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Adriatic cyanobacteria potential for cogeneration biofuel production with oil refinery wastewater remediation

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ABSTRACT

Among cyanobacteria isolated from Northern Adriatic five strains were able to grow on oil refinery wastewater. Based on the sequencing of the internal transcribed spacer (ITS region) and morphological features strains were identified as *Synechococcus* sp. MK568070, *Phormidium lucidum* MK568072, *Neolyngbia* sp. MK568073, *Pseudanabaena sp*. MK568071 and *Spirulina subsalsa* MK573248. Selected marine cyanobacteria were tested over a range of ammonium (NH_4^+) concentrations simulating oil refinery effluent conditions. Three strains demonstrated high growth potential in a range 0.8–2.0 mM NH_4^+. Maximum biomass concentrations in stationary phase were in a range 176–590 mg/l, whereas total lipid yields were in a range 7.63–21.40% of dry weight. Although all species satisfy the criteria for biodiesel production the average degree of unsaturation was lowest in *Synechococcus* sp. MK568070 and *Neolyngbia* sp. MK568073. High proportion of polyunsaturated alpha-linolenic acid found in *Spirulina subsalsa* MK573248 opens up considerations for nutritional, aquaculture or other biotechnology applications of that species.

1. Introduction

The overall growth of the population on Earth, increasing energy demand and at the same time depletion of fossil fuel reserves have put immense pressure on the consumption of non-renewable energy sources. As an answer, pioneering ideas and practices of producing biofuel were pursued since the end of the 20th century. Moreover, cultivation of oil-accumulating microbes, such as microalgae, bacteria, yeasts and other fungi which can convert substrates such as carbon dioxide, sugars, and organic acids to oils, raised great expectations in tackling the mentioned problems [1–4]. Aquaculture of microalgae has several advantages over the production of 1st and 2nd generation biofuels such as rapid biomass growth, the high proportion of lipids and high productivity per exploited area [5,6]. The production of microalgal biofuels can reduce the dependency on fossil fuels, and in addition contribute to mitigation of the atmospheric CO_2 increase and greenhouse related climate changes [7]. Hence, there is an urgent need for the methodological advancements in order to increase production efficiency of microalgal biomass and ensure its economic sustainability [8].

Microalgae, in general, have shown great growth potential by using nutrients from wastewater [9–12]. Recent studies have demonstrated that without co-products credits, the energy consumed to produce microalgal biodiesel is higher than the energy in the biofuel itself. It is demonstrated that the favorable life cycle energy consumed/energy produced ratio can be obtained by replacement of conventional biological wastewater treatment units [13]. Upendar et al. [7] have optimized the effectiveness of *Synechococcus* sp. NIT18 for biofixation of CO_2 from flue-gas in proportions as high as 10–15%. Along these lines, many studies focus on finding integrated solutions to increase the benefit/cost ratio by using the same feedstock of microalgae for multiple purposes. Interesting solutions of cogenerated production of bioethanol, methane, and electricity in a downstream of the biodiesel production at the wastewater treatment plants are significantly increasing the overall value of produced microbial biomass [14,15]. Furthermore, combining seawater and wastewater as a growth medium for marine microalgae can be even more cost-effective in comparison to using freshwater microalgae [6].

Some wild strains of microalgae can naturally produce a high proportion of valuable compounds such as lipids, proteins, and carbohydrates [16–18]. Genetic engineering opened up the possibility for production of targeted molecules in higher concentrations. These

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possibilities have benefited food, chemical, and pharmaceutical industries [19–22]. Although genetically modified (GM) strains of microalgae show higher potentials in the production of certain compounds of interest, there are some crucial advantages in the cultivation of indigenous microalgae. Mass production of indigenous microalgae avoids possible complications and risks related to the use of GM organisms. For example, a devastation of the existing fauna could occur in the case of transfer of GM microalgae resistant to viruses from the open ponds to the natural water bodies [22].

For specific products and/or purposes, microalgae can be cultivated as monoculture, mixed cultures or in consortium with cyanobacteria and/or heterotrophic bacteria [23–26]. Cyanobacteria are a group of photosynthetic prokaryotes having the ability to use a wide range of inorganic and organic substrates for their growth. Their capacity for sequestration of CO_2 while degrading e.g. phenol and other pollutants [27–29], including the crude oil, directly or in consortium with heterotrophic bacteria, and supplying the oxygen, are especially attractive traits [30,31]. Cyanobacteria are, similarly to many microalgae [32,33], especially efficient in dealing with heavy metals toxicity, and therefore used to decrease concentrations of heavy metals in the wastewater [34–37]. Cyanobacteria, in general, prefer relatively high ammonium (NH_4^+) concentration for their growth, in line with their relatively high tolerance to ammonia (NH_3) in comparison to most of the microalgae classes [38]. This makes them especially attractive for application in wastewater remediation.

Motivated by all the above mentioned, we have isolated several indigenous species of marine cyanobacteria from the highly eutrophic coastal zones of Northern Adriatic, tested their growth ability on oil refinery wastewater and analyzed their biochemical composition relevant for estimating biodiesel production potential. Here, we present the phylogenetic trees of isolated marine cyanobacteria species, followed by the rapid assessment of their tolerance to range of NH_4^+ concentrations. Finally, we give the overview of lipid, carbohydrate and protein content, as well as the fatty acid profile of the representative species of different cyanobacteria orders that showed high tolerance and/or growth under increased NH_4^+ concentrations, discussing their potential in wastewater remediation and biofuel production.

