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Biochemical, toxicological and genomic investigation of a high-biomass producing *Limnothrix* strain isolated from Italian shallow drinking water reservoir

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ABSTRACT

The presence of cyanobacteria in water for drinking and recreational purposes is considered problematic due to their bloom-forming and toxigenic potential. While most cyanotoxins are produced by extensively studied cyanobacteria, little is known about other abundant genera not usually assessed for their toxic potential. Here, a new isolate of *Limnothrix redekei* from an Italian drinking water reservoir was cultured and investigated using biological, chemical, and genomic methods. Compared to other *Limnothrix* spp., the isolate yielded high biomass productivity and was mainly constituted by proteins. The isolate had genomic features similar to those of the other 10 genomes of *Limnothrix* spp. published so far, regardless of their geographical origin. Most genes were implicated in primary metabolism (e.g. photosynthesis, nutrients transport, cell division), while <1 % of the genome was dedicated to secondary metabolism. Two regions encoding for a cyanobactin and a polyketide were found, whereas those for known cyanotoxins were absent. Toxicological investigations based on the crustacean *Artemia* sp. bioassay revealed potential toxicity of *Limnothrix* aqueous extracts (mortality >90 %). The highest toxicity was observed in a single fraction of the extract, whose UV spectrum presented carotenoid-like characteristics. Although the structure of the putative toxin was not yet elucidated, these findings pose the basis for further investigations on *Limnothrix* spp. toxicity, for which the observed toxic aqueous extracts could not exclude possible implications for ecosystems and humans through drinking water resources.

1. Introduction

Cyanobacteria are widespread prokaryotic organisms living in a wide range of habitats, such as soil and aquatic ecosystems (freshwater, brackish, or marine), as well as those having extreme conditions (hypersaline, hot springs, polar regions); thus, they can be virtually found in every environment [1]. Cyanobacteria exhibit a broad morphological diversity, ranging from unicellular and colonial organisms to filamentous ones, and show a wide spectrum of physiological adaptations to the surrounding environment (e.g., benthic vs planktonic behaviour). This great diversity may reflect their unique and plastic metabolism, which allows cyanobacteria to be highly adaptive and competitive in many ecosystems [2,3]. Cyanobacteria are capable of producing a wide array of metabolites which may help them thriving under adverse conditions [4]. The list of these compounds is constantly updated, with >2000 cyanobacterial metabolites known nowadays [5]. Cyanobacterial metabolites are characterized by very different chemical structures (i.e., peptides, polyketides, alkaloids, terpenes), and may exhibit various and potent bioactivities, including antiviral, antibacterial, antifungal, anti-inflammatory, and enzyme-inhibiting effects [4–6]. About 18 % of known cyanobacterial metabolites are toxic to animals and humans, and include the well-studied cyanotoxins (microcystins, nodularins, anatoxin-a, cylindrospermopsins, saxitoxins) as well as emerging ones [5]. Due to the taxonomic confusion of cyanobacteria, i.e., prokaryotes classified according to both the Prokaryotic and the Botanical Codes [7], the true diversity and taxon-wide distribution of such metabolites and

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toxins are difficult to interpret. Nonetheless, it is known that over two-thirds of known natural cyanobacterial compounds are produced by the genera *Microcystis*, *Lynbgya*, *Nostoc*, and *Hapalosiphon*, whereas little is known about other less-studied genera regardless of their distribution and occurrence worldwide [4]. From a common perspective, *Microcystis* is usually considered one of the most toxic genera of cyanobacteria due to its wide distribution and impact on a global scale, although the ability to produce cyanotoxins appears to be more common in filamentous forms, including well-known genera of equal importance (e.g., *Dolichospermum*, *Aphanizomenon*, and *Lyngbya*) [8]. So far, most of the studies have been focused on planktonic bloom-forming genera in freshwaters because of the risks associated with their presence for animal and human health [9]. Only recently, major attention has also been given to benthic and tychoplanktonic cyanobacteria (i.e., benthic with planktonic phases) as a potentially dangerous source of toxins, since benthic material can be detached and spread into the water column, possibly increasing the harmfulness of water in case of toxic strains [10]. For instance, filamentous cyanobacteria of the genus *Limnothrix* have received little attention in recent decades, likely due to their alleged non-toxic nature and their co-presence in shallow freshwaters with toxic and bloom-forming genera, much better known, such as *Planktothrix* [11–13]. Species belonging to the genus *Limnothrix* are traditionally considered as planktonic or tychoplanktonic and exhibit characteristic polar aerotopes localized at the ends or in the middle of cells. Despite its relatively small dimensions (trichome diameter < 2.0 μm), *Limnothrix* spp. may be co-dominant in shallow European lakes [12–15], contributing up to 59 % of the annual total cyanobacterial biomass [16]. Furthermore, the massive development of *Limnothrix* spp. can be occasionally problematic for water treatment managers [17]. Nonetheless, the identification of *Limnothrix* may be difficult due to its small size, the variability of taxon-specific morphological traits (i.e., central and polar aerotopes), and the high resemblance to genera such as *Pseudanabaena* and *Geitlerinema* [17,18]. Particularly, the morphological taxon-specific features may be lacking under environmental stress conditions or in cultures [14,19]. Indeed, tychoplanktonic-to-benthic behaviour and morphological changes in *Limnothrix* have been described both in the field and in cultured strains, suggesting an environment-driven adaptation for this genus. For instance, strains collected in the field have shown the tendency to form mats or "balls" floating on water surfaces, and when in culture they grow preferably attached to the flask surfaces losing their aerotopes [17,18]. This morphological plasticity can affect the correct identification of *Limnothrix* in freshwaters, possibly leading to misidentification if only morphological features are covered. Indeed, it has been well established that the application of a polyphasic approach, which consists in a combination of morphological, molecular, and ecological criteria, is a better option for cyanobacteria identification [20,21]. Recently, advances in cyanobacterial genomics have been of help in the resolution of taxonomical uncertainties in less-investigated or cryptic genera [7,20,21], rapidly accelerating the discovery of new metabolites and emerging toxins produced by these organisms [8,22,23]. In the field of drug and toxin discovery, genomics has played a key role, allowing a major understanding of biological dynamics that regulate the synthesis of secondary or specialized metabolites in many organisms, including cyanobacteria [23–25]. About 5–6 % of the cyanobacterial genome is usually dedicated to the synthesis of secondary metabolites, making genome-wide investigations attractive for their discovery [26]. In cyanobacteria, most secondary metabolites are encoded by groups of genes clustered together within the genome, known as biosynthetic gene clusters (i.e., BGCs). Based on the specificity existing between BGCs and their end products, the production of both known and unknown compounds and toxins could be predicted [27,28]. In this context, the data mining of sequenced genomes for the discovery of genes encoding enzymes potentially involved in the biosynthesis of metabolites (i.e., genome mining) has become a key approach to study BGCs in cyanobacterial genomes [23,29,30]. The diversity of known cyanobacterial metabolites goes beyond the enormous biosynthetic potential found in the genomes, and for some metabolites the biosynthetic pathway remains unknown [4]. The integration of data derived from analyses of cyanobacterial genomes, and from chemical and biological methods could help in linking these gaps, thus expanding the discovery of novel toxins [28,31]. Understanding the biological nature of under-investigated cyanobacteria genera through genomics techniques could also improve the monitoring of emerging toxic species in water intended for human consumption.

The main objectives of this study were to characterize a new cyanobacterial isolate from an Italian drinking water reservoir, as well as to explore the potential toxicity of a bloom-forming but less-investigated cyanobacterial genus, through the integration of biological assays, chemical methods, and whole genome sequencing.

