask™, Thermo Scientific, San Jose, California, USA) filled with 500 mL of WC media [55]. The flasks were maintained in a culture room at 20 °C ± 0.5 °C with a 16:8 light-dark cycle and a light intensity of 50 μmol photons/m/s. After 40 days of incubation, the cultures were sampled for toxicity assessment, and chlorophyll-a and pheophytin concentrations were estimated (method PN-ISO 10260:2002). We also measured the concentration of nutrients in the medium from 40-day-old cultures using a flow injection analyzer (FIA compact, MLE, Germany). Samples of soluble reactive phosphorus (PO_4^{3-}) and dissolved nitrogen ($NO_2^- + NO_3^-$ and NH_4^+) were analyzed after filtration through membrane filters (0.45 μm, Sartorius) according to ISO 15681-1:2005, ISO 13395:1996 and ISO 11732:2005, respectively. Total phosphorus (TP) was analyzed according to ISO 15681-1:2003. Digestion was carried out in an optimized version of the suggested procedure given in ISO 15681-1:2003. The total nitrogen (TN) analysis method was based on ISO 29441:2010.

The toxicity assessment was based on the *Daphnia magna* clone BDeM2, which originated from Cerbin's library of *Daphnia* clones at the Department of Hydrobiology at Adam Mickiewicz University in Poznań, and the *Daphnia pulicaria* clone DpBrH, which originated from a library of *Daphnia* clones held at the Department of Hydrobiology at the University of Warsaw. Stock cultures of *Daphnia* were maintained in glass jars filled with 500 mL of filtered and conditioned water from Kakskerranjärvi Lake (southwestern Finland; GPS coordinates: 60°21'54.25" N, 22°14'7.971" E). The cultures were incubated in a phytotron chamber at 20 °C and a light intensity of ~20 μmol photons/m/s and fed the green alga *Acutodesmus obliquus* strain SAG 276-3a thrice a week at a concentration far exceeding a level limiting early growth. In the biotests, we evaluated the effect of filtered extracts and filtrates from UNIE 1 and UNIE 2 on the survival of daphnids. The filtered extracts were obtained from 100 mL of the cyanobacterial cultures by triple freeze-thawing and sonication for 15 min (Sonorex RK156, Banelin electronics, Berlin, Germany). Then, the extracts were filtered through GF/C filters. Filtrates were obtained by filtration of 100 mL of cyanobacterial culture through GF/C filters, and no sonication or freezing processes were applied. The prepared liquids were maintained at -20 °C until analysis. Biotests were conducted in glass vials on animals not older than 48 h. Five cohorts (10 animals per cohort) of each *Daphnia* species were exposed for 48 h to either 7 mL of filtered extract or 7 mL of filtrate from each cyanobacterial strain. Five cohorts of each *Daphnia* clone were exposed to 7 mL of freshly prepared WC media in which cyanobacteria were cultured as a control in the biotests. Filtered extracts and filtrates of cyanobacteria and the control medium were aerated by bubbling air through the solution for 15 min before the start of the test. We did not feed the organisms during the test. After 48 h, the number of living and dead daphnids was counted in all vials using a stereoscopic microscope. Based on the collected data, we calculated the percentage survival of each *Daphnia* cohort.

2.12. Assessment of the productivity of the UNIE 2 strain at different thermal regimes

The biovolume-based productivity of strain UNIE 2 was checked at three temperatures (20, 26, and 36 °C). Each treatment consisted of six replicates. Incubation was initiated from single trichomes in the wells. Single filaments were picked from 3-week-old stock cultures and transferred separately to individual wells of a 6-well plate (Orange Scientific, Belgium) filled with 10 mL of WC media. Well plates with UNIE 2 isolates were placed in separate aquaria filled with distilled water. The water temperatures in the water baths were adjusted with 0.25 °C precision by using water heaters with temperature controllers. The isolates were incubated for three weeks under a 16:8 light-dark

cycle and a light intensity of 50 μmol photons/m/s. After 12 days, 1 mL samples were taken from the wells, the filaments were counted, and morphometric measurements were performed (20 measurements of trichome width and 30 measurements of trichome length in three randomly selected wells of each treatment). Morphometric data were averaged for each treatment and used to calculate trichome volume using a formula for computing the volume of the cylinder. The trichome volume data and trichome counts were then used to calculate the UNIE 2 biovolume using the following formula:

$$\text{Biovolume}\left(\text{B}, \text{mm}^3\ \text{L}^{-1}\right) = \text{trichome volume} \times \text{trichome density}$$

Biovolume-based productivity was used as a proxy for UNIE 2 growth, and it was calculated using the following formula:

$$\text{Productivity}\left(\text{P}, \text{mm}^3\ \text{day}^{-1}\right) = (\text{B}_2 - \text{B}_1) \times (t_2 - t_1)^{-1}$$

where B_1 and B_2 are the biovolumes of cyanobacteria on days t_1 (start of incubation) and t_2 (end of incubation), respectively. After three weeks of incubation, we took photos of the mats formed by UNIE 2 in the wells. Microscopic analyses were conducted and photographs were taken using a Leica DM IL LED light inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Jenoptik ProgRes Speed Xtcore3 digital camera and ProgRes image capture software (Jenoptik Optical Systems, Jena, Germany).

2.13. Statistical analyses

Statistical analyses were performed and graphs were generated using R software version 4.0.2 [58] and RStudio version 1.3 [59]. The data were visualized using the cowplot [60], ggplot2 [61], and gridExtra [62] R packages.

Ordination methods were used to determine the relationships between physicochemical factors and the biometric parameters of the cyanobacterial mats. Cyanobacteria–environment relationships were explored through redundancy analysis (RDA) [63]. Due to the skewed distributions of most variables, a square root transformation was performed. To reduce the number of variables, a forward selection procedure using the Monte Carlo test with 999 permutations was applied (variables were discarded until a significance threshold of $p < 0.05$ was reached). Variables with significance levels below $p < 0.05$ were passively projected into diagrams. Ordination was performed using the CANOCO software package [64]. Changes in the formation of cyanobacterial mats and their occurrence in habitats in response to major environmental factors were modeled using generalized additive models [65]; we used the Poisson distribution, while smooth term complexity was selected using the Akaike information criterion (AIC) [66].

One-way analysis of variance (ANOVA) was applied to test the effect of temperature on the biomass productivity of the UNIE 2 strain. Model assumptions were checked visually (by generating histograms of model residuals, residual versus fitted value plots and normal Q–Q plots) and tested with formal tests (homogeneity of variances via Levene's test using the package car [67], normality based on Shapiro–Wilk's test). The data were square-root-transformed to improve the distribution. After transformation, the data were analyzed by ANOVA.

3. Results

3.1. Habitat and structure of cyanobacterial mats

3.1.1. Environmental conditions

The geothermal water that springs directly from the borehole (GPS coordinates: 51°58'49.0" N, 18°47'12.0" E) had a constant temperature of approximately 68 °C and was colorless, with a salty taste and a slight petroleum-like smell. The water temperature at the research site (GPS coordinates: 51°59'16.0" N, 18°46'40.8" E) with cyanobacterial mats ranged from 26.8 °C (in places approximately 20 m from the discharge of geothermal water into the pool) to 55.0 °C (approximately 0.5 m from the discharge point of water; Table 3). In the subsites within the pool (Fig. 1), the pH values ranged from 6.73 to 8.27. The water current through the pool was constant in each year of study and ranged from 3.88 m^3/s directly in the pool's geothermal water flow to 0.62 m^3/s at the outflow (Fig. A.4).

The average salinity and conductivity were 56.1 ‰ and 11.26 mS/cm, respectively. The water was characterized by high chloride (3020 mg/L) and sodium concentrations (1670 mg/L). Among the biogenic elements, forms of inorganic nitrogen ($N\text{-}NO_3$ = 0.71; $N\text{-}NH_4$ = 1.73 mg/L) showed the highest levels. The phosphorus concentration was 0.014 mg/L. In terms of other essential elements for life processes, high concentrations of calcium, potassium and magnesium were noted (Table 1). It is worth highlighting the presence of micronutrients such as iron and manganese, which, together with cobalt and selenium, are also essential growth stimulators.

3.1.2. Formation of cyanobacterial mats

The morphology of the microbial mats of the Uniejów water (eco) system included several types: (i) sponge-like orange mats immersed in water; (ii) sponge-like green mats immersed in water; (iii) overlapping green mats immersed in water with a lumpy-clumpy or homogeneous structure; and (iv) mats with a pillow-sponge structure emerging above the water surface (Fig. 2A–C).

In the artificial pool, the mats formed large, flat underwater surfaces with areas of convex, pillow-spongy structures emerging above the water surface. The outer layer of the emerged surfaces of the mats was consistently orange, while the submerged layer was consistently green (Fig. 2A, B). The bottom layer was dark green or black and was loosely attached to the substrate. The mats of all types had high porosity (Fig. 2D). The numerous "tunnels" penetrating through the mat layers were the result of the release of oxygen bubbles produced during photosynthesis (Fig. 2). The thickness of the mats ranged from 0.5 to 2.5 cm depending on the location of the thermal water discharge. The most compact mats with regular layers were completely underwater and located in the areas of the pool with the highest temperatures, i.e., close to the thermal water discharge point into the pool (Fig. 3). These mats were composed of individual overlapping layers and showed vertical heterogeneity (Fig. 2F and 4A, B). All layers were primarily formed by various bent, densely interwoven cyanobacterial filaments, with mineral particles, mainly iron, manganese and sulfur compounds (Fig. 4C), attached to varying extents to the sheaths (Fig. A2).

The immersed cyanobacterial mats were found in water at depths ranging from 1.5 to 11.5 cm. The differences in parameters among the subsites influenced the structure of the cyanobacterial mats. At temperatures above 50 °C, the mats were thicker and more resilient, while at temperatures below 40 °C, the mats were thinner and had a looser structure. According to the RDA of the bioplots, only two features that characterized the cyanobacterial mats (i.e., dry mineral residue and water content) were most significantly related to the pH gradient, water depth, and electrolytic conductivity. Changes in water salinity had the least influence on the above parameters of the cyanobacterial mats (Fig. 3A). The remaining bioparameters of the mats, such as fresh and dry weight, dry and organic matter content, and mat thickness, were related to the temperature gradient (Table 4). The values of these

Table 3
Physicochemical profile of cyanobacterial microhabitats determined at the biomass sampling subsites ($n = 30$).

Parameters	Mean	Min.	Max.
Depth of water (cm)	7.65	1.50	11.50
Temperature (°C)	42.84	26.80	55.20
pH	7.55	6.73	8.27
Salinity (‰)	50.61	50.07	50.79
Conductivity (mS/cm)	11.26	11.02	11.54

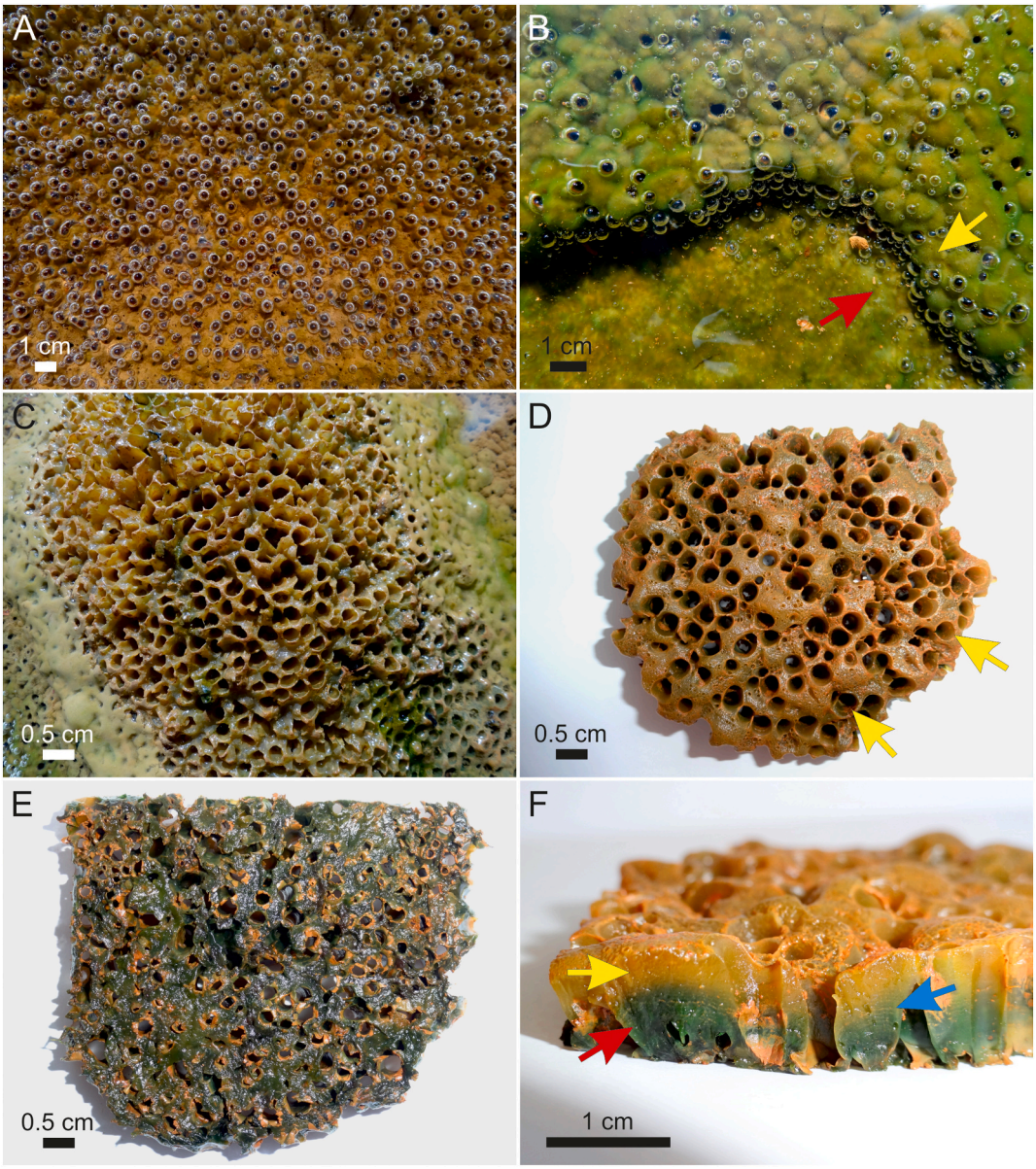

Fig. 2. Macroscopic cyanobacterial mats of the Uniejów hot spring. A – The upper side of the orange mat immersed in water (approximately 2 cm thick homogeneous layer) with numerous visible air bubbles. B – Top view of an overlapping green mat immersed in water. The upper mat shows air bubbles and a lumpy-clumpy structure (yellow arrow). The lower mat is homogeneous with fine air bubbles (red arrow), C – a mat with a pillow-spongy structure emerging above the water surface, D – a mat with a spongy structure removed from the water with visible channels across the mat (yellow arrows), E – the underside of a dark green mat, F – a cross-section of a mat with a visibly orange top layer (yellow arrow) and a green underside layer (red arrow). The blue arrow points to the layered structure of the mat. Scales: 1 cm (A, B and F), 0.5 cm (C–E). Photo credit: A.S. Rybak. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

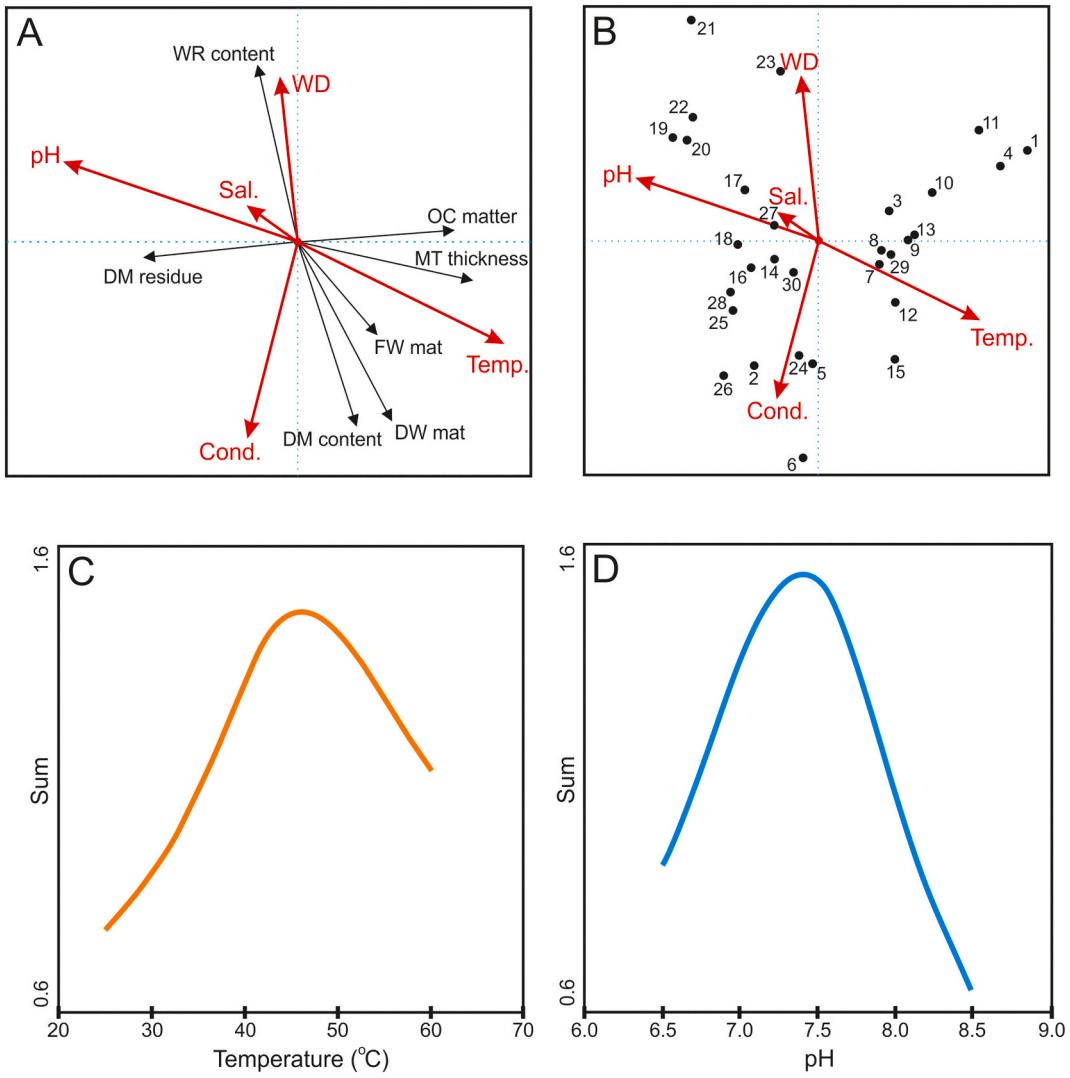

Fig. 3. Redundancy analysis (RDA) biplots for the physicochemical profile of the habitat and parameters of the cyanobacterial mats (A) and for the background of the subsamples ($n = 30$) from the research site (B). Generalized additive models – the response of cyanobacterial mats to two explanatory variables, temperature (C) and pH (D). FW mat – fresh weight of cyanobacterial mat, DW mat – dry weight of cyanobacterial mat, DM content – dry matter content, WR content – water content, DM residue – dry mineral residue, OC matter – organic matter, MT thickness – mat thickness, WD – water depth, Sal. – salinity, Temp. – temperature, Cond. – conductivity.

parameters increased with increasing temperature.