2. Materials and methods

2.1. Isolation and cultivation of cyanobacteria

In order to isolate the cyanobacterial species already adapted to disturbed environment, the surface seawater was sampled in the proximity of wastewater outlet of the INA PLC refinery plant, located in Urinj Bay, in May 2017. Samples of seawater were distributed to 0.5 l transparent glass bottles and kept under natural lighting and temperature, until growth of algae was observed (approx. 30 days). Proliferated samples were transferred to various liquid culture media enriched with 10% of untreated industrial wastewater. This test allowed for better selection of microalgae that have the ability to cope with negative impact of wastewater. After two weeks, the cyanobacteria that expressed tolerance to wastewater were purified through a series of transplantations between solid and liquid media, until separate cultures were obtained. Two different media, modified PCR S-11 [39] and BG-11 [40] were used. The base of both media was aged seawater, having salinity of 38 psu, filtered at 0.2 μm and autoclaved, with the addition of Difco BactoAgar for solid media. After purification, the isolated cultures were cultivated in the most suitable medium, with regular 2 week subculturing, under the ambient temperature of 25 °C, the cool white light flux of 70 μmol photon m^{-2} s^{-1} and 14:10 day:night light regime.

Five representative species belonging to different orders of cyanobacteria, showing wide tolerance to NH_4^+, were cultured for further biochemical analyses. The isolates were cultured in adequate liquid growth medium (BG-11 or PCR S-11) with 10% addition of untreated oil refinery wastewater. The biomass was harvested in stationary phase of algae growth.

2.2. Identification of species

The main identification tool was 16S rRNA molecular analysis based on the sequencing of the internal transcribed spacer (ITS) region. To complement and confirm the identification, cultures were observed under the inverse light (Boeco, Germany) and epifluorescence microscope (Carl Zeiss, Germany).

2.2.1. DNA extraction from cyanobacterial cultures

Total DNA was extracted from cyanobacterial cultures by using NucleoSpin Plant II kit (Macherey-Nagel, Germany) following manufacturer's instructions with slight modifications. After centrifugation of the cyanobacterial cultures for 10 min at 600*g*, 100–300 mg of the pellet obtained was further resuspended in a TEN buffer (50 mM Tris-HCl of pH = 8.00, 5 mM EDTA and 50 mM NaCl in final concentrations). 1% of N-lauroylsarcosine sodium salt (Sigma, USA) was added and tubes were incubated for 1 h at room temperature with occasional vortexing. Due to possible inhibition of subsequent PCR reactions by N-lauroylsarcosine sodium salt, pellets were washed four times with TEN buffer. Disruption of the cells was done by addition of 3 small beads to the dry pellets with subsequent shaking on a Vortex-Genie 2 (MoBio, USA) for 5 min at maximum speed. Homogenate was then resuspended in 100 μl TEN buffer and incubated for 30 min with lysozyme (0.5 mg/ml final concentration) at 37 °C. After addition of Buffer PL1 (NucleoSpin Plant II kit, Macherey-Nagel, Germany) and RNase (Qiagen, Germany) samples were incubated for additional 30 min at 65 °C. Last incubation for 30 min at 55 °C included addition of Proteinase K (Promega, USA). All further steps were carried out following NucleoSpin Plant II protocol. Extracted DNA was quantified on BioSpec-nano (Shimadzu, Japan) while its integrity was checked by electrophoresis on 1% agarose gel.

2.2.2. PCR amplification of cyanobacterial ITS region

Internal transcribed spacer (ITS) region was PCR amplified from the extracted DNA by using primers CYA106F (5′-CGGACGGGTGAGTAAC GCGTGA) and 23S0R (5′-CTTCGCCTCTGTGTGCCTAGGT) being specific for cyanobacterial ITS region [41]. PCR was done in a final volume of 25 μl containing 12.5 μl of the EmeraldAmp Max PCR Master Mix premix (TaKaRa Bio, Otsu, Japan) provided with polymerase, optimized buffer, $MgCl_2$ and dNTP mix (composition is proprietary), 1 μl of each of the primer (20 μM), 2 μl of the extracted DNA and redistilled H_2O. Amplification was carried out by following a program that included: 94 °C for 2 min; 35 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 2 min and final extension at 72 °C for 7 min [41]. Obtained PCR amplicon of 2000 bp, cleaned from the gel by using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), was cloned into pGEM®-T Vector Systems (Promega, USA) following manufacturer's instructions and transferred into *E. coli* cells. From each of the cyanobacterial cultures, clone library was constructed, and up to 10 clones were subjected to RFLP analysis by using *Alu*I (Promega) restriction enzyme. Clones showing different pattern were sent to Macrogen (Amsterdam, The Netherlands) for sequencing of the amplified ITS region with a reverse primer CYA-1380R (5′-TAACGACTTCGGGCGTG ACC) [41]. Retrieved sequences were edited manually and compared to the NCBI Genbank database using Blast tool.