2. Materials and methods

2.1. Cyanobacterial strain isolation

The cyanobacterial strain was isolated from a surface water sample collected from a small freshwater body (L1) belonging to three partially connected lakes (L1, L2, and L3) intended for drinking water processes (area 3500–10.000 m^2, perimeter 250–400 m, depth < 5 m) and located in NE Italy (43°58′17.4"N 12°24′33.4″E, locality Verucchio, Rimini, Italy), in June 2020 (Fig. S1). The three lakes originated from a previous unified water body that was separated over time and are currently managed by the local drinking water company for potabilization purposes (Romagna Acque-Società le Fonti S.p.A.). All lakes have experienced a consistent rise in cyanobacterial density in recent years, especially in warm months. Specifically, the cyanobacterial strain was isolated through micropipetting, then it was plated on 1 % agarose supplemented with BG11 medium [32] following the procedure described by Haande et al. [33]. The purified cyanobacterial inoculum was then maintained in culture using liquid BG11 medium, and grown in an Erlenmeyer glass flask at temperature 20 ± 1 °C, under a light intensity of 90–120 μmol m^{-2} s^{-1}, and with photoperiod (light: dark) 16:8 h. Based on preliminary microscopic investigation (ZEISS Axiovert 100), the cyanobacterial strain was attributed as belonging to the family Pseudanabaenaceae according to Komárek and Anagnostidis [19], and labelled as LRLZ20PSL1.

2.2. Cultivation of the cyanobacterial isolate

2.2.1. Growth

LRLZ20PSL1 strain was cultured for 18 days under the same conditions previously described (section 2.1), with an initial inoculum of 0.03 g L^{-1} and reaching a final volume of 3.5 L. The growth was followed every 2–3 days as dry weight (g L^{-1}) and turbidity (optical density at 750 nm), collecting triplicate aliquots of 25–50 mL. Measures of dry weight were obtained by filtering culture aliquots with glass microfiber filters (Whatman GF/F, Ø 47 mm, nominal pore size 0.7 μm), that were pre-washed with distilled water, dried at 105 °C for 24 h, and pre-weighted. After filtration, the filters were dried for 1–3 h at 105 °C, then cooled to room temperature and weighed. Turbidity in terms of absorbance of the cyanobacterial suspension at 750 nm was measured spectrophotometrically (UV/VIS/NIR, JASCO V-650, Tokyo, Japan). The cyanobacterial volumetric productivity (P_b, mg L^{-1} day^{-1}) was calculated according to Hempel et al. [34], as follows:

$$P_b = (DW_1 - DW_0)/(t_1 - t_0) \quad (1)$$

Where:

$(DW_1 - DW_0)$ = variation of dry weight expressed as mg L^{-1} between days t_1 and t_0.

$(t_1 - t_0)$ = cultivation period considered and expressed as days.

The cyanobacterial specific growth rate (μ, day^{-1}) was also investigated according to the equations of Li et al. [35], substituting the cell

count with dry weight measures:

$$\mu = [ln(DW_1) - ln(DW_0)]/(t_1 - t_0) \quad (2)$$

Where:

$DW_0; DW_1$ = dry weight at t_0 and t_1.

$(t_1 - t_0)$ = cultivation period considered and expressed as days.

For both P_b (mg L^{-1} day^{-1}) and μ (day^{-1}), the cultivation period considered, corresponding to the cyanobacterial exponential growth phase, was calculated between day 2 (t_0) and day 16 (t_1) of cultivation.

2.2.2. Nutrients consumption

The residual inorganic nutrients (i.e., N-NO_3, N-NO_2, P-PO_4) in the medium were determined by ionic chromatography (883 Basic IC plus, METROHM, Switzerland) according to the standardized protocol described by the Italian National Environmental Agency [36]. The uptake rate of nitrate (N-NO_3) and phosphate (P-PO_4) by the cyanobacterium throughout the cultivation was calculated according to Fiori et al. [37], using the measures of dry weight instead of cell counts, as follows:

$$Uptk = -\left([C]_1 - [C]_0\right)/(\gamma \bullet (t_1 - t_0)) \quad (3)$$

In which:

$$\gamma = \frac{DW_1 - DW_0}{ln(DW_1) - ln(DW_0)}$$

Where:

$[C]_0; [C]_1$ = inorganic nutrient concentration (either N-NO_3 or P-PO_4) expressed as mM at specific days of the cultivation, i.e., t_1 and t_0

$DW_0; DW_1$ = dry weight (g L^{-1}) at t_0 and t_1

$(t_1–t_0)$ = cultivation period expressed as days

Final values of Uptk were therefore expressed as N-NO_3 or P-PO_4 millimolar concentration per gram of cyanobacterial biomass per day (mmol g^{-1} of biomass day^{-1}).

2.2.3. Biochemical composition of the biomass

The cyanobacterial biomass was collected on days 8 and 18 through centrifugation and then freeze-dried for subsequent analyses of biomass composition in terms of proteins, lipids, and polysaccharides, expressed as percentage of biomass (%dw). The protein content of LRLZ20PSL1 cells was determined on freeze-dried material (10–15 mg) extracted with 3.0 mL of NaOH (0.5 M). The extracts were briefly vortexed, then incubated for 8 min at 90 °C under constant magnetic stirring. Afterwards testing tubes were transferred on ice for 2 min and centrifuged (2550 ×*g* for 10 min), and the supernatant was collected. The procedure was repeated three times until the cyanobacterial pellet turned colorless. Finally, the protein content was determined on the collected fraction according to the Folin-phenol reaction [38]. For lipid determination, dried cyanobacterial material (50–100 mg) was extracted with a 2:1 (*v*/v) mixture of dichloromethane and methanol at 60 °C for 90 min, under constant magnetic stirring, repeating the extraction procedure three times. Then, samples were cooled down and centrifuged (3000 ×*g* for 10 min), and the organic phase of the resulting supernatant was collected on pre-weighted glass vials. The organic fraction was dried under a gentle nitrogen flux, and the vials with the lipid fraction were weighted. For the polysaccharides fraction, aliquots of freeze-dried material (8–10 mg) were extracted according to the procedure described by Myklestad and Haug [39]. Briefly, the pellets were extracted with sulfuric acid (12 M) and incubated at 37 °C for 1 h. Afterwards, samples were diluted with distilled water, vortexed, and incubated at 100 °C for 2 h. Then, the samples were cooled down on ice for 30 min and centrifuged (3000 ×*g* for 10 min), and the polysaccharide content was determined in the supernatant by the phenol-sulfuric acid colorimetric reaction using the JASCO V-650 spectrophotometer [40].

2.3. Toxicity screening through Artemia sp. bioassay

The potential acute toxicity of the LRLZ20PSL1 strain was tested by means of the crustacean *Artemia* sp. bioassay, using the procedure of APAT and IRSA-CNR [41]. Artificial sea water at salinity 30 (SW 30 ‰) was prepared with Red Sea Salt to hatch *Artemia* sp. cysts (Vivani Animal Nutrition and Care Products BV, NL06743), that were maintained at 25 ± 1.0 °C and ambient light for 1–2 h, then incubated in the dark for 24 h. Afterwards, hatched larvae were collected in a separate glass Becker with SW 30 ‰ and rested for additional 24 h prior to the test. The assay was conducted in 24-well plates using a short-term exposure of 24 h and testing cyanobacteria on 10 *Artemia* sp. individuals in 1 mL of testing solution. The newly isolate LRLZ20PSL1 strain and other cyanobacteria were tested, namely cf. *Anabaena* sp. and cf. *Dolichospermum* sp., both isolated from Reno river [42], *Planktothrix agardhii* (CCAP 1459/16), *Planktothrix rubescens* (CCAP 1459/22), *Aphanizomenon klebahnii* (CCAP 1401/3), and *Microcystis aeruginosa* (CCAP 1450/10), all purchased from Culture Collection of Algae and Protozoa, and *Arthrospira platensis* (CCMP 1295) acquired from Provasoli-Guillard National Center for Marine Algae and Microbiota (currently known as NCMA). Cyanobacterial strains were collected by centrifugation from cultures at the exponential growth phase, then all pellets were freeze-dried and weighed. For each strain, a stock testing solution of cyanobacterial pellet dissolved in SW 30 ‰ was prepared. The solutions were sonicated for 20 min to stimulate cellular breakage and release of toxins and other metabolites, then final concentrations of 500 μg mL^{-1} were tested in duplicate. SW 30 ‰ was used as blank, while live cells of the toxic dinoflagellate *Ostreopsis* cf. *ovata* and the marine diatom *Phaeodactylum tricornutum* were used as positive and negative control, respectively, as previously reported [43]. Mortality of the *Artemia* sp. nauplii was expressed as the percentage of dead individuals with respect to the total (%dead/tot).