Of all the environmental factors analyzed, water temperature and pH had the greatest influence on the structure and composition of the cyanobacteria in the studied habitat (Monte Carlo permutation test, $p < 0.001$). According to generalized additive models (GAMs), cyanobacterial mats achieved optimum growth in microhabitats (i.e., in the pool) at temperatures ranging from 40 to 50 °C and pH levels ranging from 7.2 to 7.7 (Poisson distribution, $p < 0.001$) (Fig. 3C and D, Table 4).

3.2. Morphological and phylogenetic assessment

3.2.1. Taxonomic characteristics of the bioconsortium

Analysis of the mats revealed that filamentous cyanobacteria were the main biomass-forming group (> 98 %). A total of 6 cyanobacterial taxa were identified in fresh mats (in descending order of contribution to the mat community): *Leptolyngbya* sp. (*Leptolyngbyales*), *Desertifilum dzianense* Cellamare, Charlotte Duval, Touibi, Djediat & Cécile Bernard 2018 (*Desertifilales*), *Anagnostidinema amphibium* (Gomont) Strunecký, Bohunická, J.R. Johansen & Komárek 2017) [syn. *Geitlerinema amphibium* (C. Agardh ex Gomont) Anagnostidis (*Coleofasciculales*)], *Desertifilum fontinale* Dadheech, H. Mahmoud, Kotut, K. & Krienitz 2014, *Thermoleptolyngbya hindakiae* Panou, Kokocinski & Gkelis 2022 (*Oculatellales*), *Oxynema acuminatum* (Gomont) Chatchawan, Komárek, Strunecký, Smarda & Peerapornpisal 2012 (syn. *Phormidium acuminatum* (Gomont) Anagnostidis & Komárek 1988), and *Chroococcus* sp. (*Chroococcales*) (Fig. 5A–E). The taxonomic composition of cyanobacteria in the mats was constant through all months and years of sampling. The filaments of the cyanobacterium *Leptolyngbya* sp. were straight, flexuous, sometimes arcuated or undulated, immotile with a sheath, and slightly constricted at the cross-wall, with cells 1.5–3 times longer than wide without aerotopes and a cell width of 1.3–1.8 μm. Trichomes of *A. amphibium* were found primarily in the surface layer of the mat, and loose trichomes were recorded in the water above the mat.

Observed diatoms, such as *Navicula cryptotenella, Halamphora tenerrima, Crenotia angustior* and *Nitzschia palea,* together with coccal cyanobacteria and green algae, constituted <2 % of the mat biomass (Fig. A.2). They occurred only in colder places, where they could grow in company of cyanobacteria, as diatoms have definitely low tolerance to high temperatures.

Two different isolates of cyanobacteria (Fig. 5F–H) were obtained

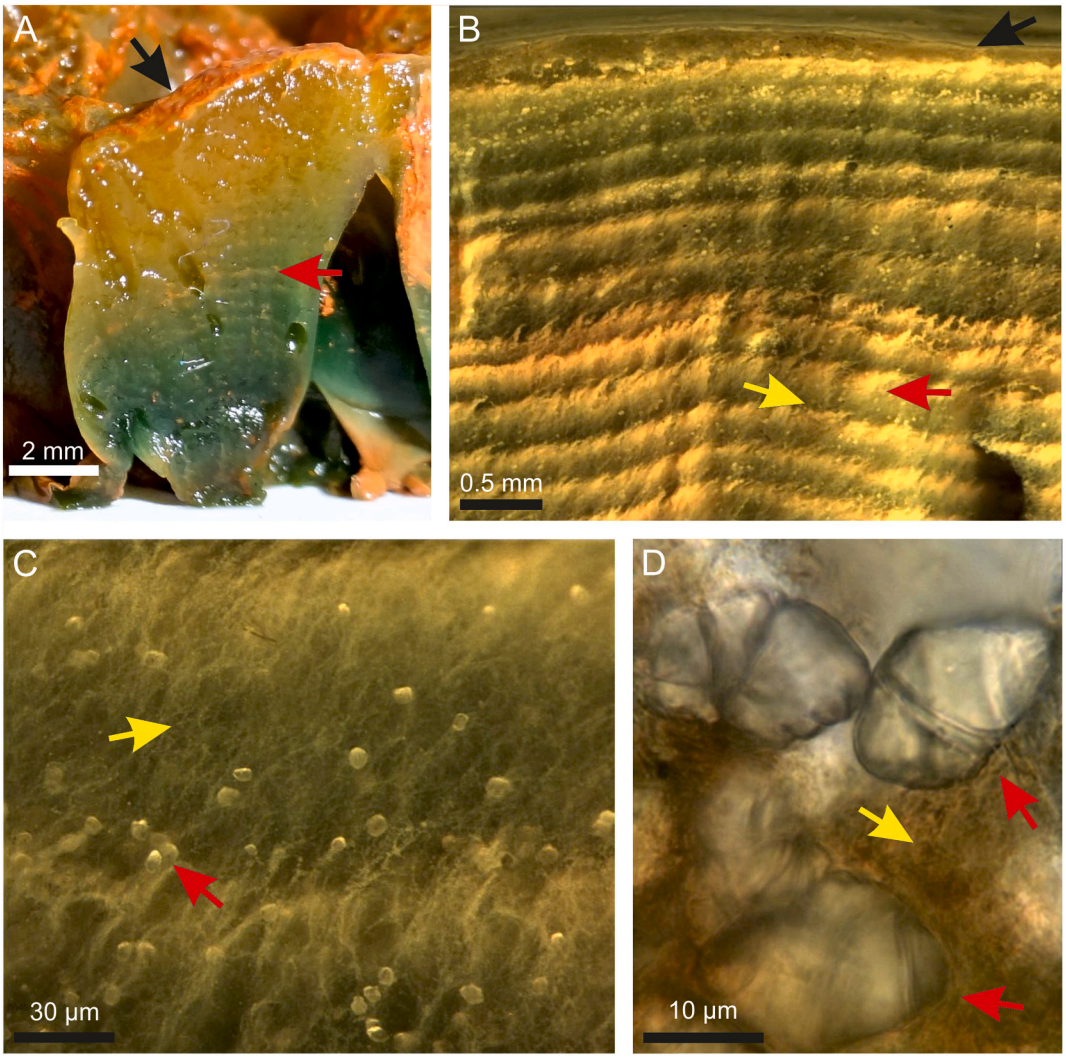

Fig. 4. Cross-sections from a fresh cyanobacterial mat. A – Layered structure of the mat visible to the naked eye. The black arrow indicates the surface, and the red arrow indicates one mineral layer with an orange colour. B – Details of the layered arrangement of cyanobacteria and precipitated mineral crystals (yellow arrow - dark layer dominated by cyanobacteria, red arrow - light layer dominated by mineral compounds). C – Magnification of one layer with visible cyanobacteria (yellow arrow) and crystals (red arrow). D – Precipitation of mineral salts in the form of crystals (red arrow) and amorphous forms (yellow arrow). Scales: 2 mm (A), 0.5 mm (B), 30 µm (C), and 10 µm (D). Photo credits: A: A.S. Rybak; B – D: A. Hindáková. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Parameters of cyanobacterial mats from the research site (n = 30).

Parameters	Mean	Min.	Max.
Fresh weight of cyanobacteria mat (kg/m^2)	7.27	2.02	12.37
Dry weight of cyanobacteria mat (kg/m^2)	1.26	0.15	2.20
Dry matter content (%)	17.07	4.85	30.51
Water content (%)	82.93	69.49	95.15
Dry mineral residue (g/kg/dry weight)	881.67	731.79	942.05
Organic matter (g/kg/dry weight)	137.76	61.51	366.52
Mat thickness (cm)	1.83	0.50	2.50

from the Uniejów mats: UNIE 1 was identified as another species of the genus *Thermoleptolyngbya* and described as *T. hindakiae*, and UNIE 2 was determined to be *Desertifilum dzianense* [12].

3.2.2. Molecular data

Sequence analysis of 16S rRNA revealed 97 % homology (query cover of 100 %) between the studied sample from the mat and the strains *Leptolyngbya* sp. S2C11 (accession no. KJ654313), *Leptolyngbya* sp. D4C9 (accession no. KJ654312) and *Geitlerinema* sp. D5C17 (accession no. KJ654310). Recently, published strains of *Thermoleptolyngbya hindakiae* UNIE1 (accession no. OK623654 and NR_176596), which were also isolated from the geothermal mats in Uniejów, showed the highest homology to the studied sample (99.3 %); however, the query coverage of the BLAST analysis was as low as 62.3 %. Differences occurred in the above results because different molecular markers (primers) were used to amplify the 16S rRNA gene (Fig. A.3).

3.3. Fatty acid (FA) composition in the cyanobacterial mats

The presence of 9 saturated and 4 unsaturated FAs was detected in the cyanobacterial mat samples. The FA compositions of lipids from the examined mats are shown in Table 5. The predominant FAs were oleic (C18:1 (*n*-9)), palmitic (16:0) and *trans*-palmitoleic acids (C16:1 (*n*-7)). The lowest levels of FAs were recorded for decanoic (C10:0), myristoleic (C14:1 (*n-5*)), and lauric (C12:0) acids. The fatty acid composition did not vary among subsamples, with relatively high quantities of saturated fatty acids. The oleic (OA), palmitic (PA) and *trans*-palmitoleic acids (TPA) in the cyanobacterial mats accounted for >36 %, > 31 % and > 26 %, respectively, of the total fatty acid content.

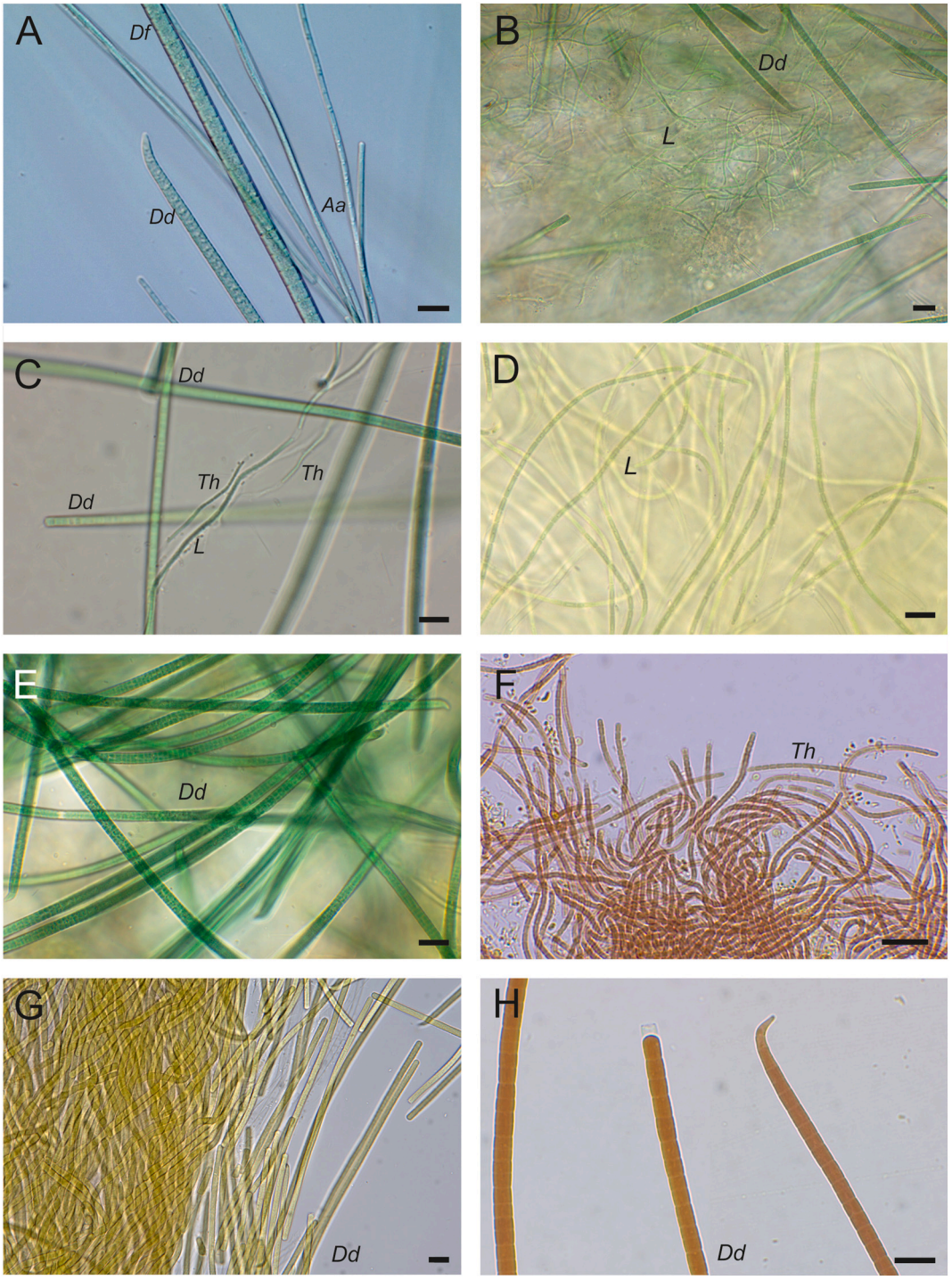

Fig. 5. LM micrographs of cyanobacteria that formed fresh mats (A–E) and isolated cyanobacterial strains: UNIE 1 - *Thermoleptolyngbya hindakiae* (F) and UNIE 2 - *Desertifilum dzianense* (G, H). Abbreviations: *Dd - Desertifilum dzianense*, *Df – Desertifilum fontinale*, *Aa – Anagnostidinema amphibium* (syn. *Geitlerinema amphibium*), *L – Leptolyngbya* sp., *Th - Thermoleptolyngbya hindakiae*. Scale bars: 10 μm. Photo credit: A. Pełechata.

3.4. Toxicological assessment and cyanotoxin contents

The cyanobacterial mat samples investigated in the present study did not demonstrate toxigenic properties in terms of their potential to produce cyanotoxins from the main group, represented by microcystins, cylindrospermopsin or anatoxins, as confirmed by the absence of the *mcy*E, *cyr*J or *ana*F genes, respectively. No MCs, CYN or ATX-a were identified in the collected material. Our analyses indicated that the tested cyanobacterial mats do not threaten the aquatic environment or humans via cyanotoxin production.

3.5. Toxicological screening of the UNIE 1 and UNIE 2 strains and assessment of their toxicity based on Daphnia biotests

ELISA revealed the presence of anabaenopeptins and a lack of anatoxin-a, β-methylamino-L-alanine and saxitoxins in the examined strains (Fig. A.1.). HPLC-DAD and LC–MS analyses confirmed the lack of anatoxin-a and did not reveal cylindrospermopsin, microcystins, or

Table 5
Fatty acid composition and amounts in cyanobacterial mats from the geothermal system in Uniejów. The number of replicates is $n = 3$ (the highest mean values for each FA are shown in bold, and the lowest mean values are underlined). 1 ppm = 0.0001 %.

	ppm			%		
Fatty acid	Min.	Max.	Mean	Min.	Max.	Mean
C10:0 decanoic acid	0.3	0.7	0.47	0.02	0.04	0.03
C12:0 lauric acid	1.7	1.9	1.83	0.1	0.11	0.10
C14:0 myristic acid	6.8	9.8	8.70	0.4	0.55	0.50
C14:1 (*n-5*) myristoleic acid	0.6	1.1	0.87	0.04	0.06	0.05
C15:0 pentadecylic acid	4.8	5.6	5.17	0.28	0.31	0.29
C16:0 palmitic acids	540.1	555	**547.60**	30.94	32.12	**31.41**
C16:1 (*n-7*) *trans*-palmitoleic acid	447	476.3	**459.90**	26.21	26.56	**26.37**
C17:0 margaric acid	4.9	6.9	5.83	0.29	0.4	0.34
C18:0 stearic acid	42.6	43.9	43.17	2.4	2.54	2.48
C18:1 (*n-9*) oleic acid	619.2	655	**635.63**	36.32	36.52	**36.45**
C18:2 (*n-6*) linoleic acid	8	9.3	8.83	0.45	0.54	0.51
C20:0 arachidic acid	7.3	13.4	10.77	0.43	0.74	0.61
C22:0 behenic acid	10.7	18.7	15.17	0.63	1.04	0.87

Table 6
The results of screening of the mesophilic cyanobacterial strains for the presence of certain toxic metabolites. The symbols (+) and (–) indicate that a given cyanometabolite was or was not detected, respectively. Other symbols indicate methods used for the detection of cyanometabolites: Ý – ELISA immunoassay, ¥ – LFIA immunoassay, and Λ – chromatographic techniques.