ITS sequences obtained from cultured cyanobacterial strains, along with those selected from the NCBI database, were aligned using ClustalX 2.1 software (http://www.clustal.org/clustal2/). MEGA 7.0.26 [42] was used to perform phylogenetic analysis. The evolutionary history was inferred using the Neighbor-Joining method [43]. According to the lowest Bayesian information criterion (BIC) scores [42] best fitting model was chosen for downstream analysis. The reliabilities of phylogenetic relationships were evaluated using a nonparametric

bootstrap analysis with 1000 replicates. Phylogenetic tree was visualized using Interactive Tree of Life software (https://itol.embl.de/) [44].

2.3. Assessment of NH_4^+ tolerance

In order to determine which of the isolated cyanobacteria have the highest potential for oil refinery wastewater remediation, all strains were subjected to the rapid assessment of the ammonium tolerance. Range from 0.8 to 2.0 mM NH_4^+ was chosen to cover the common annual range of NH_4^+/NH_3 concentration in the oil refinery wastewater, i.e. the minimum and the maximum concentration to be expected when cultured in wastewater under open pond conditions.

Four solutions of artificial seawater (ASW) with fixed NH_4^+ concentrations (0.8, 1.2, 1.6 and 2.0 mM) were prepared and arranged in microwell plates in triplicates. Dissolved inorganic nitrogen (DIN), in which ammonium/ammonia had a share of more than 99%, and dissolved inorganic phosphorus (DIP) concentrations were adjusted by addition of NH_4^+ (800 mM) and PO_4^{3-} (100 mM) stock solutions to achieve the precise concentrations of NH_4^+, as well as to simulate the DIN/DIP ratios of PCRS-11 and BG-11 media used in cultivation of isolated cyanobacteria. Each microwell contained 250 μl of respective ASW solution and was inoculated with 20 μl of cyanobacterial culture, while blanks contained 270 μl of only respective ASW solution without inoculate. According to Bower and Bidwell [45], at pH of 8.3 and ambient temperature of 25 ± 1 °C, concentrations of NH_3 (toxic form of interest) in the prepared solutions were approx. 10% of the targeted NH_4^+ concentrations. As a proxy for growth, optical density at λ = 690 nm (OD_{690}) was recorded daily using Multiskan Ascent Plate Reader (MTX Lab Systems, USA). The final daily OD_{690} measurements were calculated by subtracting the average OD_{690} of the blanks from the average OD_{690} values of inoculated wells after omitting the eventual outlier values among triplicates, detected by Modified z-score method [46].

2.4. Carbohydrate determination

Total carbohydrate content of each cyanobacterium culture was quantified using the anthrone method described in Herbert et al. [47]. Dried pellets obtained from 1 ml of culture in triplicates, were treated with 1 ml of 1 M NaOH by vigorous vortexing. The mixture was kept in the boiling water bath for 5 min and then sonicated at 40 kHz for five cycles of 30 s. The crude homogenate was centrifuged at 3000 rpm for 5 min, and 100 μl of supernatant from each replicate was used for the determination of carbohydrate content. The anthrone reagent was prepared by dissolving 0.2 g anthrone in 5 ml of absolute ethanol and addition of chilled 75% H_2SO_4 up to the volume of 100 ml. An aliquot of 100 μl of sample was added to separate test tubes, volume was topped up to 5 ml by adding the 900 μl sterile distilled water and 4 ml of anthrone reagent. After 10 min at room temperature (in the dark) samples were boiled for 10 min, and immediately ice-chilled for 10 min. Finally, the absorbance of each sample was measured at 625 nm. The corresponding absorbance of unknowns was interpolated with the absorbance of glucose standards to get the concentrations of carbohydrates produced by each species.

2.5. Protein determination

The protein content in the microalgal samples was determined by using a Micro-Biuret method described by Itzhaki and Gill [48]. Briefly, 1 ml of 0.5 M NaOH was added to the dried algal pellet, in triplicate, and the samples were incubated at 80 °C for 10 min. Pellets were cooled, centrifuged (3000 rpm, 5 min) and the procedure was repeated 3 times, applying 100 °C in the last incubation step. After supernatants were collected, aliquots of 300 μl were added to 500 μl of deionized H_2O and 200 μl of reagent (0.21% $CuSO_4x5H_2O$ in 30% NaOH). Absorbance of each sample was measured at 310 nm. The corresponding absorbance of unknowns was interpolated with the absorbance of BSA standards to get the concentrations of proteins produced by each species.