2.4. Investigation of cyanobacterial metabolites

2.4.1. Preparation of cyanobacterial extracts

The LRLZ20PSL1 culture was cultivated in a 10 L bubble column photobioreactor with BG11 medium reaching a final volume of 6 L, under the same conditions previously described (section 2.1) and with continuous aeration using filtered (0.22 μm) air at a flow rate of 1 L min^{-1}. Subsequently, whole biomass was retrieved by centrifugation, obtaining 1.5 *g* of freeze-dried material for subsequent chemical analyses. About 10 mg biomass was weighed into 2 mL test tubes and 1 mL of methanol (Honeywell Riedel-de-Haën, USA) and acid-washed glass beads (0.5 mm, Scientific Industries INC) were added. The cyanobacterial cells were disrupted using a Fastprep®-24 homogenizer set at 6.5 m s^{-1} for 30 s (MP Biomedicals, Irvine, CA, USA), and rested on ice for 5 min. The extraction was repeated, then the extracts were centrifuged (16,000 ×*g* for 5 min) and the methanolic fraction was collected. Prior to liquid chromatography analyses, two testing solutions were prepared, i.e., A and B. Solution A was a mixture 1:3 (*v*/v) of methanolic extract and acetonitrile (ACN), while solution B was obtained by firstly adding 0.1 % trifluoroacetic acid (TFA) to the methanolic extract, then preparing a 1:3 (*v*/v) mixture of the acidified methanolic extract ACN.

2.4.2. Ultra-high performance liquid chromatography analyses

Cyanobacterial extracts were analyzed by ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-QTOF), using an Acquity I-Class UPLC-Synapt G2-Si system (Waters Corporation, Milford, MA, USA), equipped with a Kinetex C8 column (50 × 2 mm, 1.7 μm, 100 Å, Phenomenex Inc.). The system was operated in gradient mode with a flow rate of 0.3 mL min^{-1}, and 1 μL of sample was injected in the column and eluted at 40 °C. The solvents of the mobile phase were water +0.1 % formic acid (A) and acetonitrile-isopropanol +0.1 % formic acid (1:1 *v*/v, B). The gradient program was set initially with 95 % of solvent A, and solvent B was increased

from 5 to 100 % in 5 min and maintained for 2 min, then B was brought back to 5 % in 0.5 min and finally kept for 2.5 min before next run. Mass spectra were acquired by the QTOF integrated in the Acquity system, that was calibrated with sodium formate and Ultramark 1621® (calibration mass range 91 to 1921 *m/z*), and leucine enkephalin was used as a lock mass reference compound. Mass spectra were recorded in positive electrospray ionization (ES+) mode, with a mass range of 50–2000 *m/z*, using the following settings: reference cone voltage 30 kV, source temperature 120 °C, capillary voltage 2.5 V, MSE trap collision energy ramp from 20.0 eV and ended at 40.0 eV.

2.5. Identification of the toxic fraction through chromatographic separation integrated with bioassay

About 50 mg of LRLZ20PSL1 freeze-dried material was extracted with 1 mL of ACN and water 1:1 mixture (*v*/v), following the same extraction procedure previously described (section 2.4.1). After centrifugation, the supernatant was collected and dried under nitrogen stream overnight, then the dried extract was eluted with 500 μL of 75 % ACN and filtered (0.22 μm). The eluted extract was subjected to Hydrophilic Interaction Liquid Chromatography (HILIC) using an Acquity UPLC system (Waters Corporation, Milford, MA, USA) equipped with a photodiode array detector (PDA eλ detector). For each run (13 min), 10 μL of samples were injected into a HILIC column x Bridge™ PREMIER BEH amide (4.6 × 150 mm, 2.5 μm, 130 Å, Waters Corporation, Milford, MA, USA), and the separation into 12 fractions was achieved using two solvents (A: water +0.1 % formic acid, and B: ACN + 0.1 % formic) in a linear gradient with 1.0 mL min^{-1} flow rate at 40 °C. The solvent gradient program was set as follows: A and B were kept for 9.5 min at 15 % and 85 %, respectively, then both solvents were brought to 50 % in 0.5 min and held for 2.0 min; finally, the concentration of A was brought back to 15 % for 0.1 min before next run. The UV spectrum of the collected fractions was acquired using a range of 210–800 nm with 1.2 nm resolution. A total of six runs with the cyanobacterial extract and one with a blank solution (75 % ACN) were performed, and the resulting 12 fractions for each run (Table S1) were collected and dried under nitrogen stream. To assess the active toxic fraction of LRLZ20PSL1 extract, the *Artemia* sp. bioassay was used. Prior to the test, each dried fraction was resuspended with 0.5 mL of SW 30 ‰, except for the blank that was directly resuspended in 1 mL. The same fractions from two different runs were combined to finally obtain triplicates of 1 mL volume for the test. The *Artemia* sp. bioassay was subsequently conducted on eluted fractions as previously described (section 2.3).

2.6. Genome analyses

2.6.1. DNA extraction

Aliquots from cultures of LRLZ20PSL1 in exponential growth phase were centrifuged and washed twice with sterile nuclease-free water, then genomic DNA from each aliquot was extracted with Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Corp., Irvine, CA, USA) according to the manufacturer instructions, and quantified with a Qubit® fluorometer using the dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). The general quality was checked by loading extracted DNA on TBE 0.8 % agarose gel and stained with BlueJuice™ Gel Loading Buffer (Invitrogen, Carlsbad, CA, USA), observing the absence/presence of smearing as indication of possible contamination.

2.6.2. Whole-genome sequencing

The extracted genomic DNA was sequenced at BMR Genomics (Padova, Italy). A shotgun library was constructed using TruSeq® DNA Sample Prep Kits and sequenced using an Illumina MiSeq platform following the 300PE approach (Illumina, CA, USA).

2.6.3. Draft genome preparation and bioinformatic analyses

Raw reads from whole-genome sequencing were checked for quality by FastQC 0.11.7 [44]. TruSeq Illumina adapters and low-quality short sequences (i.e., Phred 33 and lengths shorter than 30 bp), were removed using Trimmomatic v. 0.38 set in paired end mode [45]. The genome was assembled into contigs using SPAdes 3.14.0 [46], with "–careful" option active to minimize mismatches. Since genomic DNA was obtained from a mono-specific non-axenic culture of LRLZ20PSL1, a metagenomic binning approach was used to obtain the final genome clear of any other microbial and viral contaminant [47]. Thus, contigs of the assembled genome were taxonomically assigned by Kaiju [48], and the sequences only related to the phylum Cyanobacteria were retrieved. The quality of the final assembly was checked with CheckM v. 1.0.18 [49] and QUAST v. 4.4 [50], then the genome was first annotated using Prokka v. 1.14.5 [51] and re-annotated with RAST annotation pipelines [52–54]; each tool was accessed through Kbase platform [55]. Finally, BGCs for specialized metabolites were predicted using antiSMASH 6.0 with "loose" detection strictness [30], whereas taxonomical assignment of the final assembled genome was performed using Kaiju run in "greedy" mode [48]. The genome sequence of LRLZ20PSL1 has been deposited at DDBJ/ENA/GenBank under the accession JAZAQF000000000. The version described in this paper is version JAZAQF010000000.