Cyanobacterium	Cyanometabolite						
	Aps	ATX-a	BMAA	CYN	MCs	NOD	STXs
T. hindakiae strain UNIE 1	+ Ý	– ÝΛ	– Ý	– Λ	– ¥Λ	– ¥Λ	– Ý
D. dzianense strain UNIE 2	+ Ý	– ÝΛ	– Ý	– Λ	– ¥Λ	– ¥Λ	– Ý

Aps - anabaenopeptins, ATX-a - anatoxin-a, BMAA - β-methylamino-L-alanine, CYN - cylindrospermopsin, MCs - microcystins, NOD - nodularin, STXs – saxitoxins.

nodularin in the samples from these strains. The TRFIA confirmed the absence of microcystins and nodularin in the UNIE 1 and UNIE 2 strains. A summary of the results of the toxicological screening of the strains is provided in Table 6, while the details are presented in Fig. A.1.

The biomasses of the UNIE 1 and UNIE 2 strains, expressed as the chlorophyll-*a* concentration, after 40 days of incubation in *Daphnia* biotests were 184.19 and 766.88 μg/L, respectively. The values of several parameters in freshly prepared culture media and filtrates of 40-day-old UNIE 1 and UNIE 2 cultures are outlined in Table 7.

The survival of the *D. magna* and *D. pulicaria* animal cohorts after 48 h of exposure to the freshly prepared medium (control) was 100 %. The exposure of *D. magna* cohorts for 48 h to filtered extracts of UNIE 1 and UNIE 2 reduced animal survival to 82 % ±17.8 % (mean ± standard deviation) and 96 % ±5.4 %, respectively. The survival rates of *D. magna* in the cohorts after 48 h of exposure to the UNIE 1 and UNIE 2 filtrates were 96 % ±8.9 % and 100 %, respectively. In the case of *D. pulicaria*, all the experimental animals survived the 48-h exposure to the filtered extract and filtrate of each strain. During the inspection of vials with experimental animals using a magnifier and further observation of daphnids using a stereoscopic microscope at the end of incubation, we did not observe any inhibition of the swimming activity of living animals in the control medium or in the filtered cyanobacterial extracts or filtrates.

3.6. Productivity of UNIE 2 strains at different temperature regimes

Temperature had a significant influence on the biovolume-based productivity of the UNIE 2 strain (ANOVA: $F_{2,14} = 78.14$, $p < 0.001$). Incubation at 26 °C resulted in the highest productivity in UNIE 2 (mean = 0.0019 $mm^3\ L^{-1}\ day^{-1}$), which was significantly greater than that at 20 °C (mean = 0.00012 $mm^3\ L^{-1}\ day^{-1}$; Tukey's HSD test: $p < 0.0001$) and 36 °C (mean = 0.00044 $mm^3\ L^{-1}\ day^{-1}$; Tukey's HSD test: $p < 0.0001$) (Fig. 6). The productivity of UNIE 2 incubated at 36 °C was significantly greater than that at 20 °C (Tukey's HSD test: $p = 0.004$). Photographs of the growing strains at two monitoring times (12 days and 21 days) are presented in Fig. 7.

4. Discussion

4.1. Hot springs as an extreme habitat for cyanobacteria

Natural or artificial hot springs are thermal systems powered by geothermally heated groundwaters and are located worldwide in the Americas, Africa, Asia, Europe, and Australia, as well as on islands such as Iceland or New Zealand [68–72]. This fact influenced the widespread use of thermal waters in medicine (balneotherapy) and for recreation in antiquity by communities living in present-day Greece, Rome, Turkey, and Japan [73,74]. This demand remains to date, and modern technology enables deep mining boreholes to be made for geothermal water

Table 7
Biomass yield of the examined cyanobacterial strains and values of several culture medium parameters measured after 40 days of incubation at 20 °C and in freshly prepared culture medium.

Variable	Freshly prepared culture medium	*T. hindakiae* (strain UNIE 1)	*D. dzianense* (strain UNIE 2)
Chlorophyll-*a* (μg/l)	–	184.19	766.88
Pheophytin (μg/l)	–	8.87	136.92
pH	7.5	7.83	8.43
N-NO_3 + NO_2 (mg/l)	14.0	12.2	6.79
N-NH_4 (mg/l)	0.0	0.06	0.09
TN (mg/l)	26.0	19.45	14.22
P-PO_4 (mg/l)	2.2	1.31	0.82
TP (mg/l)	2.2	1.44	0.82

N - nitrogen, P - phosphorus, TN - total nitrogen, TP - total phosphorus.

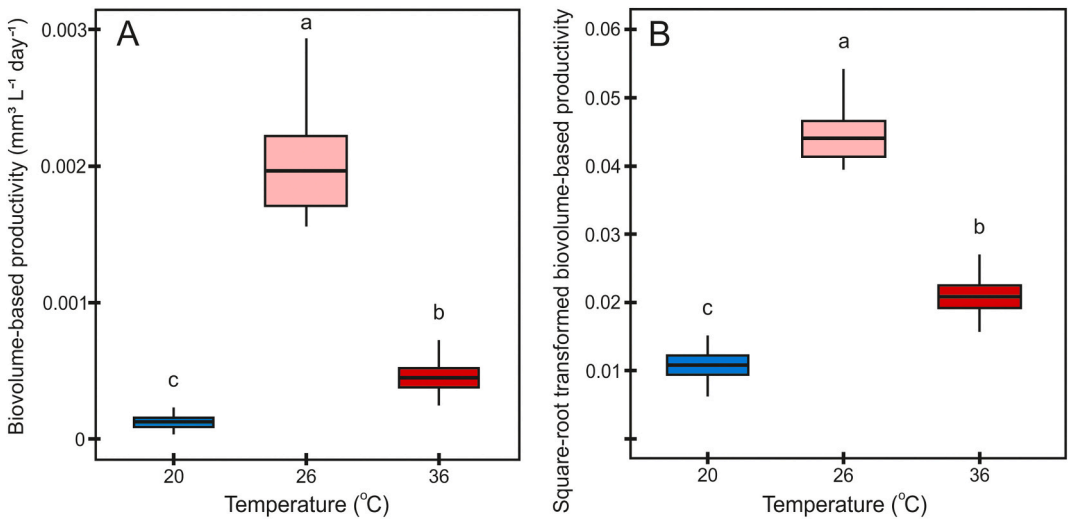

Fig. 6. Biovolume-based productivity (A – untransformed data, B – square-root-transformed data) of the strain UNIE 2 - *Desertifilum dzianense* after 12 days of incubation in WC media under three thermal regimes (20, 26, and 36 °C). Incubation was started with a single trichome of length 200-300 μm. The means, standard errors, minima, and maxima are shown. The letters above the whiskers indicate homogenous groups revealed by post hoc Tukey's honestly significant difference (HSD) test for pairwise comparisons ($p < 0.05$).

deposits that do not naturally rise to the Earth's surface [75]. Thus, it is possible to create artificial hot springs and other hydro/ecosystems powered by geothermal waters, e.g., water reservoirs and channels.

The chemical composition of geothermal waters largely depends on the geological origin of the rocks and on whether the water arises and outflows naturally or through artificial, deeply drilled boreholes. The water chemical parameters are also correlated with the reaction of water with minerals deposited in rocks/sediments [70]. The pH of geothermal waters ranges from very acidic (pH < 1) to strongly alkaline (pH > 10). The chemical composition of water from hot springs is often dominated by sulfates, carbonates, or silicates [73,76–78]. Based on the chemical composition of the thermal water from the Uniejów hot spring, it is classified as the Cl−Na type, indicating that it originated from deep aquifer structures [79]. Particular attention is given to the fact that the water from many hot springs can be highly radioactive, while others show radioactivity levels no different from that of normal groundwater [11,73,80,81]. The temperature of this type of water, which can range from 30 °C to >100 °C, is regarded as the most crucial determinant [73]. The water temperature in the artificial hot spring in Uniejów is constant and reaches approximately 70 °C at the point of outflow to the surface. On the other hand, global data analysis indicates that hot springs often have water temperatures ranging from 40 to 50 °C, with a median value of 42 °C [72]. However, the water temperature between individual hot springs located in small areas (e.g., Icelandic, Yellowstone or Yunnan hot springs) can vary significantly due to geological conditions, hydrostatic pressure and other factors [82,83]. The high content of mineral compounds influences the salinity and electrolytic conductivity and, together with temperature, allows for the classification of this habitat as extreme.

The specificity of habitat conditions affects the types of organisms inhabiting hot springs. Thus, many organisms classified as extremophiles can be found there, i.e., organisms that tolerate at least one environmental parameter that reaches extremely high/low values, e.g., temperature, salinity, or hydrostatic pressure [84,85]. These extremophile representatives belong primarily to the domains Bacteria and Archaea, with only a few examples of those in Eukarya (e.g., algae, fungi, and protozoa) [86]. Living organisms recorded in hot springs are classified into different ecological groups that achieve optimal growth along a temperature gradient. In this type of ecosystem, thermophiles (with optimum water temperature between 35 and 70 °C), extreme thermophiles (with optimum temperature 55–80 °C), and hyperthermophiles (with optimum temperature 75–113 °C) can be found [87]. In our case, a complex of cyanobacterial species inhabiting a hot spring in Uniejów developed at a water temperature gradient from 26.8 to 55.2 °C, with optimal *in situ* growth of mats occurring between 40 and 50 °C. According to these data, we can consider this biocomplex of microorganisms to be thermotolerant. However, we found that the cyanobacterial strain UNIE 2 grew fastest at 26 °C (Figs. 6 and 7). These results are in line with a separate study in which the strain UNIE 2 achieved the highest biomass (expressed by growth curves) at temperatures ranging from 20 to 40 °C [12]. On the other hand, in the previous study, the authors presented the order of growth rate of the UNIE 1 strain at different temperatures as 30 > 20 > 40 °C. Our results are consistent with these observations; therefore, we can refer to *Thermoleptolyngbya hindakiae* and *Desertifilum dzianense* as mesophilic species. Interestingly, these two strains, under *ex situ* conditions, achieved the lowest biomass growth at temperatures ranging from 50 to 60 °C [12]. These results may indicate that the cyanobacterial bioconsortium growing in geothermal waters from the Uniejów hot spring is probably more resistant to high temperatures than individual living species. However, further research is needed to elucidate this aspect of interspecies interactions (i.e., protocooperation).

In addition, based on the level of water minerals (5.82 %), the species of cyanobacteria were classified as moderate halophiles. This group of cyanobacteria achieves optimal growth in waters with salinities ranging from 5 to 20 % (0.85–3.4 M NaCl) [88,89]. Cyanobacterial species were found in geothermal habitats with site temperatures up to 74 °C. Most of these species occur at pH > 6.0 and do not fix N_2 [90]. Consequently, cyanobacterial mat formation was facilitated in the waters of Uniejów by the presence of nitrogen and phosphorus ions, as well as micronutrients. A possible obstacle to the survival of organisms could be the concentration of chloride and sodium ions. However, cyanobacteria are resistant to high concentrations of these elements [91,92]. Even under unfavorable environmental conditions, cyanobacteria are able to survive due to physiological adaptation mechanisms (i.e., acclimatization) supported by modifications in gene expression [93].

4.2. Cyanobacterial diversity in the hot springs

The species composition of photo- and chemotrophic microorganisms in mats formed in hot springs can be very diverse and unique. The biota is regulated by different factors, particularly temperature, light,

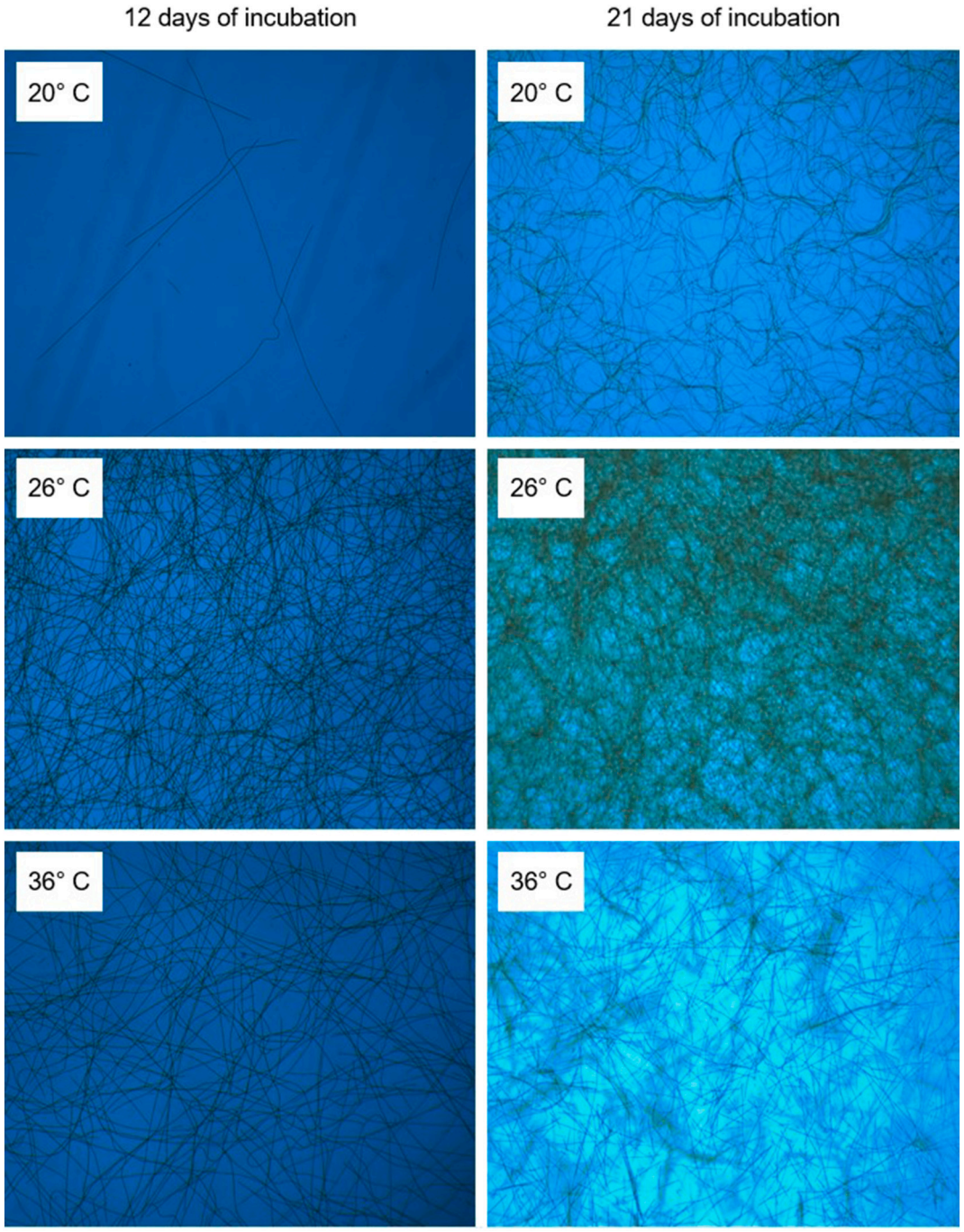

Fig. 7. Micrographs showing the state of the mats of the *D. dzianense* strain UNIE 2 after 12 days (before sampling for microscopy) and 21 days of incubation in WC media at three thermal regimes (20, 26 and 36 °C). Incubation was started with a single trichome of length 200-300 μm. Photo credit: Ł. Wejnerowski.

oxygenation, salinity, and ground structure [94,95]. For this reason, critical internal and external processes (i.e., photosynthesis rate, nitrogen fixation, denitrification, metal/sulfate reduction, and methanogenesis) result in the formation of specific microhabitats in laminated microbial mats. A network of metabolic relationships and biological interactions (e.g., symbiotic, neutral, or amensalistic interactions) between individual species of prokaryotes within the given mat layers is also essential for maintaining the stability of entire bioconsortia [96]. Thus, the species composition of mats can be variable or uniform in single layers. Microbial mats may consist of several hundred or even thousands of bacterial species. Cyanobacteria, as phototrophic organisms, commonly colonize geothermal springs; nevertheless, in some cases, they may be represented by only a few phylotypes/taxa. For example, analysis of microbial mats from the Garga hot springs in the Baikal rift zone, Russia (sulfate-sodium water, 45–70 °C, salinity 1 %, pH 8.1), revealed a similar cyanobacterial species composition with stratification of the mat, with a dominance of representatives of the genus *Leptolyngbya* [97]. In another case, metagenomic analysis of cyanobacterial mats obtained from the Mushroom and Octopus hot springs in Yellowstone, USA (temperature gradient of 60–65 °C), revealed several ecotypes of the genus *Synechococcus*-like in the upper layer of the mats [94,98]. Interestingly, subsequent studies conducted on these hot springs revealed that members of photomixotrophic bacteria from the genus *Roseiflexus* were most common in the microbial community of the mats. This finding indicates the dominance of various taxonomic groups in individual layers of microbial mats.

Representatives of the genus *Leptolyngbya*-like are among the most common filamentous cyanobacteria found in microbial mats in thermal springs. *Leptolyngbya*-dominated mats have been observed in several thermal springs, e.g., in Australia, China, Russia, India, Italy, Romania, Algeria and Chile [82,97,99–105]. According to Keshari et al. [82], *Leptolyngbya*-like is the most diverse genus of cyanobacteria in the thermal springs of Yunnan Province (China). Species of the genus *Leptolyngbya* ssp. were highly thermoadaptive and occurred over a wide temperature range of 38–85 °C. The cyanobacterial community of Uniejów mats had low species diversity. The community structure was comparable to that of mats from the Innot Hot Springs in Australia [99]. In this case, the dominant components of the mat were *Leptolyngbya*-like species, with a low proportion of other species of filamentous cyanobacteria (mainly *Oscillatoriales*-like) and chroococcal cyanobacteria. Unlike the mats from the Innot Hot Spring, we did not observe any heterocytous cyanobacteria in our study pool.

The cyanobacterial mats in the examined Uniejów hot spring also had filaments of *Anagnostidinema amphibium* (syn. *Geitlerinema amphibium*). This species is distributed worldwide and is a mat-forming freshwater or thermal cyanobacterium [106]. It is a permanent component of summer microbial mats in the southern Baltic Sea [107] and has a good ability to acclimatize to dynamically changing environmental conditions [108]. This ability to acclimatize is achieved by changes in pigment content and proportional adjustments in photosynthetic rates, among other mechanisms. In Iran, this species was recorded in thermal springs in the temperature range of 34–57 °C [109], i.e., in a similar range as the geothermal waters in Uniejów in the present study.