2.6. Lipid extraction and fatty acid analysis

For total lipid and fatty acid analysis, 50 ml of samples were filtered on pre-combusted GF/F filters (Whatman). Prior to extraction, filters were mechanically disrupted with a tissue homogenizer in dichloromethane:methanol i.e. DCM:MeOH (2:1, vol/vol) mixture. Total lipids were extracted according to Bligh and Dyer [49]. Extraction was performed in ultrasonic water bath at 34 °C with emitting ultrasonic power of 200-400 W, in 3 × 30 min cycles. Extracts were saponified, methylated and analyzed according to Miller [50]. After addition of 1.2 M NaOH in a 50% aqueous methanol solution, the tubes were placed in a boiling bath for 30 min. After cooling, the saponificate was acidified with 6 M HCl (pH < 2), then 12% BF_3 (in MeOH) was added in saponificate that was immediately heated for 5 min in a boiling water bath. After cooling, the fatty acid methyl esters (FAME) were extracted in DCM. FAME were analyzed by gas–liquid chromatography (GLC) on a 6890 N Network GC System equipped with a 5973 Network Mass Selective Detector with a capillary column (30 m/0.25 mm/0.25 mm; cross linked 5% phenylmethyl siloxane) and ultra-high purity helium as the carrier gas. The oven temperatures were programmed as follows: 70 °C for 5 min, then ramped up to 205 °C by 4 °C min^{-1}, holding for 4 min at 205 °C, then ramped up to 270 °C by 4°Cmin^{-1}. Column pressure was constant at 15 psi. Retention times, peak areas and mass spectra were recorded on the ChemStation Software. FAME were identified by mass spectral data and the family plots of an equivalent chain length (ECL) data for GC standards for the GC column were used. FAME mix C18-C20, PUFA1, PUFA3 standards (Supelco, USA), C4-C24 FAME standard mix, cod liver oil and various individual pure standards (Sigma, Germany) were applied.

2.7. Biodiesel properties

FAME profiles were used for calculation of the ratios between total unsaturated and saturated FAME (UNS/SAT), and the average degree of unsaturation by summing up all the products of mass fractions of each unsaturated fatty acid with their number of double bonds. The obtained values were used to predict the biodiesel properties of microalgae lipid compositional profiles as proposed by Hoekman et al. [51] and the references therein. Biodiesel characteristics, described by kinematic viscosity (KV), specific gravity (SG), cloud point (CP), cetane number (CN), iodine value (IV) and higher heating value (HHV) were calculated by the empirical equations developed for fast assessment of compositional profile of biodiesel from algal lipids [52–54] and compared to EN 14214 European Standards [55].

3. Results and discussion

3.1. Phylogenetic analysis of cyanobacterial ITS sequences

Following isolation process, 26 cultures were sent for molecular identification. As a result of the amplification and sequencing, partial nucleotide sequences of the targeted internal transcribed spacer (ITS) region were obtained, with an average length of 600 bp. Based on the sequencing results and morphological features 5 different cyanobacteria strains were identified. Sequences of the identified strains have been deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) under accession numbers MK568070-MK568073 and MK573248.

Phylogenetic analysis presented in Fig. 1 suggested that sequence of the strain named MK568070 shows high homology (> 99%) to different cultured and uncultured strains belonging to picocyanobacteria