2.6.4. Phylogenetic and phylogenomic analyses

The 16S rRNA sequence was extracted from the draft genome, then the best-fitting hits were preliminary assessed through BLAST. Afterwards, a phylogenetic tree was constructed using the first 50 hits, *Gloeobacter violaceus* PCC 7421 (NR_74282) that was used as an outgroup, and representative sequences of species belonging to the main cyanobacteria families that were retrieved from the curated database CyanoSeq v. 1.1.1. [56]. Briefly, all selected 16S rRNA sequences and the query isolate were aligned using MUSCLE in MEGA11 [57]. Phylogeny was constructed on W-IQ-TREE Web Service [58], using maximum likelihood approach (ML). The best model for DNA sequences was assessed by the integrated model-selection test in W-IQ-TREE, i.e. ModelFinder [59]. The tree was generated using the substitution model K3P with options to allow a proportion of invariable sites (+I), discrete Gamma distribution (+G4), and 1000 ultrafast bootstraps replicates [60]. Finally, a phylogenomic tree was also constructed based on genomic data, utilizing 164 concatenated single-copy proteins common to cyanobacterial genomic sequences. The protein sequences were extracted from the genome using Prokka v. 1.11 [51], and subsequently selected with BUSCO v. 5.4.4 [61], using cyanobacteria_odb10 as the lineage parameter. Sequence alignment was done using MAFFT v. 7.525 [62]. The maximum likelihood phylogenetic tree was inferred with IQ-TREE v. 1.7 using 1000 ultra-fast bootstraps and the automatically selected substitution model LG + F + I + G4. The generated tree was edited in iTOL v. 5 [63].

2.7. Data analysis

Statistical analyses on cyanobacterial biochemical composition and *Artemia*'s mortality rates were performed on PAST v. 4.17 [64]. Homoskedasticity and normal distribution of the data were evaluated by Levene's and Shapiro-Wilk's tests, respectively. For normally distributed data, significant differences between groups were assessed by one-way analysis of variance (ANOVA, $p < 0.05$), followed by Tukey's post-hoc test when appropriate.

3. Results

3.1. Growth and biochemical composition of the isolate

The isolate LRLZ20PSL1 (Fig. 1) was a filamentous cyanobacterium characterized by slightly curved thin trichomes (diameter < 2.0 μm). The strain had the tendency to clog-up into soft mats and "balls" free-floating in culture. Trichomes consisted of isopolar cylindrical cells

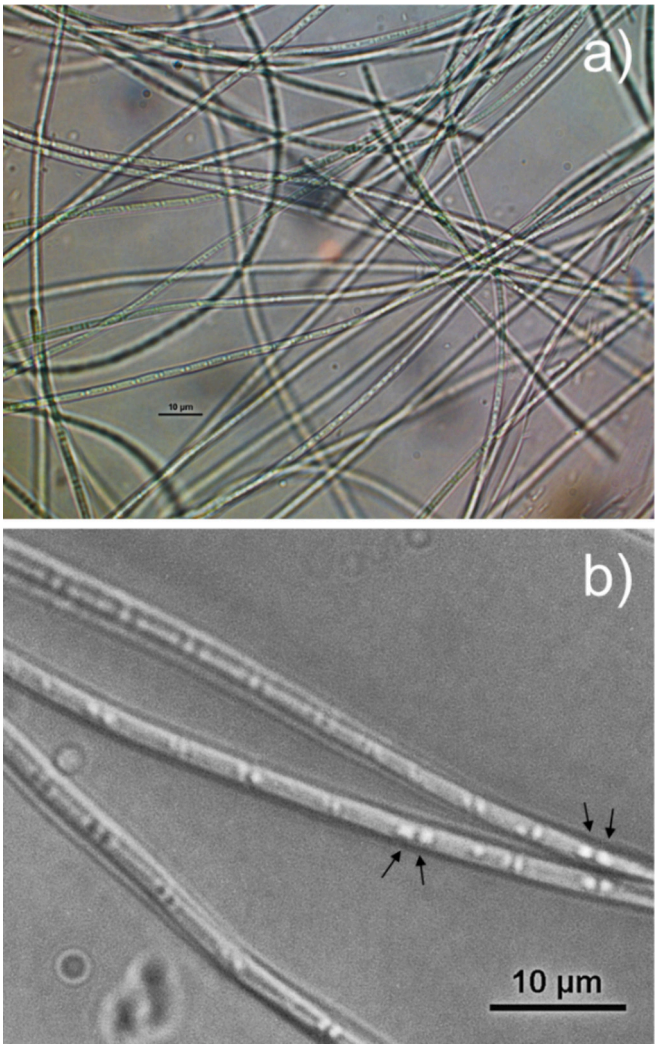

Fig. 1. Microscopic observation under 1000× magnification of the isolated strain LRLZ20PSL1, a) high-biomass culture, b) details of cell structure, arrows indicate polar aerotopes (black and white image).

longer than wider (1.8–2.2 µm wide × 4.3–5.0 µm long, $n = 30$) with very small aerotopes, i.e., gas vesicles, that were sometimes lacking, particularly in filaments at the bottom of culture flasks or attached to flask's surfaces. Trichomes were slightly constricted at the cross walls and ended with untapered cylindrical cells. Specialized cells, i.e., heterocytes and akinetes, were absent. Under tested laboratory conditions (BG11 growth medium, 20 ± 1 °C, light intensity of 90–120 µmol m^{-2} s^{-1}, photoperiod light:dark 16:8 h), fresh cultures were of deep blue-green color, while shifted to a yellow-green coloration in 1–2 months old cultures.

The culture of LRLZ20PSL1 strain was scaled-up and followed for 18 days. The main growth parameters are reported in Fig. 2. The cyanobacterial biomass in culture constantly increased in terms of dry weight (Fig. 2.a), reaching a maximum value of 0.13 g L^{-1} on day 16. This value corresponded to a volumetric productivity (P_b) of 6.5 ± 0.1 mg L^{-1} day^{-1} and a growth rate (µ) of 0.092 ± 0.001 day^{-1}. As for the residual concentration of macronutrients (Fig. 2.b), the content of nitrate (N-NO_3) and phosphate (P-PO_4) decreased in the culture over time, while nitrite (N-NO_2) increased particularly after day 8. LRLZ20PSL1 consumed N-NO_3 faster than P-PO_4 (uptake values 0.84 vs 0.16 mmol g^{-1} day^{-1}), however both macronutrients were still available on the final day of cultivation, especially nitrates (day 18, > 240 mg L^{-1}), suggesting that the culture presumably never reached the stationary growth phase; this was also attested by the general coloration of the culture, that did not shift to yellow-green color, a common finding under nitrogen-replete conditions supporting a high-pigments content. This evidence was further confirmed by the biochemical characterization of the biomass, collected on cultivation days 8 and 18 (Fig. 2.c), which revealed that proteins were the major constituents (38–52 %dw), with a significant 1.4-fold increase from day 8 to day 18 (ANOVA, $p < 0.05$). The second most abundant macromolecules were polysaccharides (32–23 %dw), which slightly decreased over time (ANOVA, $p > 0.05$), whereas the content of lipids remained unchanged (11–15 %dw).