It is worth emphasizing that microbial mats can be built by endemic groups or species found only in a given hot spring and nowhere else globally because of the unique habitat conditions of hot springs [110,111]. These findings align with the general concept of endemic taxa as presented by Padisák [112], who noted that endemics adapt to extreme environmental conditions when the rate of evolution is faster than the rate of species diversification. For example, in the Kotel'nikovskii hot spring near Lake Baikal (Russia Federation), researchers discovered a new endemic cyanobacterium of the genus *Pseudanabaena* (named *Pseudanabaena* sp. *nov.*, strain 0411). These organisms inhabit specific niches of hot springs consisting of fluoride–bicarbonate–sodium sulfate water with a salinity of 0.032 % (320 ppm), a pH of 9.5, and a temperature of 80 °C [113]. Even at higher taxonomic levels, such as at the genus level, organisms may be endemic to specific geothermal springs. In the Talaroo hot spring (Australia), a new subaerophytic cyanobacterium was identified and described as the novel genus *Ewamiania* G.B. McGregor & B.C. Sendall 2017. It was reported in the study that an unknown species, *E. thermalis,* developed in a fluoride-sodium-chloride type of water with low salinity (0.083 % = 839 ppm), pH 7.9, and a temperature gradient from 48.5 to 62.7 °C; thus far, this species has not been found anywhere else in the world [114]. *Thermoleptolyngbya hindakiae* (correct "*hindakii*" see AlgaeBase [42]), the species we studied, occurs only in the artificial hot spring in Uniejów, Poland [12]. *Desertifilum dzianense*, another species coexisting in the cyanobacterial mats, was known from a site in Dziani Dzaha Lake on Petite-Terre Island (Comoros archipelago). In an isolated crater lake ecosystem, *D. dzianense* grew as a stromatolite in water with high salinity (> 50 PSU), pH 9–9.5, and temperature 29–35 °C [41,42]. Although the habitat parameters recorded in the Uniejów hot spring were slightly different (i.e., lower salinity and pH or higher temperature), this indicates a wide tolerance range of *D. dzianense* with respect to these environmental parameters. A second species belonging to the genus *Desertifilum*, *D. fontinale,* was recorded in small numbers in mats from Uniejów. Among the cyanobacteria observed, most of which were "thin," it had a considerably larger size (width of filament). This species was previously recorded only in warm springs near Bogoria Lake in Kenya, where it formed freely floating mats [33]. Intriguingly, the known sites of *D. dzianense and D. fontinale* in the Uniejów hot spring and the Dziani Dzaha and Bogoria Lakes are separated by more than six and eight thousand kilometers, respectively. It is unclear how these two species colonized the artificial hot spring in Uniejów. It is likely that unknown sites of *D. dzianense* and *D. fontinale* still exist in Southeast Africa and Asia Minor. Therefore, the organisms present in the Uniejów hot springs might have spread from these sites with the help of vectors, e.g., wetland birds migrating in spring and summer to Europe, such as the white stork (*Ciconia ciconia*), the common crane (*Grus grus*), and others. These two species of water birds fly long distances between Europe and Africa during their annual migrations and, therefore, may be responsible for the spread of microorganisms, including phytoplankton [115,116]. Recent papers on zoochory mechanisms confirmed that phytoplankton can be transported in the excrement of waterbirds over considerable distances [117]. In the paper, the authors showed that cyanobacteria constitute as much as 13 % of the phytoplankton in the excrement of waterbirds. It is also worth noting that cyanobacteria can adhere to the feathers and feet of birds, which increases the possibility of cyanobacterial dispersal in surviving forms (akinetes) and as entire microorganisms during the migration of water birds to wintering sites (i.e., from Europe to Africa) or feeding grounds (from Africa to Europe) [118]. The dispersal of mesophilic cyanobacteria to new sites is not a short-term process; it probably occurs gradually and over the long term, facilitated by local populations of water birds. In the case of *D. dzianense*, expansion mechanisms can involve nomadic birds inhabiting Madagascar, the Comoros Islands, and the eastern parts of Africa, such as lesser (*Phoeniconaias minor*) and greater flamingos (*Phoenicopterus roseus*) [119,120]. Moreover, it was proven that greater flamingos are highly specialized feeders that subsist on microscopic algae, including cyanobacteria from the genera *Arthrospira*, *Oscillatoria*, *Phormidium*, and *Synechococcus* [121]. We assumed that the direction of *D. dzianense* dispersal was from the South–East Africa/Comoros Islands to Central Europe, i.e., from a much older and natural ecosystem (such as a volcanogenic lake on Comoros Island, with an estimated age of 500,000 years) to an artificial (eco)system (powered by geothermal water) built approximately 15 years ago. The same is probably true for the direction and source of *D. fontinale*, i.e., it migrated from an original habitat consisting of a small warm spring on the shoreline of Lake Bogoria in Kenya to Central Europe. Notably, Bogoria Lake hosts many water birds, including millions of lesser flamingos each year [122].

Unfortunately, the natural origin (if it exists) of the cyanobacterium *T. hindakiae* in the Uniejów hot spring is unknown. So far, this species

has only been identified in the examined artificial ecosystem. This mystery may be resolved if *T. hindakiae* is found in other natural and seminatural ecosystems in the Eastern Hemisphere.

4.2.1. Cyanobacterial taxonomy (classical and molecular)

The genetic analysis performed here was in line with classical morphological analyses and confirmed microscopic observations showing the presence of *Leptolyngbya* and *Thermoleptolyngbya* members in the examined cyanobacterial mats from the Uniejów hot spring. According to the sequence analysis of 16S rRNA, the studied samples showed close homology to strains belonging to *Leptolyngbya* (244 bp). However, the highest homology was observed for *Thermoleptolyngbya hindakiae* UNIE1 (144 bp), which was isolated from the studied geothermal hot spring.

4.3. Mat structure

The key organisms in microbial mats are generally cyanobacteria, as they are capable of forming macroscopic structures even under extreme conditions. [123] Cyanobacteria, along with other organisms, have a specific function in adding new layers during mat growth. Due to the process of formation of new layers and the decomposition of old layers, microbial mats are vertically stratified with different functional groups of microorganisms [123]. Cyanobacterial mats are formed by both coccal and filamentous cyanobacteria, among which the genera *Synechococcus*, *Chroococcus*, *Leptolyngbya*, *Thermoleptolyngbya*, *Oscillatoria* and *Phormidium* are the most commonly mentioned in the literature [9,82,124–126]. Due to the potential applications of meso/thermophilic cyanobacteria in biotechnology and industry, intensive research has been conducted in recent years on the structure, physiology and genetics of strains isolated from cyanobacterial mats and hot springs [127]. Notably, the diversity of thermophilic cyanobacteria seems to be severely underestimated. Recent papers have focused on the taxogenomics and systematics of thermophilic cyanobacteria [128–131].

The cyanobacterial mats from Uniejów were composed of filamentous cyanobacteria that were interwoven in various ways with each other. In colder parts of the artificial pool, the conditions were suitable for the survival of diatoms and green algae within this structure/complex. Most of the cyanobacterial filaments were relatively thin (up to 3 μm wide), with morphological characteristics that in the past would very likely have placed them in the genus *Leptolyngbya*. Due to numerous and continuous taxonomic changes based on the polyphasic approach, followed by an updating of the classification of orders and families [42,131], determining the identity of "thin" filamentous cyanobacteria based on light microscopy is rather difficult. Notably, the originally defined genus *Leptolyngbya* appears in the current taxonomic system in several classes: *Pseudanabaenaceae*, *Nodosilineaceae*, *Oculatellaceae*, *Leptolyngbyaceae*, *Trichocoleusaceae*, *Oscillatoriaceae* and *Microcoleaceae* [132]. In addition, recent studies of cyanobacteria inhabiting thermal springs may yield new representatives of the family *Leptolyngbyaceae*, e.g., the new genus *Copellandiella* J.R. Johansen, Kaštovský & M.U. Akagha 2023 with new species, such *as C. thermalis* Kaštovský, J.R. Johansen & M.U. Akagha 2023, and *C. yellowstonensis* J.R. Johansen, Kaštovský & M.U. Akagha 2023 [133].

4.4. Fatty acids from cyanobacteria - composition and applications

The presence of extremely unfavorable environmental conditions for living organisms (typical of ancient Earth 3.5 billion years ago) contributed to several adaptations in thermophilic cyanobacteria at the cellular and molecular levels [134,135]. High temperature mainly affects the stability of cell membranes, causing an increase in membrane fluidity [85]. Thus, to preserve its integrity, the lipid membrane of cyanobacteria contains more saturated and straight chain fatty acids than that of mesophiles (those living in a thermal optimum in the range from 15 to 40 °C) [136,137]. In addition, polyunsaturated fatty acids (PUFAs) are known to protect cyanobacterial cells against oxidative stress by stabilizing free radicals [138]. It has also been observed in these microorganisms that ester bonds link cell membrane lipids to the cell wall, further strengthening the cell membrane [85]. Other known mechanisms of prokaryotic extremophile protection against high temperatures are based on increased stability in proteins through the activity of chaperones and the addition of monovalent and divalent salts to enhance the stability of nucleic acids [139,140].

In recent years, thermotolerant cyanobacteria have been a frequent subject of research, and they have attracted particular interest due to the possibility of using their biomass in agriculture, pharmaceuticals, nutraceuticals, and as a source of biofuel [18,141]. The reason for this popularity is that the cells of these organisms accumulate valuable bioproducts for these industries, such as thermostable enzymes and fatty acids [18,142]. PUFAs are particularly interesting due to their numerous human health benefits (e.g., they act as free radical scavengers) [143]. Interestingly, fluctuations in the proportions of FAs are observed in cyanobacterial cells, mainly associated with changes in habitat temperature [144,145]. This relationship is particularly evident in species occurring both in cold- and warm-water ecosystems, for example, in the cyanobacteria *Arthronema africanum* (Schwabe and Simonsen) Komárek and Lukavsky 1988 [146,147]. Under *ex situ* conditions, the percentage of linolenic acid in the cells of this species decreased from 33 % to 0.5 % as the temperature increased from 16 °C to 46 °C. These observations prove that the cells of *A. africanum* maintain relatively constant cell membrane fluidity by adjusting the FA composition at various temperatures. Notably, in mats built by filamentous cyanobacteria of the genus *Leptolyngbya* sp. found in Arctic ecosystems, a high content of unsaturated fatty acids (UFAs) was found [148]; the percentage of linoleic acid reached an average of 60 %, that of palmitoleic acid reached 10 %, and that of oleic acid reached 10 %. Contrary to these observations, the average percentages of these UFAs in cyanobacterial mats from the Uniejów hot spring were 0.51, 26, and 36 %, respectively (Table 5). Therefore, the composition of these UFAs was different from that of the *Leptolyngbya* mats from cold Arctic waters. Additionally, low levels of linoleic acid were detected in FAs of the filamentous thermophilic cyanobacterium *Phormidium laminosum* Gomont 1892 (strain IPPAS B-407, syn. *Leptolyngbya laminosa* (Gomont) Anagnostidis and Komárek 1988)) [149]; under culture conditions of 47 °C, linoleic acid comprised approximately 6 % of the total FAs, unlike the hexadecenoic acid content, which exceeded 30 %. However, in the case of the cyanobacterial mats from the Uniejów hot spring, the average percentage of decanoic acid among the total FAs was very low at 0.03 %. A clear dominance of one of the unsaturated fatty acids (average percentage among all FAs) was detected in the cyanobacterial mats inhabiting the geothermal waters of Uniejów. Oleic acid achieved the highest average content of >36 %, followed by saturated palmitic acid (> 31 %) and monounsaturated transpalmitoleic acid (> 26 %). This is in line with research performed on two species of thermophilic cyanobacteria from the genus *Synechococcus* (i.e., *S. elongatus* (Nägeli) Nägeli 1849, strains B-267 and *S. vulcanus* J.J. Copeland 1936 (syn. *Thermosynechococcus vulcanus* (J.J. Copeland) H. Katoh, S. Itoh, J.R. Shen and M. Ikeuchi, *nom. inval.* 2001, strain B-453), which were grown at 47 °C, and the accumulation of palmitic acid reached high levels of 53 and 66 wt%, respectively [149]. These observations and our results are confirmed by research performed on the freshwater and mesophilic cyanobacterium *Anacystis nidulans* (Richter) Drouet and Daily, *nom. illeg.* 1952 (syn. *Aphanothece nidulans* P. Richter 1884) [150]. These experimental data also indicate that the FA profile of *A. nidulans* grown at 28 °C is dominated by palmitic acid (on average > 41 %), palmitoleic acid (> 30 %) and oleic acid (11 wt%). These three fatty acids predominate in cyanobacterial mats from the Uniejów hot spring (Table 5). Oleic acid (OA) has value due to several unique properties [151]. This mono-unsaturated omega-9 fatty acid is often used in pharmaceuticals; it emulsifies and stabilizes aerosols. From a medical point of view, oleic acid may inhibit the progression of adrenoleukodystrophy (a disease of the brain and adrenal glands) and

cholesterogenesis, lower blood pressure, and improve memory [152–154]. Oleic acid can be found in various vegetables, and its percentage in cyanobacterial mats from Uniejów is comparable to that in corn oil, where oleic acid constitutes up to 37.4 % of all FAs or palm oil (39 %) [155]. Palmitic acid (PA) is another FA with content above 31 % in the examined cyanobacterial mats from the hot geothermal spring. This biomolecule is the most common saturated FA in animals, plant tissues, and microbial cells [156,157]. Its content in palm oil exceeds 44 %, while the lowest contribution of this FA is recorded in Canola (4 %) and Safflower oils (7 %) [155]. Salts of palmitic acids, such as sodium palmitate, are used to produce cosmetics and industrial mold-releasing agents [158]. However, it should be remembered that consuming large amounts of palmitic acid (commercially available mainly in palm oil) may be associated with an increased risk of coronary heart disease and some tumors [159]. Conversely, uncontrolled endogenic biosynthesis of PA caused by atherosclerosis and neurodegenerative diseases can disrupt the homeostatic balance of FAs regardless of the amount of these FAs in the diet. The last of the three FAs with the highest percentage in the tested mats with cyanobacteria (above 26 % compared to all other analyzed FAs) was *trans*-palmitoleic acid (TPA). This *trans*-monounsaturated fatty acid is an isomer of palmitoleate, and it is mainly used for nutritional purposes [160]. TPA is not synthesized endogenously by human cells and must be obtained from dairy products; therefore, TPA can be used as a biomarker for a dairy-based diet [161–164]. Moreover, clinical studies indicate that high TPA concentrations in blood plasma correlate with decreased insulin resistance, decreased onset of type 2 diabetes disease, and regulated cholesterol metabolism [165]. Unfortunately, this FA has low commercial availability on the market. For this reason, TPA is being synthesized from natural sources containing high amounts of *cis*-palmitoleic acid (*cis*-9 C16:1, or *cis*-C16:1 *n*-7) [161,166].

To realize the potential of the cyanobacterial biomass from Uniejów, subsequent industrial research should be focused on effective extraction and purification methods for bioactive molecules, particularly FAs, such as OA and PA. Notably, due to the very high content of ions and mineral salts in the mats, especially Cl^- and Na^+, using this cyanobacterial biomass without desalination is not recommended as a direct human food source. It must be noted that the biomass of thermal cyanobacteria that can be used in the food industry, e.g., as dietary supplements, must only be derived from strictly controlled breeding conditions (including controlled sanitary conditions). In this work, we studied cyanobacterial mats grown in a fenced-in but not covered pool, where various pollutants could have fallen with dry and wet precipitation. In addition, animals, such as birds, may be vectors of pathogenic organisms.

4.5. Toxicity

Cyanobacterial communities living in extremely hot aquatic ecosystems can also contain genotypes that can exert toxic effects on aquatic biota. Krienitz [167] reported the presence of hepatotoxins such as microcystins and neurotoxins such as anatoxin-a in cyanobacteria-dominated (*Phormidium terebriformis*, *Oscillatoria willei* (syn. *Phormidium willei*), *Spirulina subsalsa* and *Synechococcus bigranulatus*) mat samples from Kenyan hot springs at Lake Bogoria at water temperatures ranging from 35 °C to boiling and linked the mortality of birds occurring at the lake to cyanotoxins. Microcystins were also detected in water samples and cyanobacterial isolates of *Oscillatoria limosa* and *Synechococcus lividus* from hot springs of volcanic origin that play numerous roles for humans in Gazan, Saudi Arabia [168]. The endotoxic potential of cyanobacterial lipopolysaccharides was also found in the studied hot springs and confirmed for both cyanobacterial cell-free water samples as well as mats of cyanobacteria and isolates of several species (e.g., *Calothrix thermalis*, *Chroococcus minor*, *Fischerella thermalis*, *Microcoleus erectiusculus* or *Schizothrix calcicola*; see the full list of species in Mohamed et al. [168]). The potential of cyanobacteria occurring in hot springs to produce toxins was also demonstrated by Moreira et al. [169], who detected the genes responsible for the synthesis of microcystins (*mcy*A, *mcy*E) and cylindrospermopsin (*PS*, *PKS*) in epilithic samples (hot springs in S. Miguel Island, Azores) dominated by the taxa *Pseudanabaena*, *Gloeothece* and *Microcoleus*. In the artificial pool in Uniejów with hot groundwater studied here, the cyanobacterial mats did not show toxigenic potential for the synthesis of the most commonly studied cyanotoxins, including anatoxin-a, cylindrospermopsin and microcystins. Neither these cyanotoxins, saxitoxins nor nodularin, were detected in two strains of cyanobacteria isolated from this thermal pool, *D. dzianense* and *T. hindakiae*. However, in both strains, ELISA revealed the presence of bioactive compounds such as anabaenopeptins. To our knowledge, this is the first evidence of the presence of anabaenopeptins in mesophilic cyanobacterial strains. These bioactive compounds can exhibit inhibitory activity toward carboxypeptidases, phosphatases and proteases [170,171], and in concert with other compounds, they can affect the survivorship of some zooplankton species [172]. Although anabaenopeptins were detected in the strains and despite the high cyanobacterial biomass used to prepare extracts and filtrates for biotesting with daphnids, the survival of the *D. magna* and *D. pulicaria* cohorts was not significantly reduced in response to extracts and filtrates of *D. dzianense* and *T. hindakiae*. Overall, our toxicological survey indicated that human-made outdoor hot hydrosystems can be overgrown by cyanobacteria, which exhibit no, or at least negligible, potential for cyanotoxin production. Therefore, these systems are worth exploring for potentially nontoxic cyanobacterial strains that could be useful for biotechnological applications.