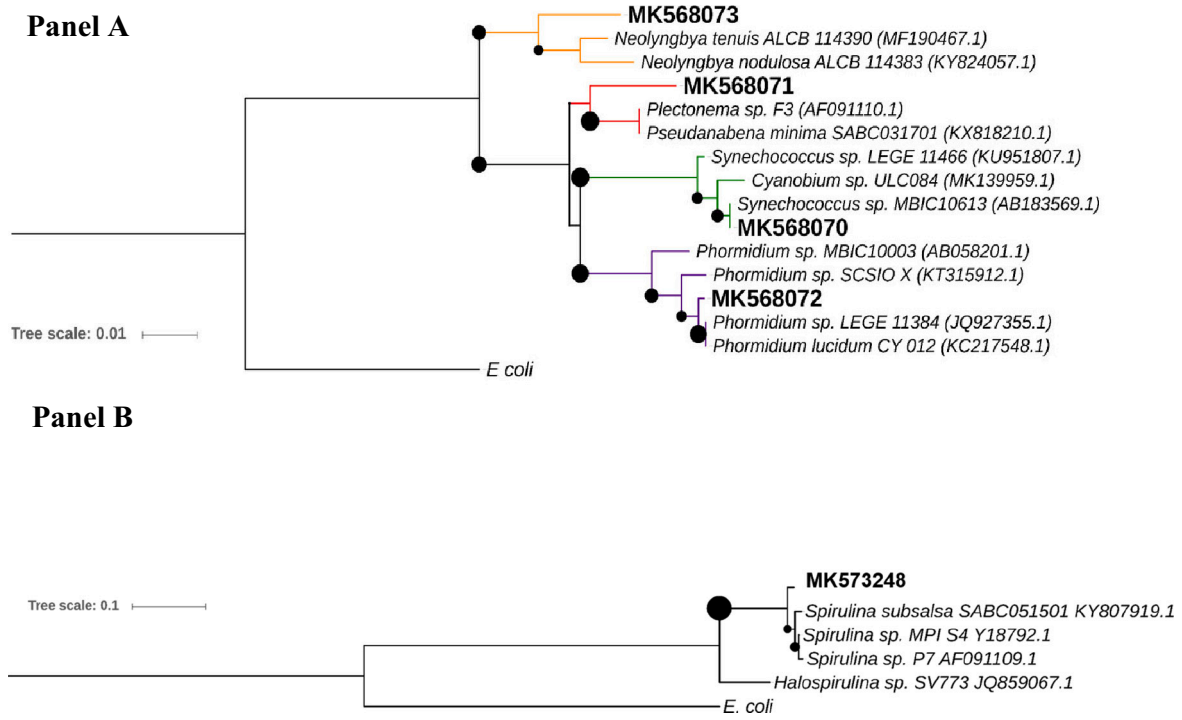


Fig. 1. Rooted phylogenetic trees constructed based on the ITS sequences obtained from cyanobacterial strains cultured in this study (shown in bold) and most closely related strains selected from the NCBI database. Phylogenetic tree for the cyanobacterial strain closely related to *Spirulina* species is shown separately in Panel B. For both trees evolutionary analyses were conducted in MEGA X using the Neighbor-Joining method and by applying Kimura 2-parameter modeled with (Panel A) or without (Panel B) gamma distribution (shape parameter = 1). The reliabilities of phylogenetic relationships were evaluated using a nonparametric bootstrap analysis with 1000 replicates. Black circles near nodes indicate bootstrap values – larger circle represents higher bootstrap value. Panel A - bootstrap values display: 0.352–1; Panel B - bootstrap values display: 0.752–0.991.

Synechococcus (*Synechococcales*; *Synechococcaceae*) as well as *Cyanobium* (*Synechococcales*; *Synechococcacea*) strains.

Phylogenetically, marine *Synechococcus* and *Cyanobium* form a tight clade within the cyanobacteria [56]. Sequence of the cyanobacterial strain MK568071 showed highest homology level (98%) to *Plectonema* (*Oscillatoriales*; *Oscillatoriaceae*) and *Pseudanabaena minima* (*Synechococcales*; *Pseudanabaenaceae*). Since *Plectonema* species are known to be distributed in clear (usually katharobic to oligosaprobic) water basins, springs or creeks, in metaphyton or periphyton, we identified the isolated culture strain as a *Pseudanabaena* species that are found to live planktic in oligotrophic, mesotrophic up to slightly eutrophic water (http://www.cyanodb.cz/). Strains MK568072 and MK568073 were identified as *Phormidium lucidum* (> 99% identity) (Oscillatoriales; Oscillatoriaceae) and *Neolyngbya* species (*N. nodulosa*, *N. tenuis*, *N. irregularis*; *Oscillatoriales*; *Oscillatoriaceae*), respectively. Strain MK573248 was phylogenetically closest related (> 97%) to the genera *Spirulina* (*Spirulinales*; *Spirulinaceae*). The sequence of this strain was likewise closely related to an array of uncultured cyanobacterial clones.

By using solely molecular identification, for some of the cultured cyanobacterial strains, we were not able to provide a clear identity. This could be partially due to problems with public databases (e.g. GenBank) for which it has been shown to contain a large number of misidentified cyanobacterial sequences with many sequences from reference strains still missing [57]. As stated by Ramos et al. [58] cyanobacterial taxonomy is currently under revision making it difficult to obtain a reliable identification at the species or genus level for some organisms. In order to provide the full species identity, it is necessary to include several molecular markers and likewise use the polyphasic approach that includes besides molecular also morphological and ecophysiological identification approaches.

As shown by more recent cyanobacterial species concepts [59,60] molecular approaches are used in combination with morphological criteria to overcome poor taxonomic resolution and identify cyanobacteria cultured in the laboratory conditions. These authors suggested that some cyanobacterial species that are morphologically indistinguishable actually do not share a common evolutionary history. Moreover, ITS region analyses clearly indicate a polyphyletic nature of *Synechococcus* [61] suggesting the existence of three marine sub-clusters of *Synechococcus* (namely 5.1, 5.2 and 5.3). Therefore, for their full identity, some other marker gene such as phycocyanin gene sequence (*cpc*) could be more useful.

Morphological features of the two fastest growing strains, *Phormidium lucidum* MK568072 and *Synechococcus* sp. MK568070, are observed using epifluorescence microscope and presented in Fig. 2. While *Synechococcus* sp. MK568070 grew as single cells, the *Phormidium lucidum* MK568072 grew as filamentous, chain-forming structures, finally forming sheets on the surface of medium and a biofilm at the surface of the flask. The morphological features of the isolated *Spirulina subsalsa* MK573248, *Neolyngbya* sp. MK568073 and *Pseudanabaena* sp. MK568071 are presented in Fig. 3. Those three species are characterized by filamentous growth as well. Morphologically the most distinct cells, bright red colored and forming long, continuous spirals, were identified as *Spirulina subsalsa* MK573248 (Fig. 3a). Same species morphology was described by Włodorska-Kowalczuk et al. [62] as *Spirulina subsalsa* Oersted ex Gomont [63], growing in large red mats at the bottom of the brackish bay in the Baltic Sea. Both *Neolyngbya* sp. MK568073 (Fig. 3b) and *Pseudanabaena* sp. MK568071 (Fig. 3c) were green pigmented and filamentous, though the latter formed shorter and less compacted chains.

3.2. Assessment of ammonium (NH_4^+) tolerance

The results of the NH_4^+ tolerance assessment are presented in Fig. 4. None of the species demonstrated radical decrease in OD_{690}, i.e.