3.2. Preliminary toxicity assessment

The potential toxicity of LRLZ20PSL1 strain was tested upon the bioassay based on the crustacean *Artemia* sp., in parallel with other cyanobacteria and microalgae, and the results are reported in Table 1. A distinct mortality of *Artemia* sp. nauplii after 24 h exposure to LRLZ20PSL1 aqueous extract was observed, to a similar extent as the toxic positive control (95 vs 100 %, respectively); this consisted in the

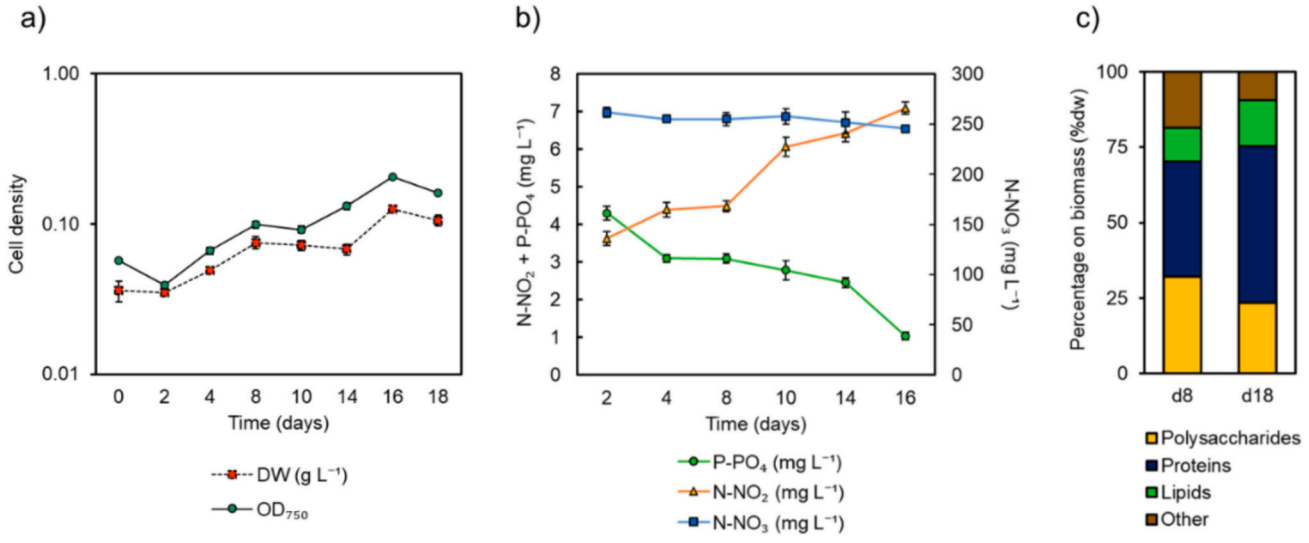

Fig. 2. Cultivation and biomass characterization of LRLZ20PSL1, a) cyanobacterial growth as dry weight (DW, g L^{-1}) and turbidity at 750 nm (OD_{750}) in logarithmic-scale; b) macronutrients consumption of nitrogen (N-NO_2; N-NO_3), and phosphorous (P-PO_4); c) biochemical composition of the biomass as lipids, proteins, and polysaccharides (%dw).

Table 1
Preliminary toxicity assessment per means of *Artemia* sp. bioassay.

Strain	Toxin production	%dead (24 h exposure)
cf. *Anabaena* sp.	No	0.0 ± 0.0
cf. *Dolichospermum* sp.	No	0.0 ± 0.0
Arthrospira platensis	No	0.0 ± 0.0
LRLZ20PSL1 strain	Unknown	95.0 ± 7.0
Planktothrix agardhii	Unknown	5.0 ± 7.0
Planktothrix rubescens	Unknown	0.0 ± 0.0
Aphanizomenon klebahnii	Unknown	0.0 ± 0.0
Microcystis aeruginosa	MCs	0.0 ± 0.0
Ostreopsis cf. *ovata*	OVTXs	100.0 ± 0.0
Phaeodactylum tricornutum	No	0.0 ± 0.0
Blank	–	0.0 ± 0.0

Data are expressed as the average percentage of dead individuals on the total in the tested volume (1 mL). Positive toxic control = *O.* cf. *ovata*; negative control = *P. tricornutum*; blank = artificial sea water (SW 30 ‰). Production of toxins: No = known cyanotoxins not detected in the sample, namely microcystins, anatoxin-a, cylindrospermopsins, and saxitoxins, Unknown = the strain was not subjected to chemical analyses for the determination of the major cyanotoxins (i.e., microcystins, anatoxin-a, cylindrospermopsins, saxitoxins), therefore the production of cyanotoxins is undetermined; MCs = hepatotoxic microcystins; OVTXs = ovatoxins.

marine benthic dinoflagellate *O.* cf. *ovata* known to produce palytoxin-like compounds (i.e., ovatoxins) highly toxic for this crustacean [43]. None of the prepared aqueous extracts of the other tested cyanobacteria affected *Artemia* sp. since no mortality was observed, including a microcystin-producing strain of *M. aeruginosa*; these results suggest that the toxic effect caused by the isolated LRLZ20PSL1 strain could be related to the production of hydrophilic metabolites, perhaps previously not identified in cyanobacteria. To deeply investigate the LRLZ20PSL1 strain toxicity, methanolic extracts of the collected biomass have also been subjected to UPLC-QTOF analysis in search for the major cyanotoxins' classes, nonetheless no matches were found for microcystins, nodularins, anatoxin-a, saxitoxins, and cylindrospermopsins.

3.3. Genome analyses

An in-depth characterization of the new isolate LRLZ20PSL1 strain was performed through genomic DNA extraction and sequencing. The summary statistics of the assembled genome produced are reported in Table 2. The final genome of approximatively 4.5 Mb had a completeness of 94.64 %, a relatively low contamination rate of 0.31 %, and it was characterized by a GC content of 55.01 %.

After re-annotation with RAST pipeline, a total of 3609 protein coding sequences (CDS) were found, whose functions are shown in Fig. 3. Genes involved in the synthesis and in the metabolism of carbohydrates, proteins, and pigments cumulatively accounted for 50 % of the total, whereas those for lipids only contributed to <3 %. As for cyanobacterial growth, 35 % of the genes were implicated in cell division, DNA/RNA metabolism, and photosynthesis plus respiration, while genes for macro- and micronutrients uptake and metabolism, as well as membrane transporters, accounted for 12 % of the total. Finally, <1 % of the annotated genome was dedicated to secondary metabolism, nevertheless about 2.5 % of the total CDS found were domains of unknown function (DUF).

As expected from the morphological observations, the isolated strain fitted into the cyanobacterial family of Pseudanabaenaceae, as the genome-based investigation revealed that the strain was closely related to the genus *Limnothrix*, with 89 % of the reads assigned to "unclassified *Limnothrix*" (Fig. S2). Phylogenomic analysis, encompassing all currently sequenced genomes of *Limnothrix* spp., revealed a polyphyletic distribution among the genomes classified within this genus (Fig. S3). The strain LRLZ20PSL1 is included in a coherent subclade, in close phylogenetic proximity with nine other *Limnothrix* genomes. Based on 16S rRNA extracted from the whole genome, a phylogenetic tree was produced using the first 50 best-fitting BLAST hits (Fig. 4), showing that the cyanobacterium here isolated clustered together with strains of *L. redekei* from the Mediterranean area; in particular, the closest related hits were originated from Lake Trasimeno in Italy (FM177493.1, 100.00 % identity, 98 % coverage, based on BLAST) and from Lake Kastoria in Greece (AJ505941.1, AJ505942.1, and AJ505943.1, both with 99.8 % identity, 96 % coverage). Therefore, the isolate of the present work was classified as *L. redekei* and represents the first strain within this species whose entire genome was sequenced.

As for secondary metabolites biosynthesis, a total of 33 BGCs were predicted (Table S2), although only four regions contained well-defined clusters, namely two BGCs related to the synthesis of terpenes (46.3 and 56.1, possibly carotenoids), one for cyanobactin, i.e. a class of cyanobacterial peptides (51.2), and one for *trans*-AT polyketide synthetase (24.1). Among these well-defined clusters, only the region encoding for the cyanobactin found a 71 % similarity to a known compound from the MIBiG data repository, i.e., limnothamide, a ribosomally synthesized and post-translationally modified peptide (RiPPs) produced by a Brazilian strain of *Limnothrix* sp. (i.e., CACIAM 69d).

The presence of a cyanobactin, highlighted by the genome-mining approach, was analytically confirmed based on its class characteristics and mass spectra in the *Limnothrix redekei* LRLZ20PSL1 extract and thus named "limnobactin". This compound had a molecular formula of $C_{77}H_2N_{21}O_{22}$, corresponding to a molecular weight of 1682.82 Da with a main ion at 841.915 *m/z* (Fig. 5).