5. Conclusions

The examined hot spring in Uniejów, where a bioconsortium in the form of mats dominated by cyanobacterial species developed, belongs to the Szczecin – Łódź geothermal district. An essential underground water basin is located in this area (where the water temperature does not exceed 70 °C), which accounts for approximately 50 % of all available geothermal energy in Poland [173,174]. Geological surveys show that Poland dominates low-temperature geothermal resources related to Mesozoic sediments, which are between 145 and 66 million years old [175,176]. Currently, in Poland, geothermal source applications involve mainly heating, balneotherapy, recreation, and, to a lesser extent, electricity production. The availability of such a source of energy (outside active volcanic areas, e.g., Iceland) is particularly valuable in context of the global energy crisis and the wars currently raging, for example, in Eastern Europe, which results in the limited or completely blockage of the import of fossil fuels from the Russian Federation. Thus, a country's energy policy should primarily be independent of external factors and based on local fossil fuels and renewable energy sources [177,178]. Thus, in 2022, the Polish government (i.e., The National Fund for Environmental Protection and Water Management) allocated >118 million dollars to develop geothermal energy in small municipalities [179].

Resources related to thermal waters lying at low depths have potential for the local economy [176]. The range of applications of such waters increases substantially when they contain dissolved mineral salts [173,174]. This is the case in Uniejów, where local authorities and companies managing geothermal waters are very active [112]. Based on the mineral-rich thermal waters, an aqua park, SPA, and rehabilitation center were created in the city [180].

Cyanobacteria often constitute a dominant group of organisms in the phycosphere of different types of habitats. Some are evolutionarily programmed to form dense mats on solid substrates or accumulate near the water surface. Cyanobacterial cells contain a wide spectrum of biocompounds with potentially unique properties, making these prokaryotes a "blue–green gold" on the market. Hydrogeothermal springs are a particularly interesting source of cyanobacterial biomass because they facilitate the growth of mats of unique thermohaline/mesohalophilic cyanobacteria. Considering the above facts, our results open

up new possibilities for managing cyanobacterial biomass, which grows quickly in geothermal water discharge locations in Poland. We have proven that blue–green algae that build sponge-shaped mats in systems fed saline geothermal waters from the Uniejów intake do not contain the most common cyanobacterial toxins. Toxicity tests of isolated and identified species of blue–green algae (i.e., *T. hindakiae* and *D. dzianense*) with invertebrates from the genus *Daphnia* were also performed. We found that cyanobacterial mats from geothermal springs in Uniejów are free of cyanotoxins and do not affect the vitality of the two *Daphnia* species. The above results allowed us to conclude that the tested cyanobacterial taxa are safe biomass sources for use in various branches of the economy. Moreover, this biosource increases the ability of the examined cyanobacteria to accumulate valuable bioproducts such as fatty acids.

Informed consent and human/animal rights statements

No conflicts, informed consent, or human or animal rights are applicable to this work.

CRediT authorship contribution statement

Andrzej S. Rybak: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Marcin Dziuba:** Writing – review & editing, Methodology, Investigation. **Aleksandra Pełechata:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Conceptualization. **Michał Rybak:** Software, Methodology, Investigation. **Sultana Akter:** Writing – review & editing, Resources, Methodology, Investigation. **Anna Czerepska:** Investigation. **Tamara Dulić:** Writing – review & editing, Investigation. **Maciej Gąbka:** Resources, Investigation. **Alica Hindáková:** Writing – review & editing, Visualization, Resources, Investigation. **Tomasz Jurczak:** Software, Resources. **Aysu Kendir:** Investigation. **Joanna Mankiewicz-Boczek:** Writing – review & editing, Visualization, Methodology, Investigation. **Jussi Meriluoto:** Writing – review & editing, Software, Resources, Methodology, Investigation. **Łukasz Wejnerowski:** Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data will be made available upon request.

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Appendix A

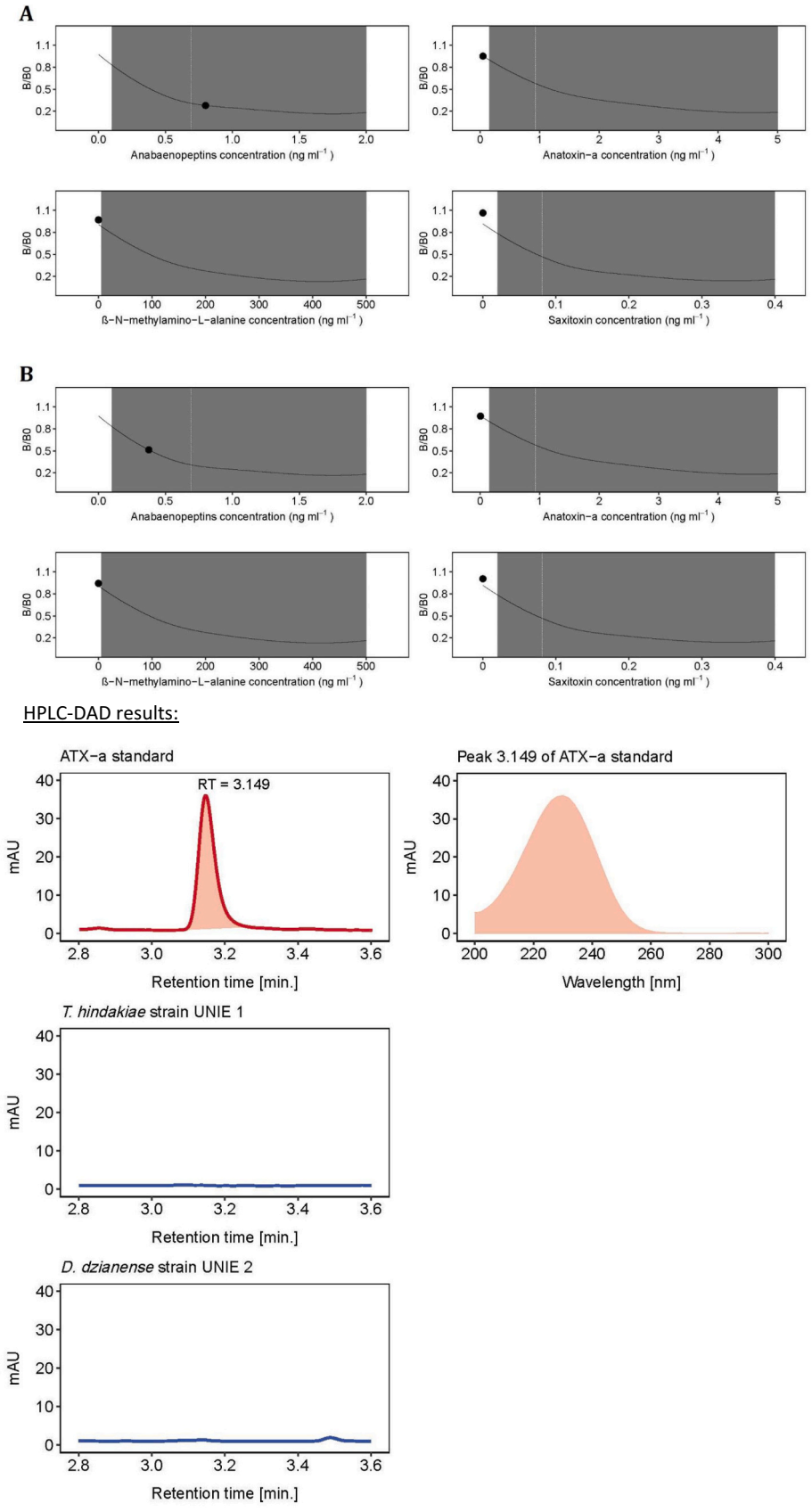
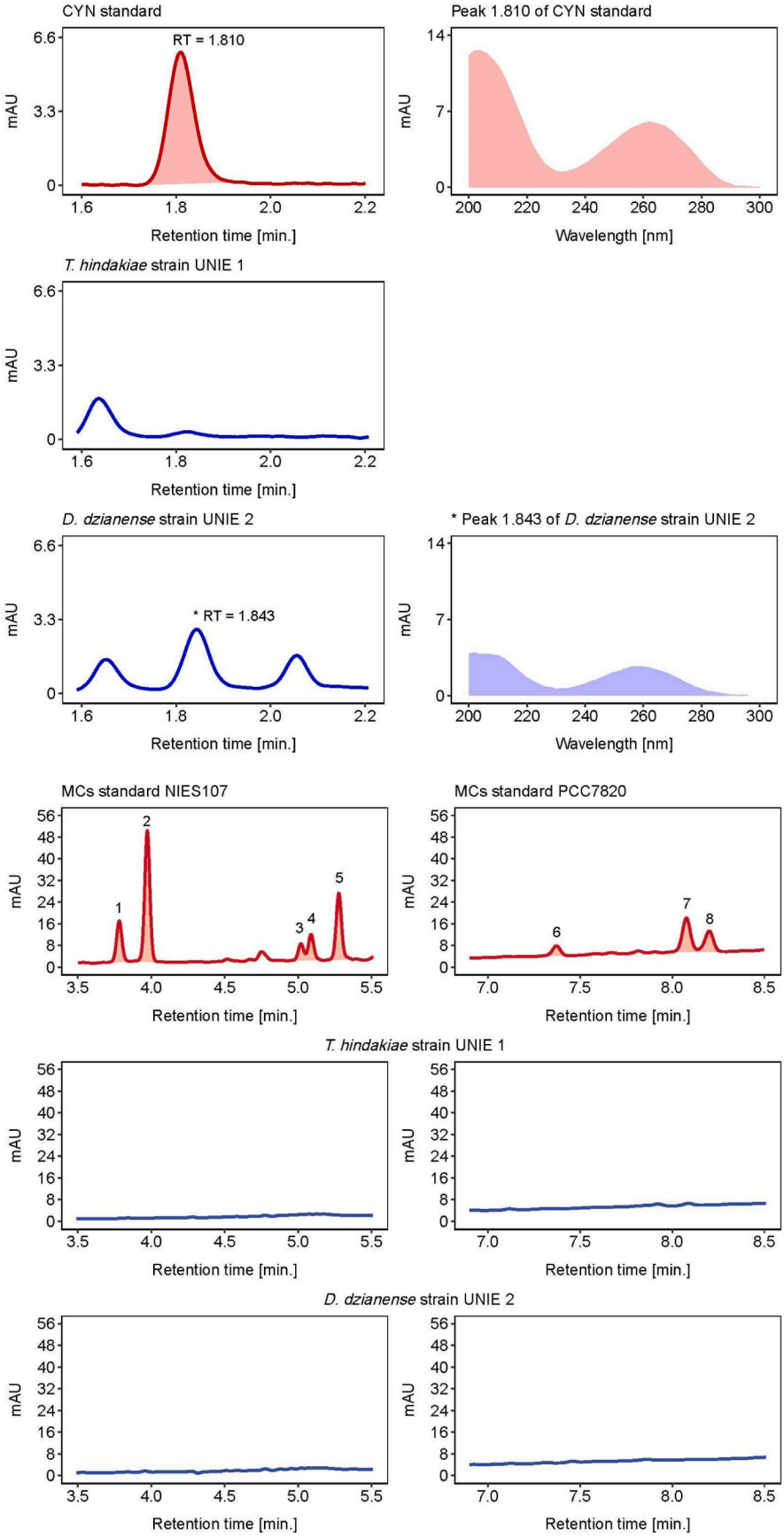


Fig. A1. The results of the toxicological screening of toxic metabolites in *T. hindakiae* and *D. dzianense* strains. ELISA immunoassay: The concentrations of anabaenopeptins, anatoxin-a, BMAA, and saxitoxins (black dots) detected in extracts of *T. hindakiae* strain UNIE 1 (A) and *D. dzianense* strain UNIE 2 (B) by ELISA. The gray area indicates the detection limit for a given cyanometabolite. The black curve is a loess-fitted standard curve constructed based on standards provided by the manufacturer. The white vertical lines indicate the concentration of a given cyanometabolite in the positive controls provided by the manufacturer.



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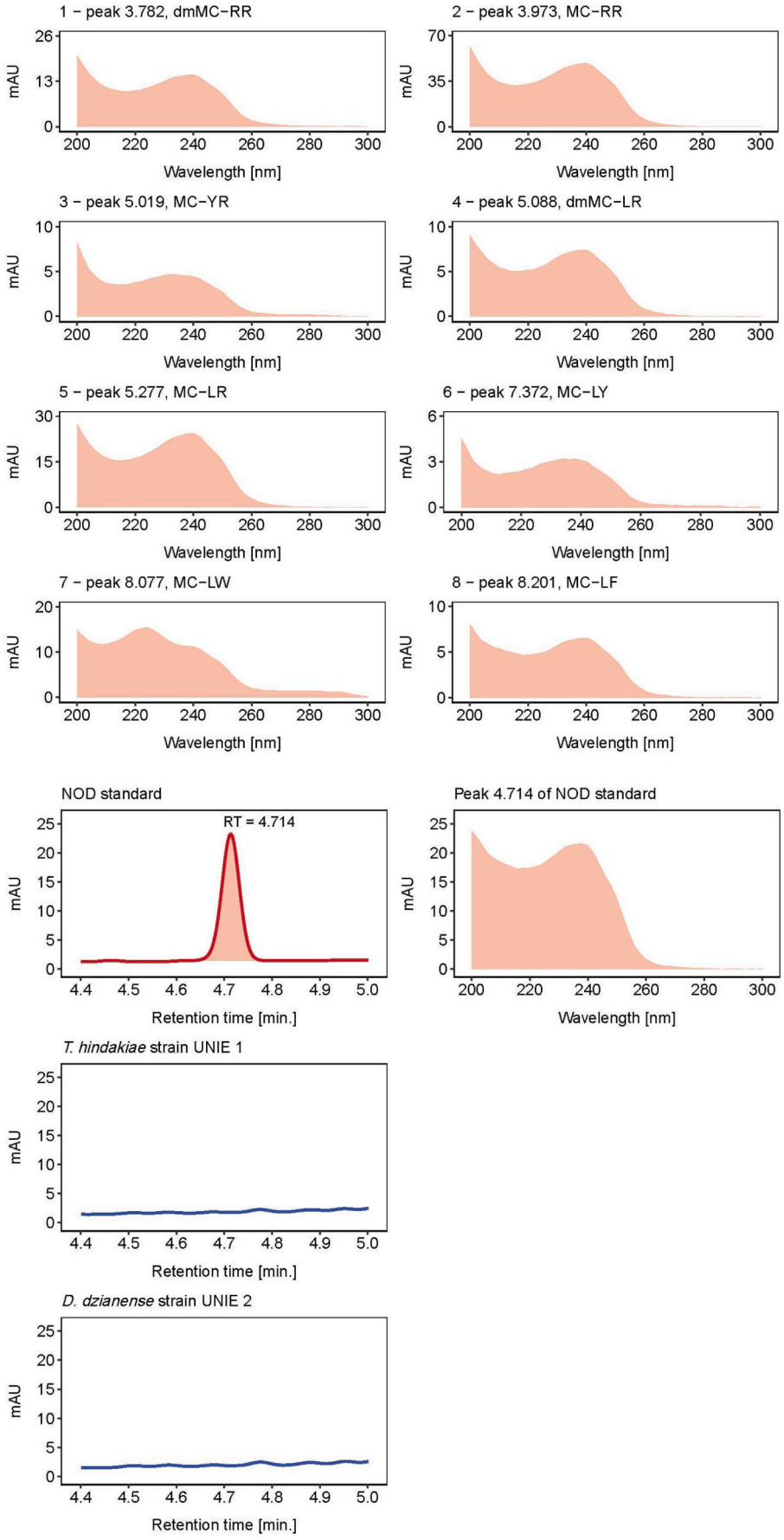

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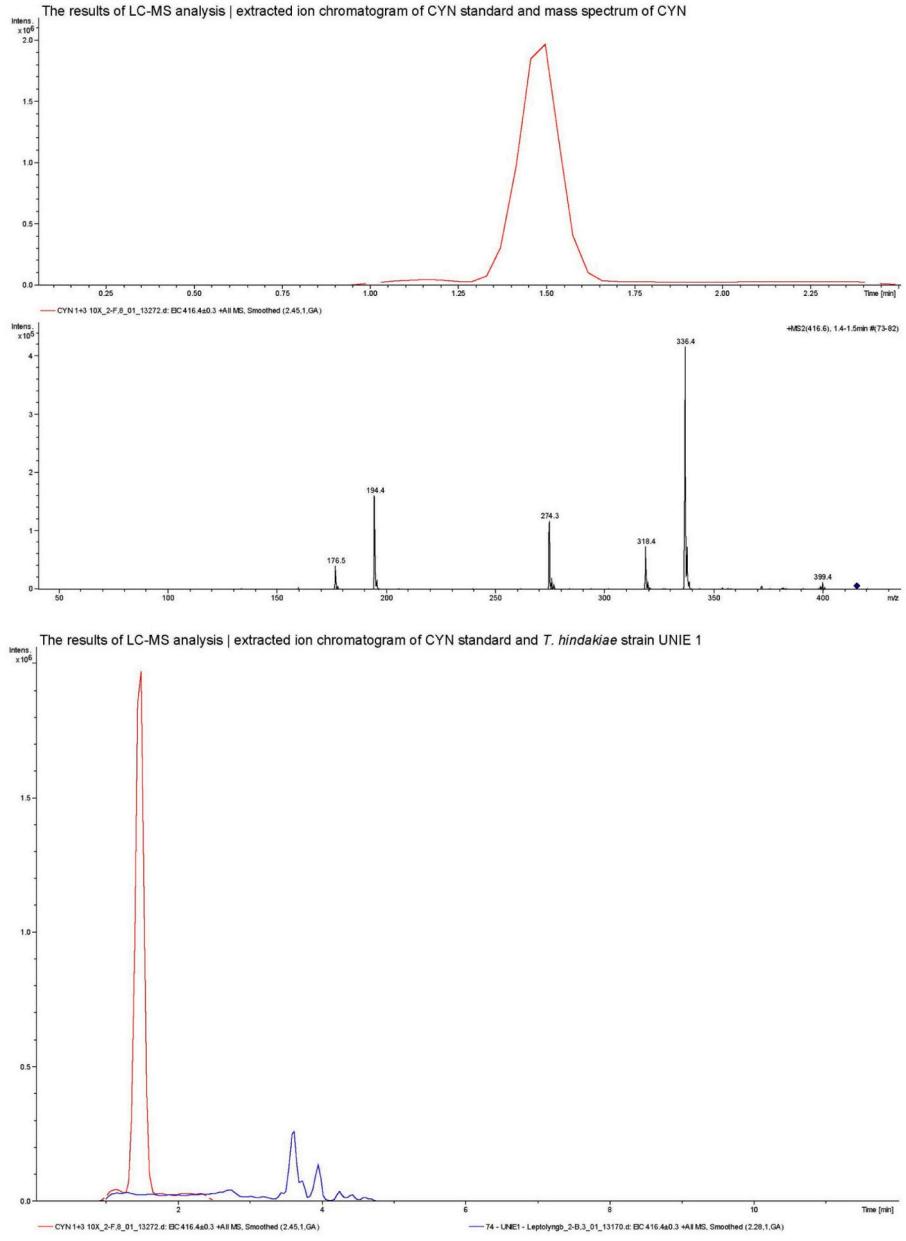
LC-MS results:



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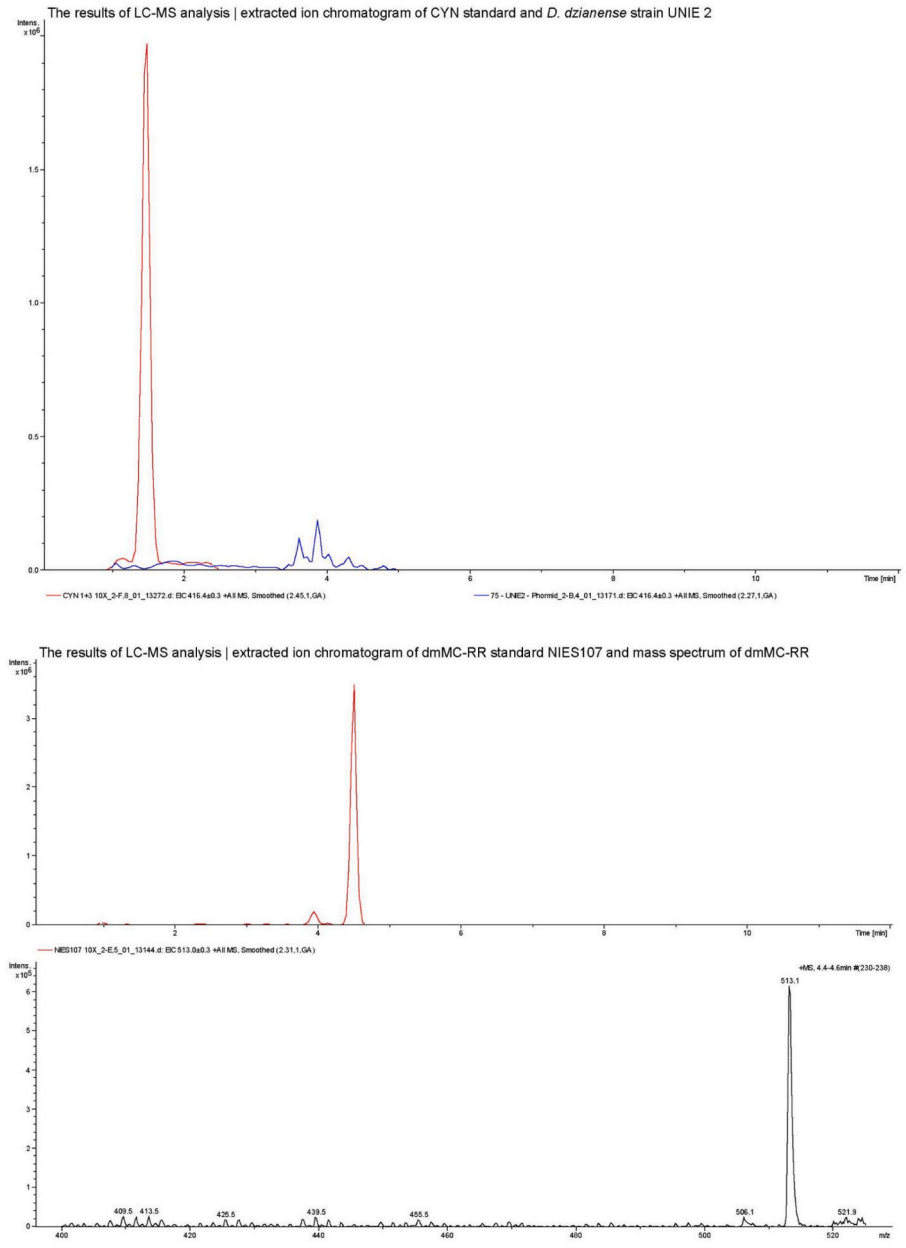

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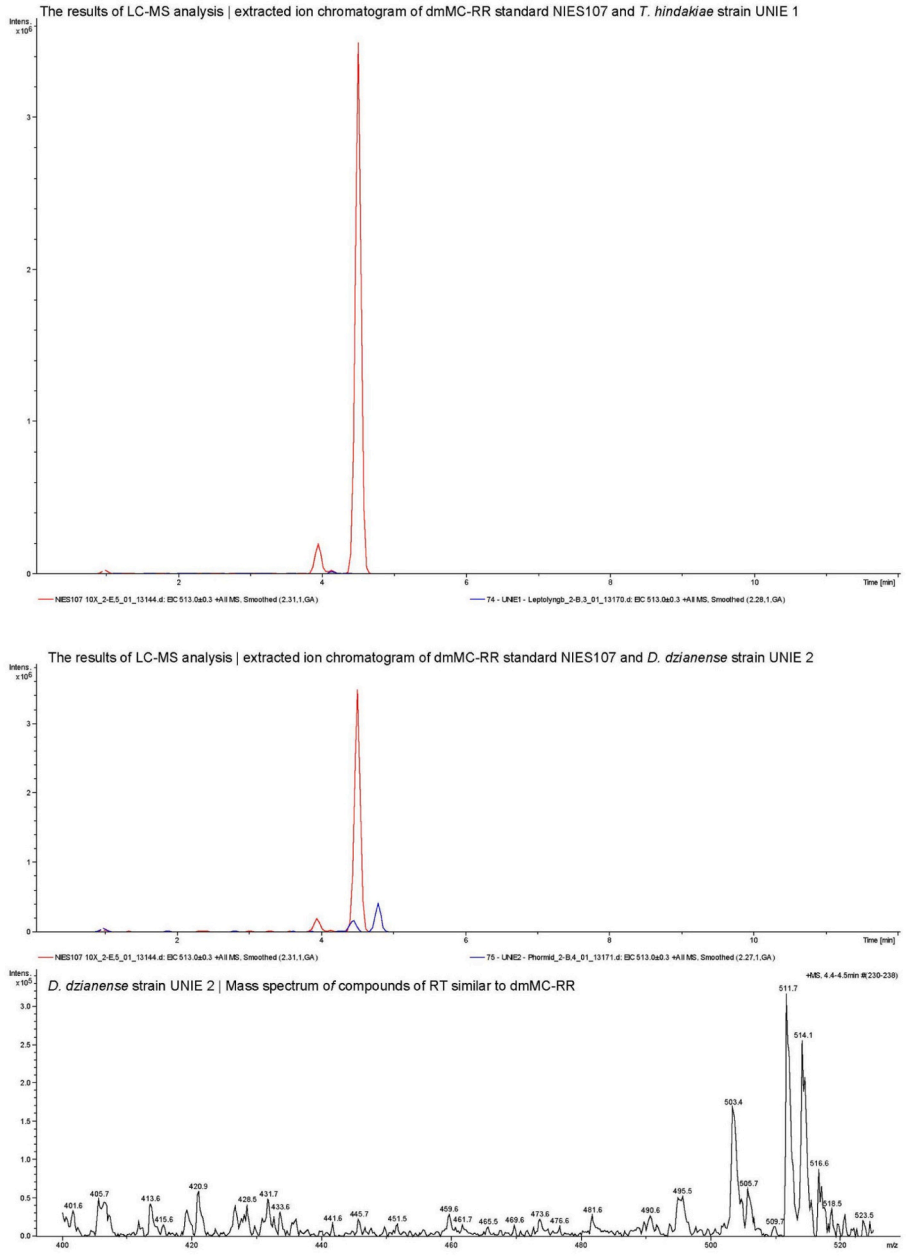

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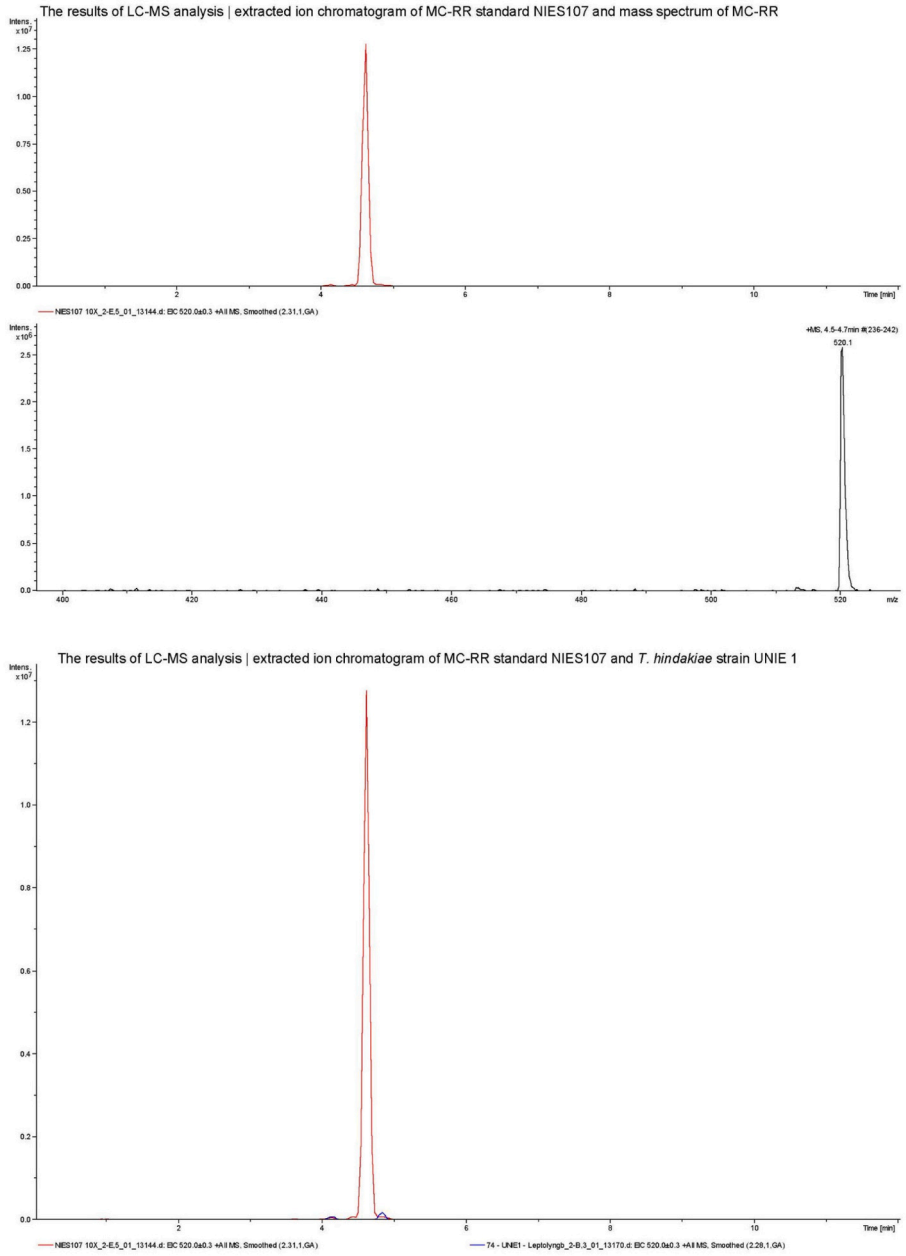

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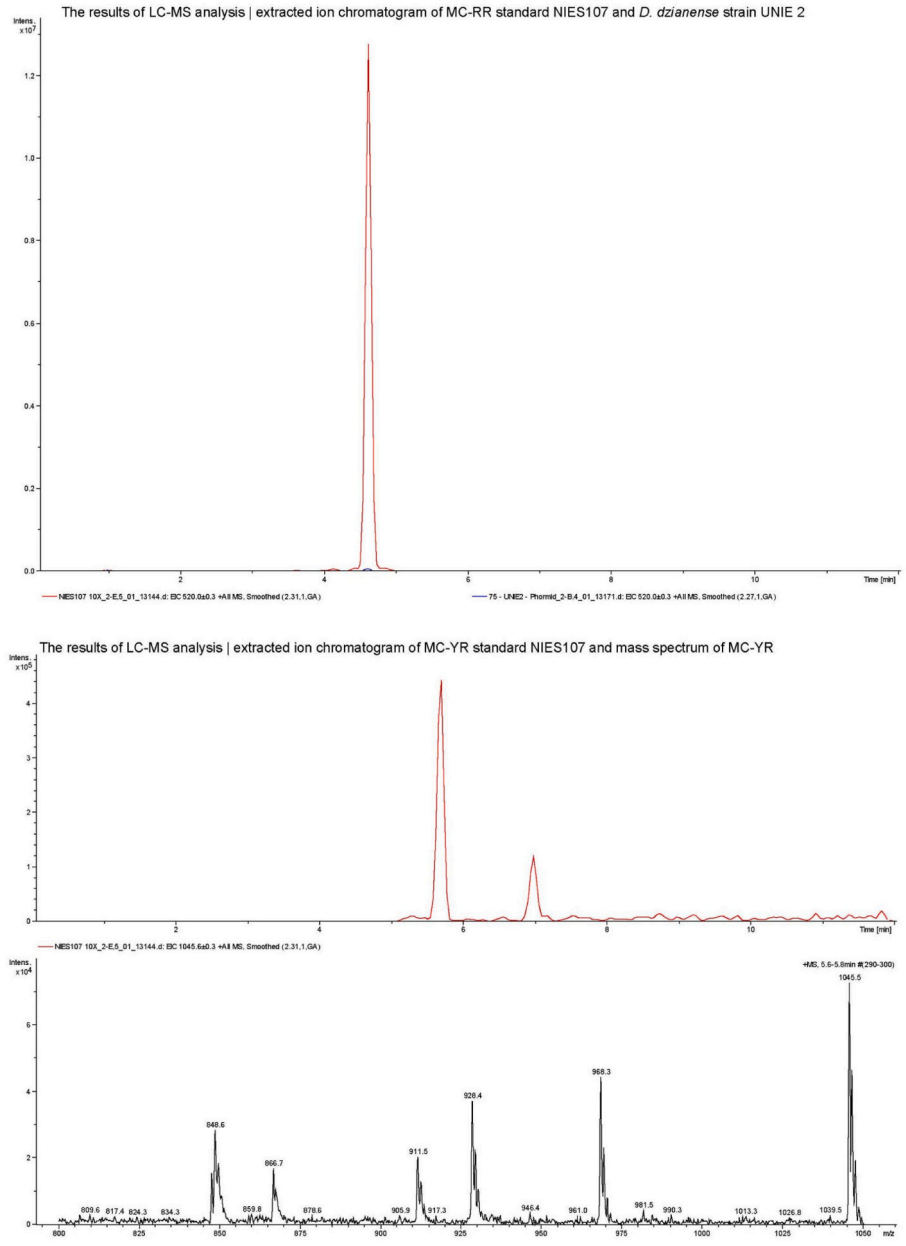

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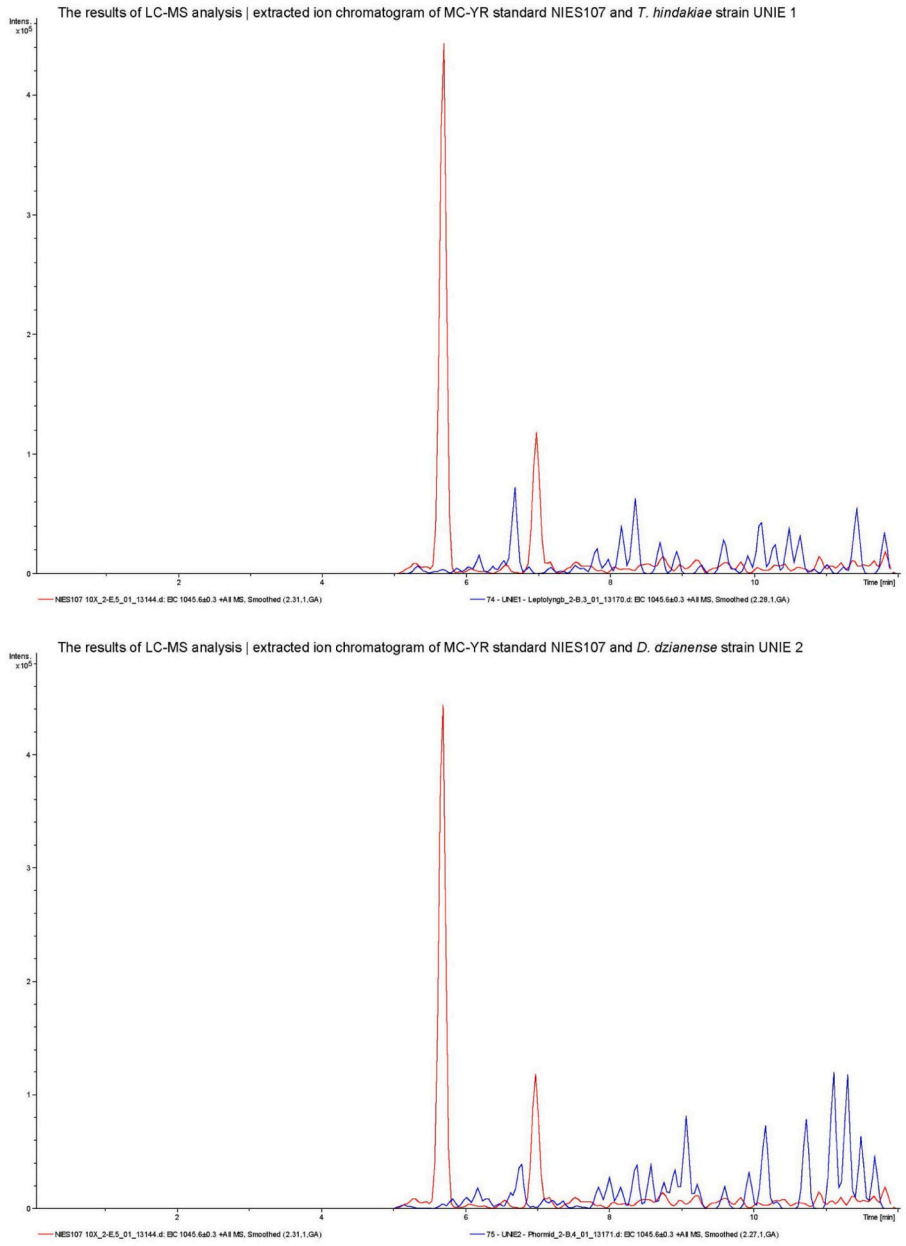