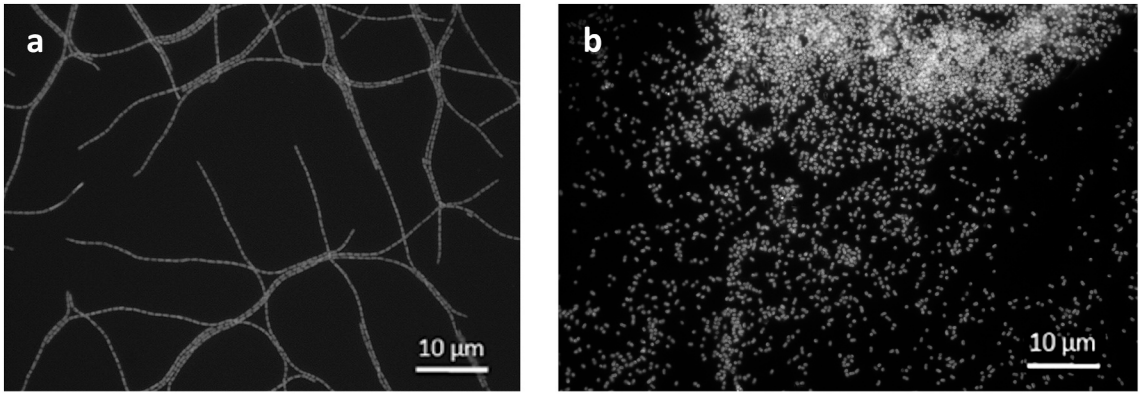


Fig. 2. Morphology of *Phormidium lucidum* MK568072 (a) and *Synechococcus sp*. MK568070 (b), visualized by epifluorescence microscope (Carl Zeiss), 1:100.

proxy of biomass concentration, which was expected, as they were initially isolated and preselected based on the tolerance to disturbed environmental conditions, achieved by addition of wastewater during isolation.

Synechococcus sp. MK568070 showed constant growth in the whole range of ammonium concentrations, increasing its OD_{690} threefold even at the highest NH_4^+ concentration. Among *Oscillatoriales* order, *Phormidium lucidum* MK568072 demonstrated growth on 1.6 and 2.0 mM NH_4^+concentrations, with the highest increase and maximum OD_{690} value at 1.6 mM NH_4^+. *Neolyngbya* sp. MK568073 increased its biomass under all conditions, reaching maximum OD_{690}also at 1.6 mM NH_4^+. *Spirulina subsalsa* MK573248 expressed threefold growth under lower ammonium concentrations (0.8 and 1.2 mM), while stagnating under higher ammonium concentrations (1.6 and 2 mM). *Pseudanabaena* sp. MK568071 showed tolerance to all NH_4^+ concentrations, but did not express any noticeable increase in biomass.

Although all inoculums contained same biomass concentration, the growth features of the culture impacted the initial OD_{690} measurements. It was observed that all species, except *Synechococcus sp*. MK568070, grew in patches and tended to adhere to the surface of microwells. These features explain why the 1st hour OD_{690} measurements differed notably among the tested cyanobacteria species except *Synechococcus sp*. MK568070. Nevertheless, the same error also occurred in later measurements, indicating that, although the OD_{690} value might not be the reflection of absolute biomass value, the slope between the measurements for same NH_4^+ concentration still manifests biomass change, i.e. growth or decay. Indicated problem did not occur for *Synechococcus* sp. MK568070 which is a well suspended culture and does not form any clusters.

There are a few studies of microalgae growth on wastewater enriched with NH_4^+ as sole source of N (with sufficiency of P-source), mostly focused on eukaryotes, such as *Scenedesmus* and *Chlorella* species, also showing successful growth [64]. Although preference towards NH_4^+ in cyanobacteria is well known [65] the difference in optimum growth concentration is visible among the herein tested species. While *Synechococcus* sp. MK568070 grows equally well throughout a whole range of tested NH_4^+concentrations, *Phormidium lucidum* MK568072 achieved the highest biomass yield at 1.6 mM NH_4^+that was significantly higher from those of *Pseudanabaena* sp. MK568071 and *Spirulina subsalsa* MK573248. The modest yield of the two latter species can be attributed to their tendency to grow in extreme environments, such as alkaline geothermal springs [66].

3.3. Biochemical composition

Final biomass concentration and biochemical composition in the stationary phase of growth of five strains of Northern Adriatic cyanobacteria are shown in Table 1.

The highest biomass concentration in the stationary phase of growth was achieved by *Pseudanabaena* sp. MK568071and *Phormidium lucidum* MK568072. The lipid content was very low in both strains, whereas the highest carbohydrate content of 60.6% was reccorded in *Phormidium lucidum* MK568072. Highest protein content of 60.9% was observed in *Spirulina subsalsa* MK573248, with a moderate final biomass concentration (251 mg/l). Both *Synechococcus* sp. MK568070 and *Neolyngbia* sp. MK568073 demonstrated to be equally rich source of lipids with at least 21% of biomass dry weight. However, high biomass yield and resulting final high lipid concentration (82.4 mg/l) in *Synechococcus* sp. MK568070 indicated its higher potential as a biodiesel feedstock in comparison to other cyanobacteria tested in this study.