As for the *trans*-AT PKS cluster, this region encoded for a polyketide synthetase in the *trans*-acetyl transferase conformation. Interestingly, the *trans*-AT PKS cluster found in the genome seemed to be highly conserved within *Limnothrix* genus, showing a similar modular composition (Fig. S4). Overall, although a product could not be found for this region, this evidence may suggest that the end product of the *trans*-AT PKS could be important for the secondary metabolism of *Limnothrix* species.

3.4. Bioassay-guided fractionation of isolate extracts tested on Artemia sp. nauplii

In an attempt to identify the bioactive compounds present in the biomass extract of LRLZ20PSL1 strain (50:50 *v*/v, solvent/water), the extract was fractionated by HILIC chromatography, and the toxicity of the resulting 12 fractions (F1–12) was assessed by *Artemia* sp. bioassay. The main absorbance peak was observed in F1, which likely corresponded to highly solvent-miscible photosynthetic pigments, whereas other unknown peaks were found in F2, F4, and F5 (see UV spectrum in Fig. S5). The toxicity of each fraction was then tested through *Artemia* sp. bioassay, and the results are reported in Fig. 6. The majority of the toxicity was retained in fraction 2, corresponding to 60–75 % mortality of the tested organisms. Lower percentages of dead individuals, in the

Table 2
Main characteristics and summary statistics of the assembled genome obtained from cultured LRLZ20PSL1.

Genome Size (Mb)	Completeness (%)	Contamination (%)	Total length (bp)	N. of contigs	G + C (%)	N50 (bp)	L50	CDS
4.5	94.64	0.31	4,530,684	81	55.01	178,346	9	3647

G + C (%) = percent content of guanine-cytosine; N50 = length of the shortest contig among the longest ones that cover the 50 % of the total assembly length, such that contigs of longer or equal lengths include half the bases of the assembly; L50 = number of contigs longer or equal to N50 length that therefore include half the bases of the assembly; CDS = number of predicted protein coding sequences.

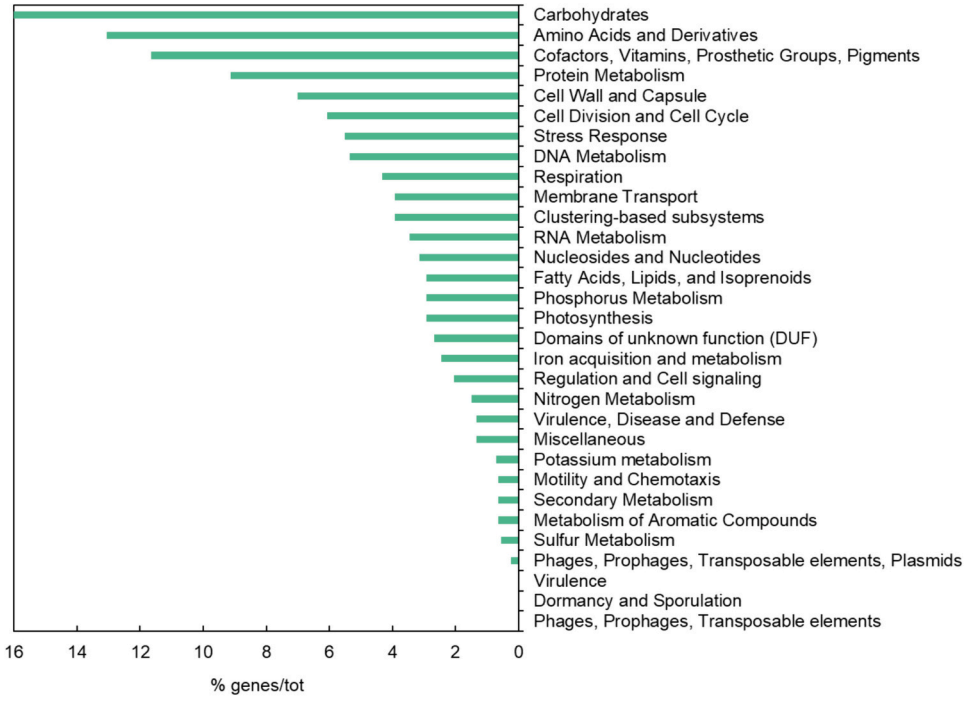

Fig. 3. Functions of the genes and percentage on the total CDS of the annotated LRLZ20PSL1 genome.

range 3–10 %, were reached in tested replicates of fractions 1, 7 and 8, whereas no mortality was observed for all the other fractions. Neither the negative control (*P. tricornutum* cells) nor the blank (dried ACN 75 % resuspended in artificial sea water 30 ‰) exerted lethal effects on *Artemia* nauplii, with neglectable mortality rates (0–5 %). It was hypothesized that, since the "limnobactin" eluted at 2.63 min (Fig. 5), this compound would have been in fraction F3 (i.e., 2.5–3.5 min, Table S1); thus, its possible implication in the extract's toxicity was excluded. Conversely, based on a spectrophotometric investigation of the peaks found at 1.65 and 2.01 min, the main components of the toxic fraction F2 appeared to have carotenoid-like characteristics, whereas other components could not be discerned (Fig. S6).

4. Discussion

4.1. Biochemical and genomics characteristics

Compared to other well-studied cyanobacteria, *Limnothrix* is a less investigated genus (222 vs 9534 hits in Scopus for "*Limnothrix*" and "*Microcystis*", respectively as of June 2024), and few data are available regarding its growth and biomass composition. In the present work, the productivity of the isolated strain under the tested laboratory conditions resulted higher than those reported for another *Limnothrix* sp. strain, i.e., 6.5 vs 1.91 mg L^{-1} day^{-1} [65], possibly as a consequence of optimized growth conditions. The biochemical composition of the biomass here observed did not differ much from the composition of two other *Limnothrix* strains (protein 21–36 %, 18–26 % carbohydrates and 5–7 % lipids [66]), confirming that this cyanobacterium may yield a higher amount of proteins with respect to the lipid content. The fact that *Limnothrix* is an under-investigated cyanobacterium is even more evident in relation to the data available on its genome. As of June 2024, >5500 cyanobacterial assembled genomes were available on the NCBI database, of which only 10 were related to the genus *Limnothrix*. Interestingly, the main characteristics of the sequenced genome in this study were similar to those of the other *Limnothrix* genomes available (i.e., size 4.5 Mb, GC content of 55 %), despite the geographical origin of the strains (Italy vs Brazil, Singapore and China). Nonetheless, the closest 16S rRNA BLAST hits for the isolate were for strains of *L. redekei* derived from Lake Trasimeno (central Italy) and Lake Kastoria (Greece), both described as shallow lakes with a variable depth (5–6 m on average [14,67]), suggesting a possible environmental separation of strains collected in different macroscopic areas. Other common characteristics to the *Limnothrix* genomes deposited in public databases were found in the genes that they harbour. For example, similarly to the other *Limnothrix* spp., this cyanobacterial strain was found to have genes for nitrate, cyanate and ammonium transporters, as well as for cyanophycin and polyphosphate, intracellular polymers used by cyanobacteria to store nitrogen and phosphorus during nutrient deficiency [68–70]. Although the cyanobacterial growth was here followed for only 18 days, this evidence could partially explain why the cyanobacterium did not consume all the nutrients, also suggesting a possible ecological advantage in nutrient-limited environments.