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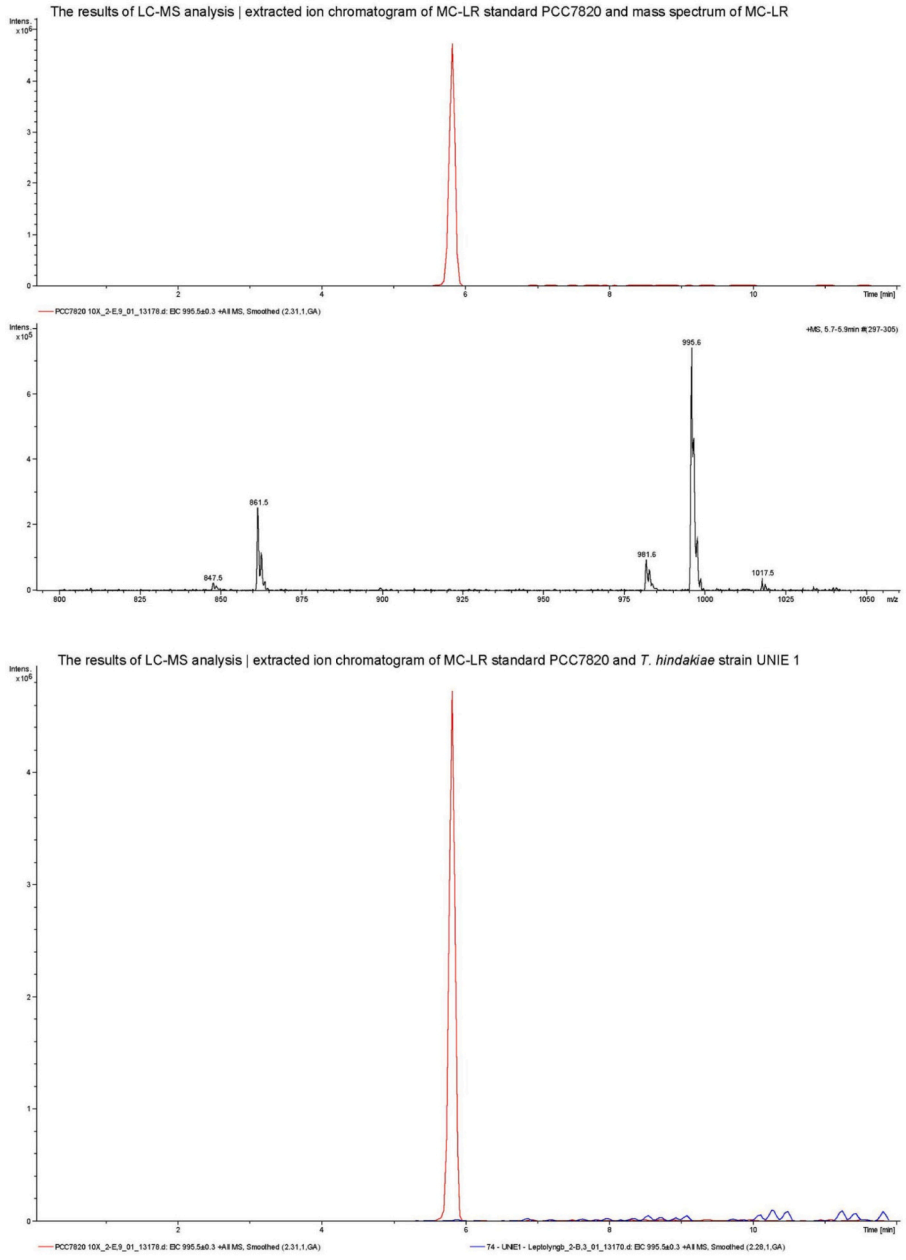

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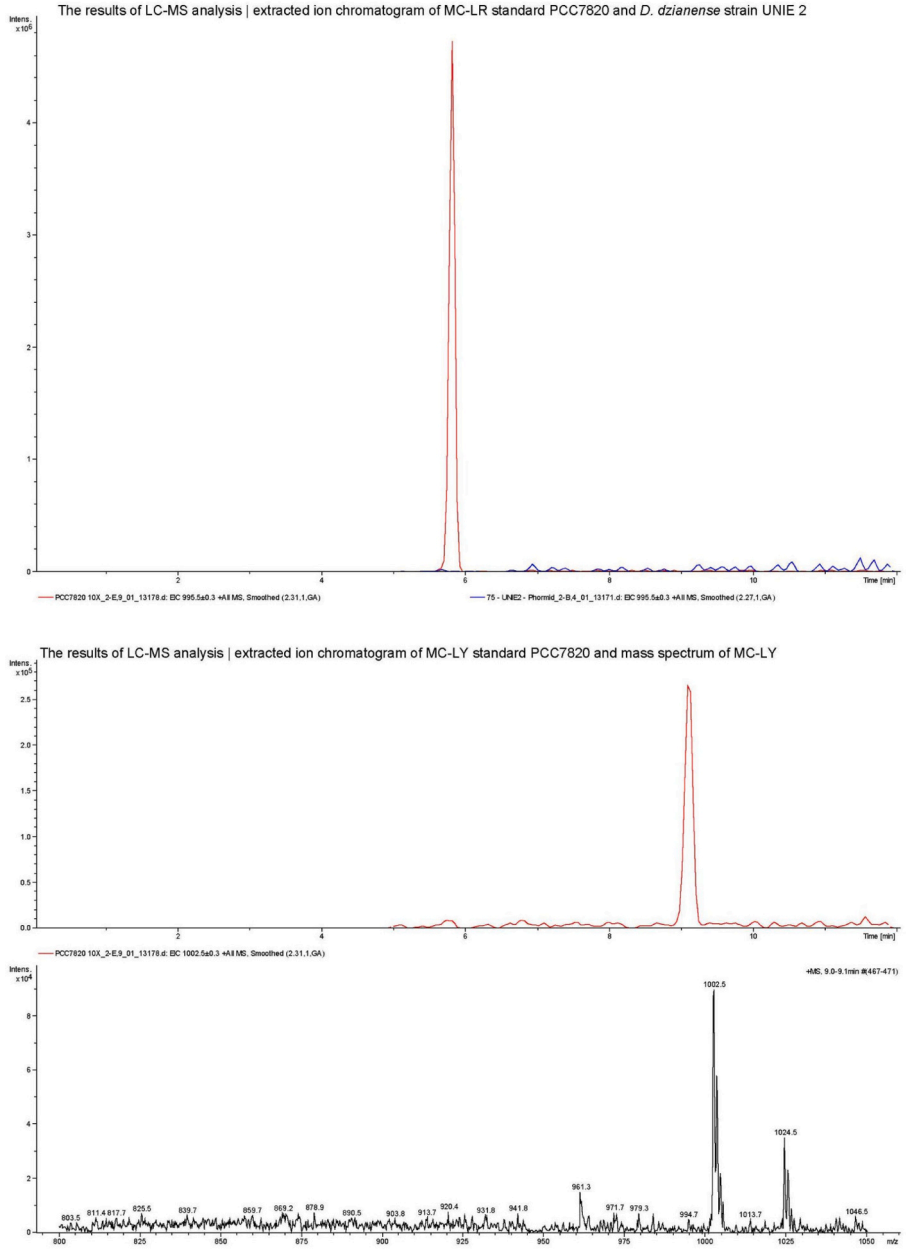

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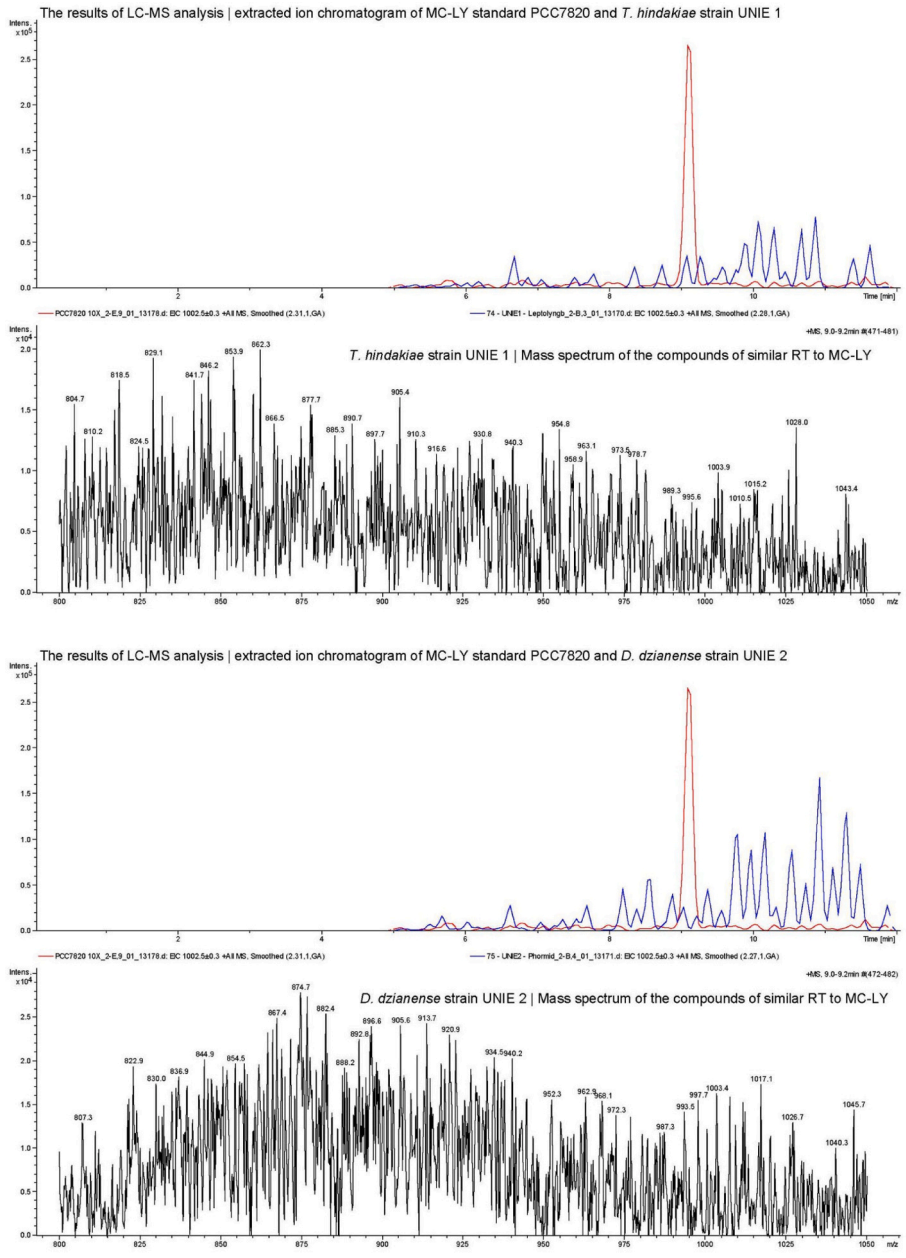

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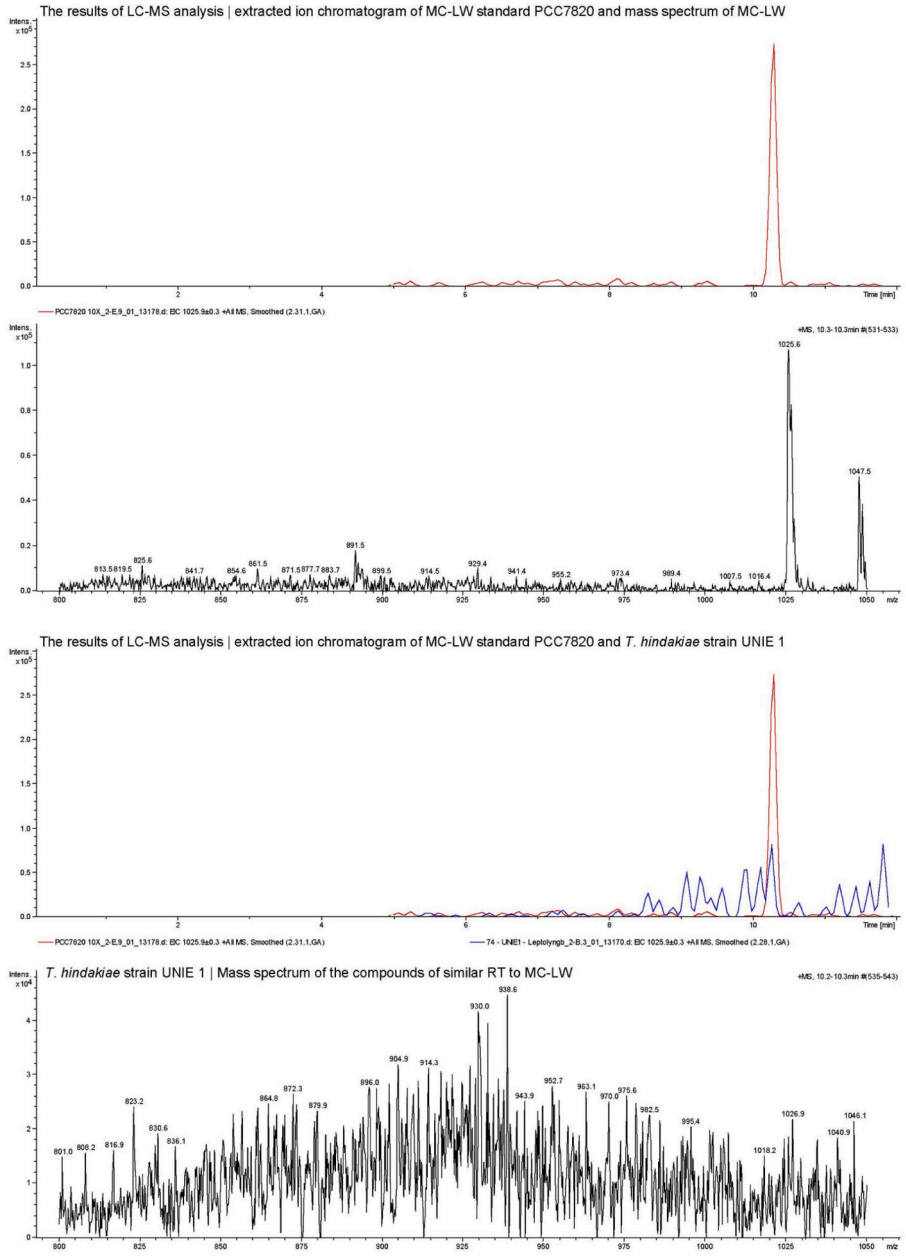

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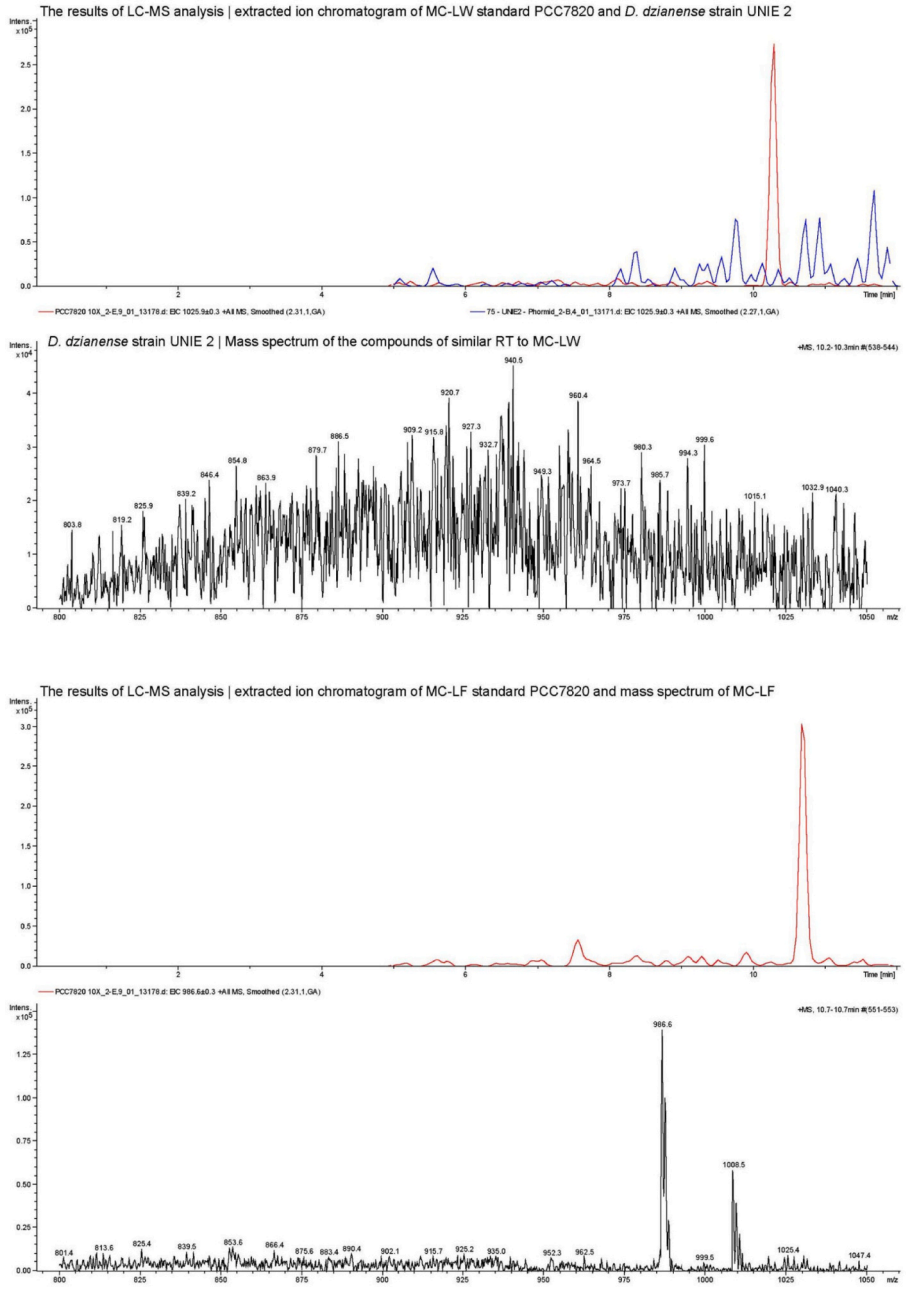