3.4. Fatty acid profiling and biodiesel properties

The fatty acid profile, total lipid content and relevant proportions of FA and ratios between saturated (SAT) and unsaturated (MUFA and

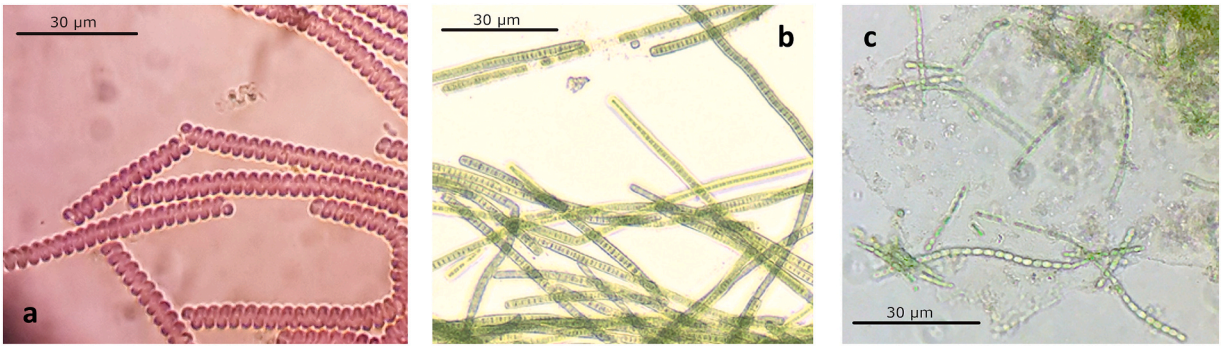
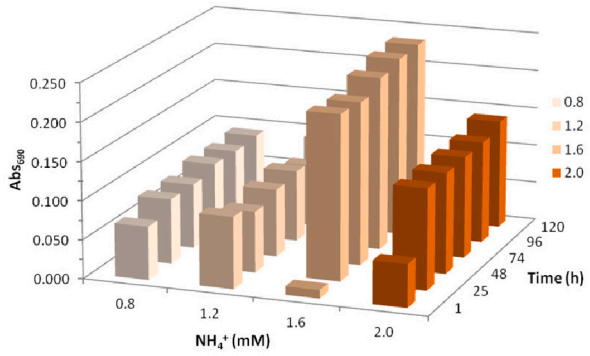
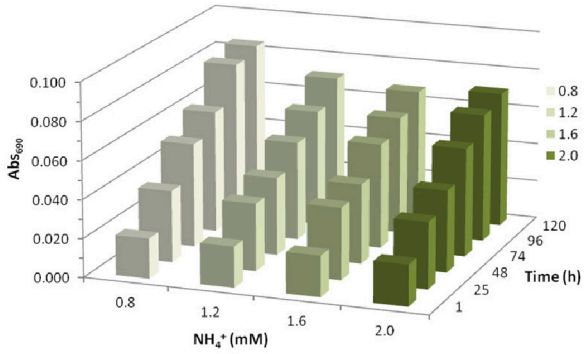


Fig. 3. Morphology of *Spirulina subsalsa* MK573248 (a), *Neolyngbia* sp.MK568073 (b) and*Pseudanabaena* sp. MK568071 (c) visualized by inverted light microscope (Boeco), 1:40.

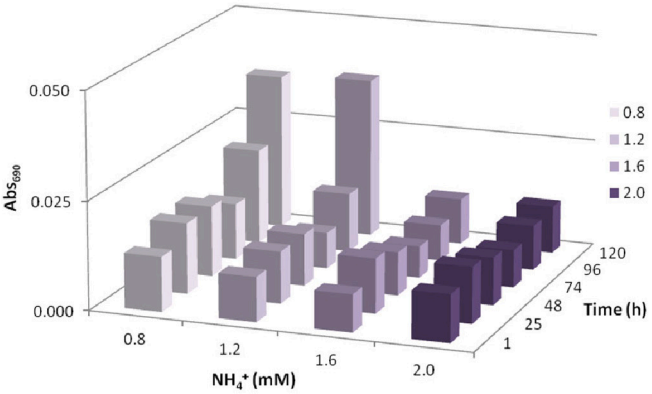
Phormidium lucidum MK568072



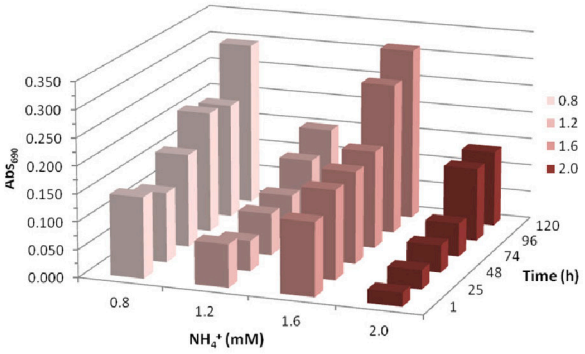
Synechococcus sp. MK568070



Spirulina subsalsa MK573248



Neolyngbia sp. MK568073



Pseudanabaena sp. MK568071

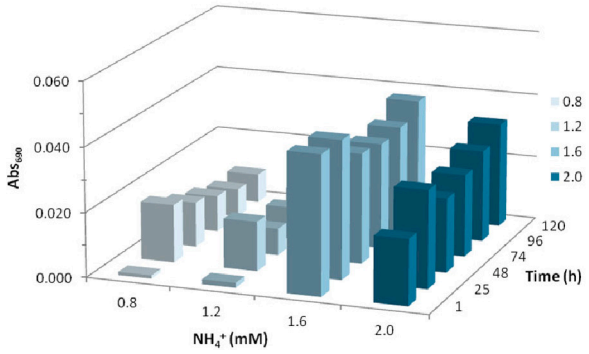


Fig. 4. Rapid assessment of the ammonium (NH_4^+) tolerance in microwell plates for 5 cyanobacteria isolated on refinery wastewater enriched medium.