4.1.1. Taxonomic issues within Limnothrix genus

As previously reported, the correct identification of *Limnothrix* spp. can be sometimes challenging because of their small dimensions, high morphological plasticity and similarities with other Pseudanabaenaceae, as well as their diverse behaviour under environmental or culture conditions [14,17–19]; all aspects that may lead to misclassification of this genus, especially among non-taxonomists. Similarly, the resolution of taxonomic uncertainties at molecular level can be also affected by the presence of sequences deposited in reference databases (e.g., GenBank) that were originally misidentified by submitters [71]. For instance, the deposited sequence of *Planktothrix* sp. FP1 (AF212922.1) was found to be likely misclassified, as it shared common characteristic with *Limnothrix* according to both morphology and molecular data [14]. The strain NIVA CYA 277/1 originating from Sweden was firstly described as the type species for *L. redekei* [72]. Nevertheless, it is worth mentioning that, based on 16S rRNA phylogeny reported in the present work, the NIVA CYA 277/1 strain diverged from many deposited sequences of *L. redekei*, including the isolate LRLZ20PSL1. This was also observed for three isolates from Lake Katoria in Greece, while the NIVA CYA 277/1 strain clustered in the "Pseudanabaena" clade [14]. Similarly, several other *Limnothrix* isolates from Donghu Lake (China) clustered together in another clade, identifying them as *L. planctonica* [73]. In this work, our strain clustered with several *L. redekei* of the Mediterranean area,

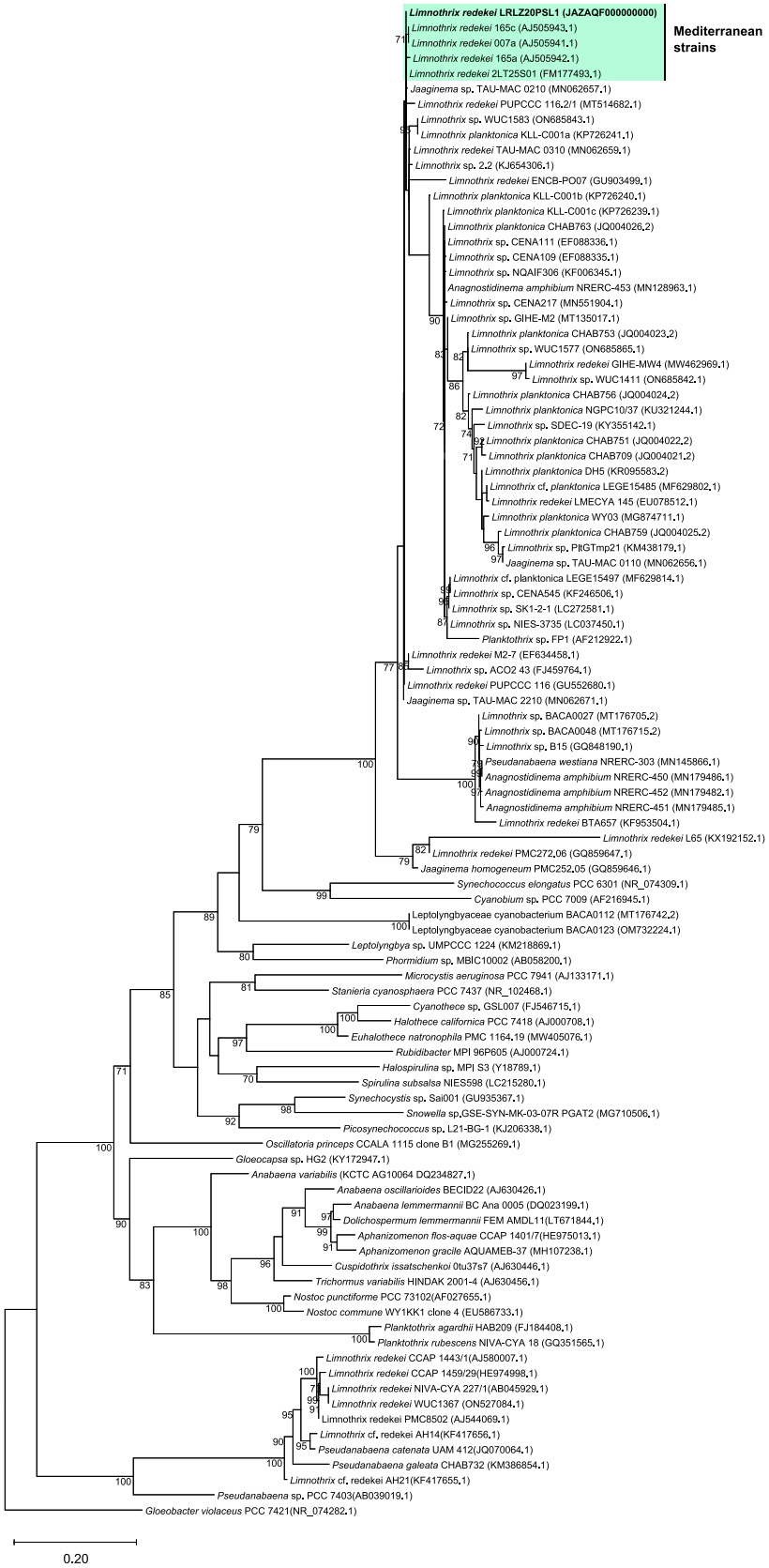

Fig. 4. Phylogenetic tree of 16S rRNA sequences of the isolated *Limnothrix redekei* LRLZ20PSL1 in the present study. *Gloeobacter violaceus* PCC 7421 (NR_74282) was used as an outgroup. Only bootstrap values above 70 % are shown. Accession numbers of sequences available in public database are reported in parentheses.

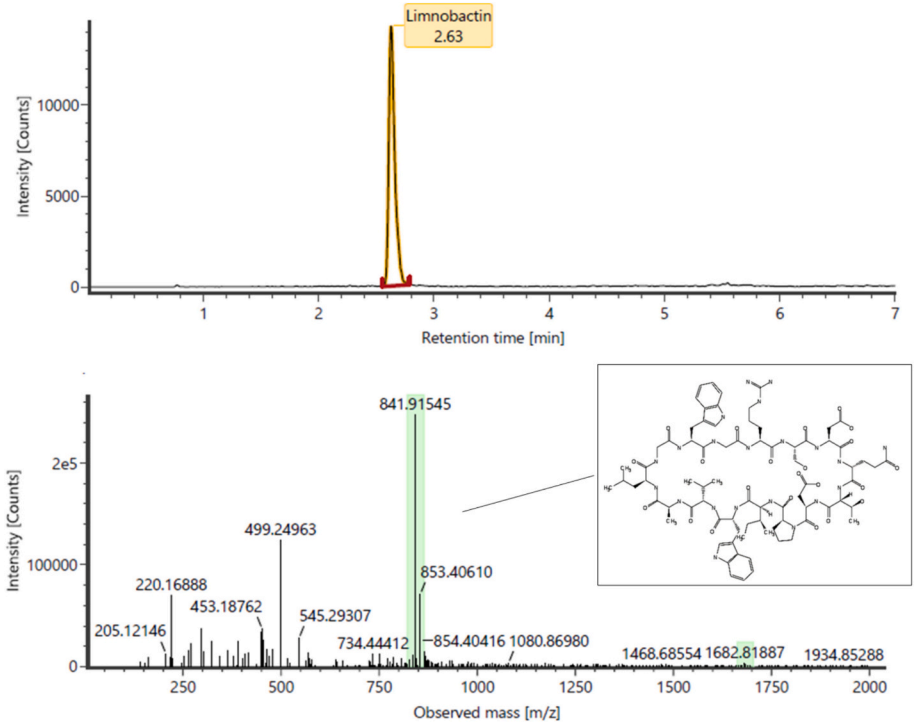

Fig. 5. Chromatogram peak, related mass spectrum and chemical structure reconstruction of the cyanobactin produced by the isolated LRLZ20PSL1 strain from Lakes Zaganti ("limnobactin").