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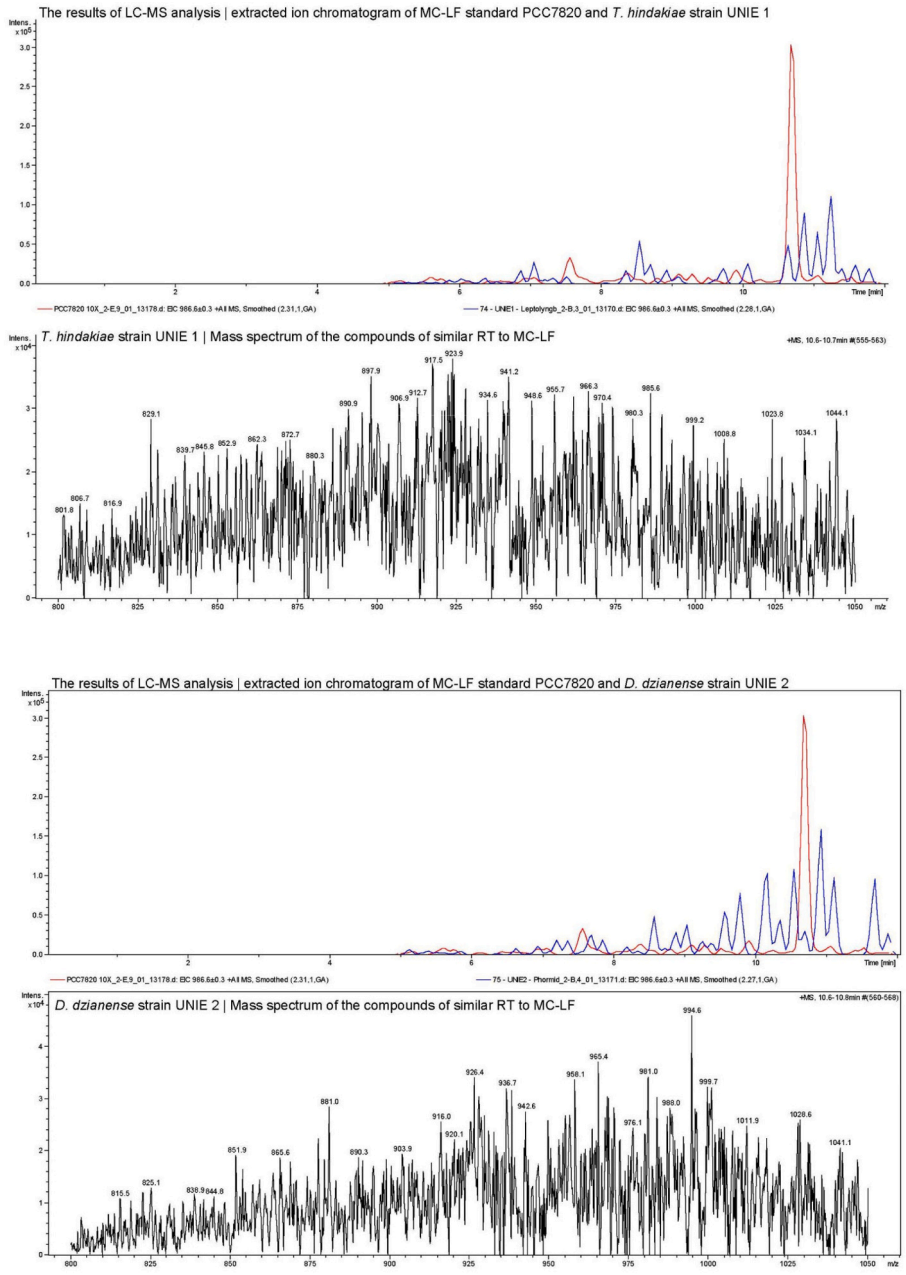

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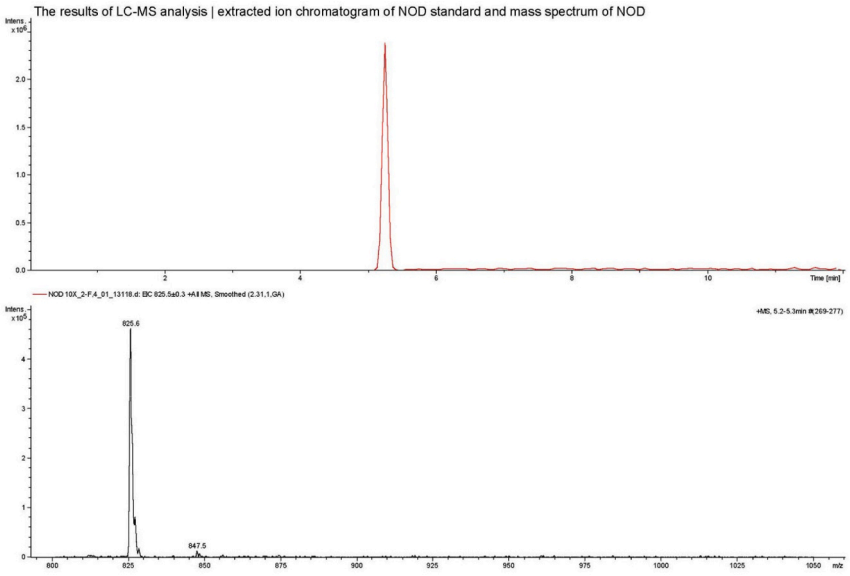
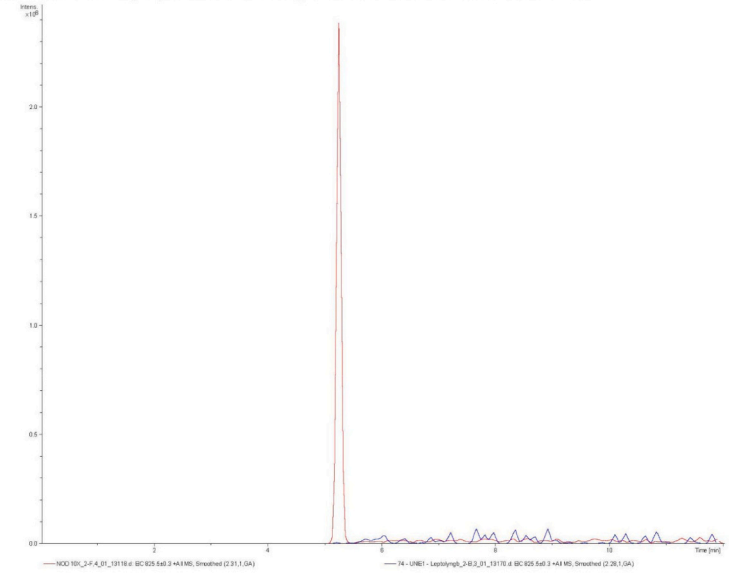
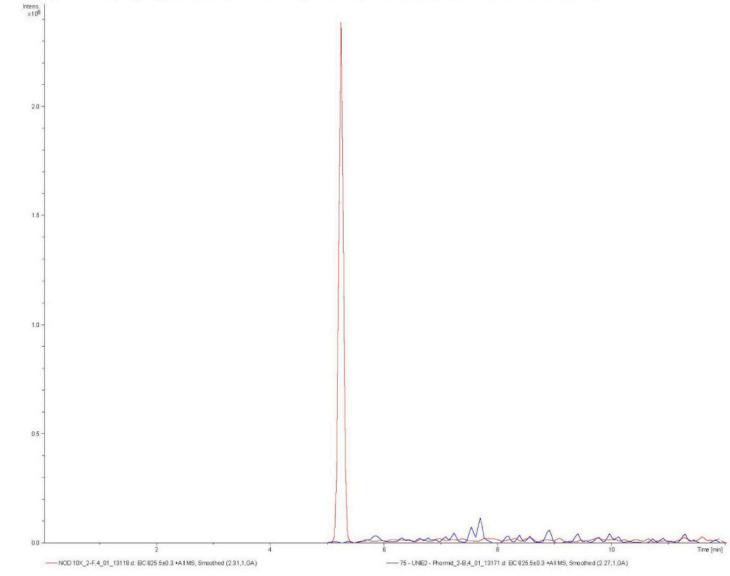


The results of LC-MS analysis | extracted ion chromatogram of NOD standard and *T. hindakiae* strain UNIE 1



The results of LC-MS analysis | extracted ion chromatogram of NOD standard and *D. dzianense* strain UNIE 2



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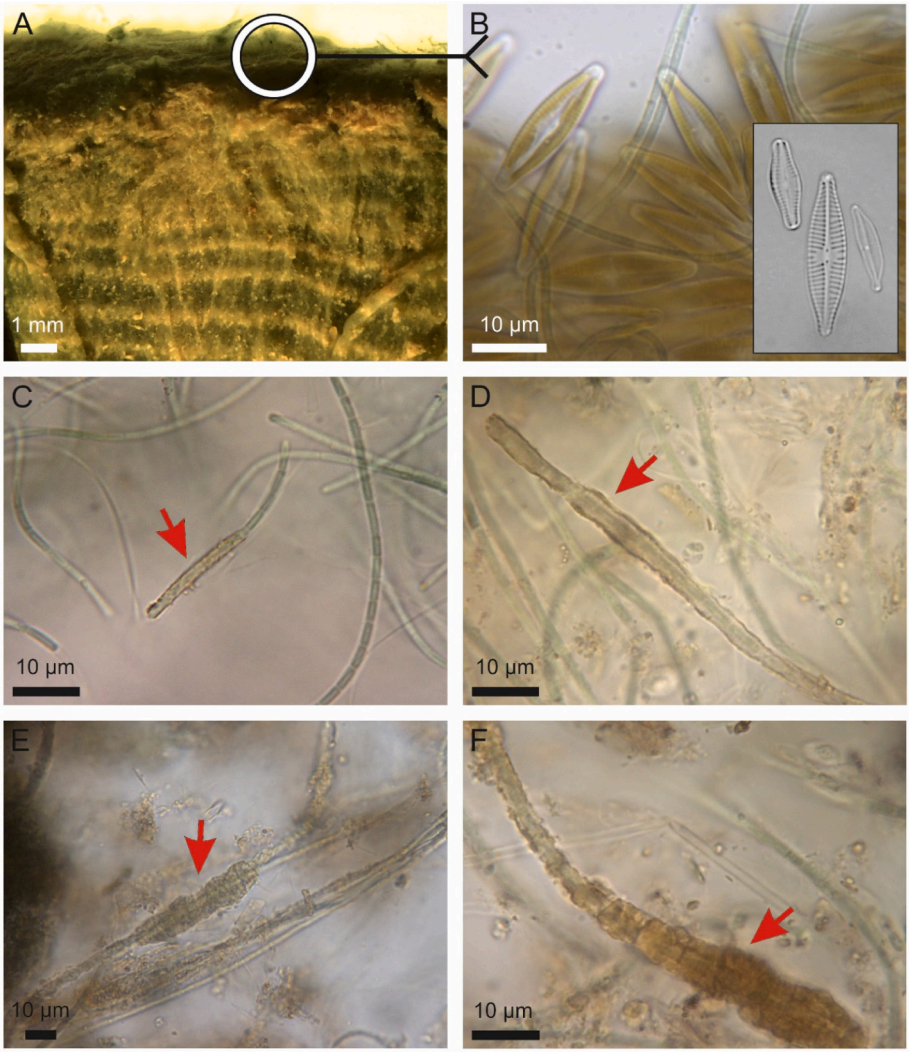

Fig. A2. The upper green layer of the cyanobacterial mat (A) occupied by diatoms (B); the dominant taxa were *Navicula cryptotenella* Lange-Bertalot, *Crenotia angustior* (Grunow) Wojtal and *Halamphora* cf. *tenerrima* (Aleem & Hustedt) Levkov. Filamentous cyanobacteria in mats with different degrees of encrustation by iron compounds (C - F, red arrows). Scale bars: 1 mm (A), 10 µm (B–F). Photo credit: A. Hindáková. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

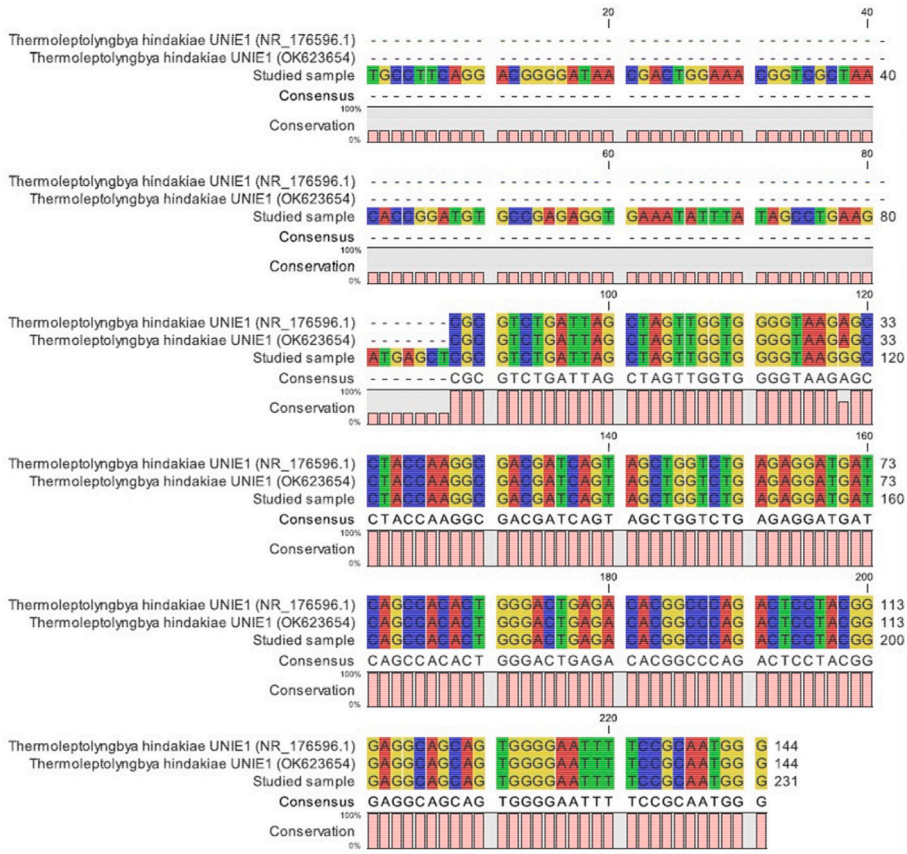

Fig. A3. Sequence alignment of the 16S rRNA gene DNA fragment between the studied sample and the most similar published strains belonging to the genus *Thermoleptolyngbya*. Accession numbers are within the brackets. Sequence alignment was performed in the software CLC Genomics Workbench 23.0.2 (QIAGEN).

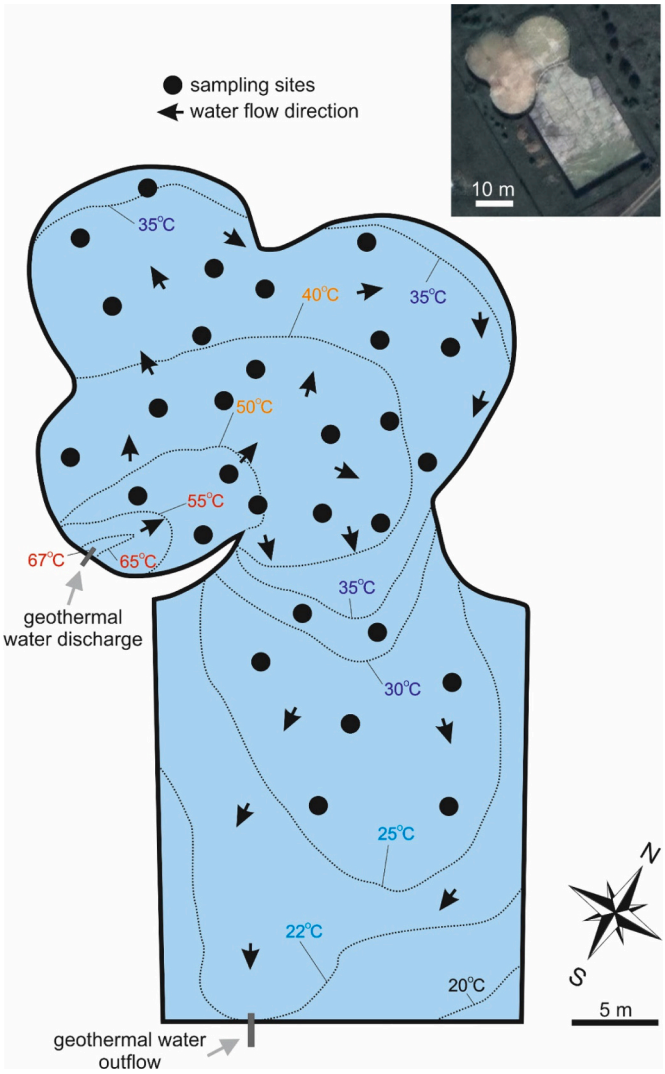

Fig. A4. The distribution of subsites (black dots) at different temperatures of geothermal water that flowed through the experimental pool inhabited by examined cyanobacteria. The black arrows indicate the direction of water flow.

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