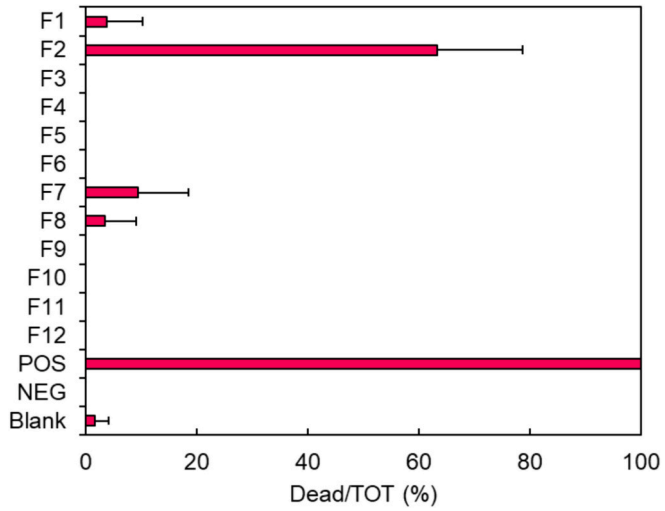

Fig. 6. Results of the *Artemia* sp. bioassay after the exposure to the fractions obtained from the cyanobacterial isolate. Data are reported as means ± standard deviations of triplicates. F1–12 = fractions collected from 1 to 12; POS = positive toxic control (*O.* cf. *ovata*); NEG = negative control (*P. tricornutum*); Blank = dried elution solvent (75 % ACN) resuspended in artificial sea water (SW 30 ‰).

together with some *L. planctonica* mainly from China [73]. In previous works, it was highlighted how the geographical origin of isolates could determine a separation among *Limnothrix* clades, including for instance Asian, Brazilian and European strains [14,73]. Thus, considering the geographical proximity of our isolate and the morphological features observed, we concluded that the strain LRLZ20PSL1 could be identified as *L. redekei*. However, the taxonomy of *Limnothrix* genus remained to be further investigated to resolve its polyphyletic nature.

4.2. Toxicity of *Limnothrix* aqueous extracts

Ecotoxicological investigations here performed, highlighted a putative toxicity of the isolated strain, with particular reference to aqueous extract of the biomass. Similarly, toxicity of aqueous extracts of freshwater, brackish, marine, and symbiont cyanobacteria towards *Artemia* nauplii were previously reported, and in most of the cases it was observed in species closely related to the Pseudanabaenaceae family [74–78]. Conversely, species belonging to the genus *Limnothrix* are traditionally considered non-toxic, although they may co-dominate shallow water bodies alongside toxic strains of *Planktothrix* sp. that, on the other hand, is more often capable of producing microcystins [11,13]. Consequently, investigations on toxins and toxic effects are only diverted to established toxic species and well-known cyanotoxins. The cyanobacterium here studied did not harbour biosynthetic gene clusters for the major cyanobacterial toxins microcystins, saxitoxins, anatoxins, and cylindrospermopsins, as also previously observed for other *Limnothrix* spp. [79]; on the contrary, it was recently reported that a *Limnothrix* sp. strain isolated from a freshwater lake in Iran was capable of producing cylindrospermopsin [80]. Interestingly, a strain of *Limnothrix* sp. (AC0243) isolated from a dam in Australia has been reported as potentially toxic [18,81,82]. The toxicity of aqueous extracts of the Australian strain was investigated by means of *in vitro* bioassays and resulted in the inhibition of cell-free protein synthesis and the reduction of cellular ATP in kidney cells [18]; additionally, toxic effects were also tested on animal models, finding that the cyanobacterial extract determined damages to the liver, lungs and gastrointestinal tract of mice, and shortened the life-span of tadpoles likely associated to general histopathological injuries [81,82]. The *Limnothrix* AC0243 strain was not capable of producing known cyanotoxins that could explain the toxic effects observed (i.e., cylindrospermopsin), thus the authors hypothesized the synthesis of a putative new water-soluble toxin, named "limnothrixin". Similarly, the toxic effects observed in the present study were also associated with aqueous extracts of the cyanobacterium, since the *Artemia* sp. bioassay was conducted by testing aqueous extracts of the isolate. Therefore, *Limnothrix* LRLZ20PSL1 may be capable of

producing similar toxic compounds as those observed for the Australian strain. Nonetheless, there is no resolved structure for the putative toxin "limnothrixin" [82], so that a comparative analysis per means of chemical methods was not possible in the present study.

4.3. Insight into the putative toxic metabolites in Limnothrix fractions

Cyanobacterial toxins belonging to different chemical classes (polyketides, alkaloids, lipopolysaccharides) can be produced via several pathways, including more frequently PKS pathways [4,27,28]. Here, a *trans*-AT PKS gene cluster was found in the studied strain, which seemed to be highly conserved within the *Limnothrix* genus, although no end product is known yet. This evidence was previously reported by Lima et al. [79], who also suggested that the *trans*-AT PKS region may be a potential candidate for the synthesis of "limnothrixin". This hypothesis may be supported by the fact that polyketides are often involved in the toxicity of various organisms, including fungi, marine dinoflagellates, and cyanobacteria [27,83,84]. At the same time, given that a structure for the *trans*-AT PKS product is not available, it cannot be excluded that the observed toxic effects could be due to a variety of compounds present in the aqueous extracts and contributing to the observed toxicity. In addition to polyketides, cyanobactins are small and cyclic peptides ribosomally synthesized by cyanobacteria via post-translational modifications of precursor proteins, and some of them may exhibit a broad range of toxic effects (for a detailed list, see [85]). Nevertheless, the cyanobactin found in the present study ("limnobactin") was eluting in a fraction that did not exert lethal effects on *Artemia* sp. nauplii and was not involved in the observed toxicity. Conversely, the main absorption peaks of the toxic fraction F2 had carotenoid-like characteristics. It is known that a carotenoid-rich diet can improve the quality and health of *Artemia* and other aquatic organisms [86], therefore this component was unlikely responsible for the observed toxicity. However, it cannot be excluded that other compounds were present in F2, whose absorption spectra may have been overshadowed by those of the pigments. Because the fractions were investigated by UV absorption, a technique strongly influenced by these shading effects, further analytical investigations by LC-MS methods are necessary to identify the toxic compounds of *Limnothrix* extract. Although the discovery of novel toxic compounds can be challenging, the identification of the fraction responsible for the observed toxicity may be used to guide future investigations aimed at solving its structure and toxicity, for instance by testing and increasing the biomass of the cyanobacterium under different growth conditions and integrating in-depth chemical analyses onto the revealed toxic fraction.

5. Conclusions

A potentially toxic *Limnothrix redekei* from a shallow Italian drinking water source was here studied by an integration of biological, ecotoxicological, chemical and genomics methods. To the best of our knowledge, this is the first fully sequenced *Limnothrix redekei* from the Mediterranean area and Europe in general. Indeed, the Paseudanabaenaceae family lacks many sequenced genomes and the other publicly available *Limnothrix* genomes are not classified at the species level. Therefore, these results could provide useful insights on an underinvestigated but ecologically important genus of freshwater cyanobacteria. Although the putative toxic metabolite produced by the isolate was not yet elucidated, genome-wide investigations had generally improved previous analyses carried out, and a new cyanobactin was found. Since toxic effects on the test organism (i.e., crustaceans *Artemia* sp.) were observed in aqueous extracts of the biomass, potential implications for drinking waters should not be excluded and further characterization of the putative toxin(s) should be performed. These findings highlight the importance of integrated methodologies for the study of a new cyanobacterial strain, from isolation and cultivation to genomics, chemistry, and ecotoxicology, to overcome the limitation of a single approach and to expand the knowledge of less-investigated species revealing their potential valorization together with their toxigenic risks.

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

Data will be made available on request.

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CRediT authorship contribution statement

MS: Conceptualization, Investigation, Visualization, Data Curation, Formal Analysis, Writing – Original Draft, Writing - Review & Editing; **TKS**: Investigation, Writing - Review & Editing; **ED**: Investigation, Visualization, Formal Analysis; **MW**: Investigation; **DF**: Resources, Writing - Review & Editing; **KS**: Resources, Writing - Review & Editing; **LP**: Conceptualization, Data Curation, Supervision, Writing - Review & Editing; **RP**: Conceptualization, Supervision, Resources, Funding acquisition, Writing - Review & Editing. All authors approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103648.

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