Table 1
Comparison of the biomass concentration (mg/l) and biochemical composition (% of dry weight) of five Northern Adriatic cyanobacteria species grown on refinery wastewater enriched medium.

	Biomass	Lipid	Protein	Carbohydrate
Phormidium lucidum MK568072	490	8.5	19.2	60.6
Synechococcus sp. MK568070	385	21.4	24.7	24.4
Spirulina subsalsa MK573248	251	15.3	60.9	21.7
Neolyngbia sp. MK568073	176	21	27.2	18.9
Pseudanabaena sp. MK568071	590	7.6	34	12.2

PUFA) FA of *Synechococcus* sp. MK568070, *Phormidium lucidum* MK568072, *Spirulina subsalsa* MK573248, *Pseudanabaena* sp. MK568071 and *Neolyngbia* sp. MK568073 were analyzed and the results are presented in Table 2.

The fatty acid profile of *Synechococcus* sp. MK568070 was dominated by C14 and C16 fatty acids, whereas *Spirulina subsalsa* MK573248 and *Phormidium lucidum* MK568072 were dominated by C16 and C18 FA. The FA profile of *Neolyngbia* sp. MK568073 is the most saturated among the five tested strains, followed by *Synechococcus* sp. MK568070. Profiles of the two latter strains contain the highest proportions of C16:0 contributing > 40% to the FA profile. The highest proportion of C16:0 (55.61%) is shown in *Spirulina subsalsa* MK573248 FA profile.

Table 2
Total lipid content, fatty acid profile and relevant FAME classes' proportion of five indigenous Northern Adriatic cyanobacteria in stationary phase of growth on refinery wastewater enriched medium.

	Phormidium lucidum MK568072	*Synechococcus* sp. MK568070	*Spirulina subsalsa* MK573248	*Neolyngbya* sp. MK568073	*Pseudanabaena* sp. MK568071
C11:0			0.32		
C12:0	0.25				
C13:0					
C14:1					
C14:0	1.54	13.26	0.53	11.25	18.77
C15:1	0.88				
C15:0i	1.90		0.11		
C15:0	0.83	1.23	0.23		
C16:1c(n-9)	8.62	21.27	0.53		
C16:1c(n-7)	0.60	1.75	3.37	5.87	
C16:0	33.61	43.04	55.61	52.04	15.51
C17:1	0.87				
C17:0	2.71	2.51	0.99		
C18:3(n-3)			30.91	9.00	
C18:2(n-6)	3.62		2.72		
C18:1c	26.26	5.94	1.40	10.63	38.35
C18:1	4.05	2.15	0.73		9.83
C18:0	7.19	8.77	2.27	11.21	17.53
C20:2(n-6)	1.09				
C20:1	1.19				
C20:0	0.68		0.29		
SAT	48.70	68.82	60.34	74.50	51.82
MUFA	42.47	31.11	6.03	16.50	48.18
PUFA	4.71		33.63	9.00	
DETRIT	7.18	3.74	1.33		
C4-14	1.79	13.26	0.85	11.25	18.77
C15-C18	91.14	86.67	98.86	88.75	81.23
C19-24	2.96		0.29		
UND	1.07	0.45	1.73	0.58	0.93
UI (n-6)	0.09		0.05		
UI (n-3)			0.93	0.27	

This strain has extremely rich content of polyunsaturated FA, containing 33.63% of PUFA and highest proportion of C15-C18 FA, being the most unsaturated among five strains. Also, major contribution (30.91%) of C18:3 (n-3), one of the essential FAs in animal nutrition, to the whole profile of FA in *Spirulina subsalsa* MK573248 opens considerations of this species for biotechnology purposes other than biodiesel production. Richness of *Spirulina subsalsa* MK573248 in alpha-linolenic acid (ALA), high content of lipids (15.30%) and its growth on wastewater makes it suitable for aquaculture feed production and phycoremediation of aquaculture farms [67]. A closely related freshwater strain *Spirulina platensis* studied by González-Fernández et al. [68] has revealed promising potential in anaerobic digestion of NH_4^+-enriched digestate.

Some of the main characteristics determining suitability of lipids for biodiesel are the chain length and degree of saturation. The average degree of FA unsaturation (ADU) was calculated as proposed by Islam et al. [54] to estimate the suitability of cyanobacteria biomass for biodiesel production. Biodiesel properties are estimated from the molecular structure of the FAME taking into account the acyl chain length and quantity and position of unsaturated bonds. Results are presented in Table 3.

All 5 cyanobacteria strains have satisfactory kinematic viscosity (KV), cetane number (CN) and iodine value (IV). The properties of all tested strains roughly adhere to the EN 14214 standard criteria, though *Synechococcus* sp.MK568070 showed kinematic viscosity on the border high value of 5.01. The cetane number is an important parameter indicating time delay in the ignition of fuel for diesel cycle engines. According to the EN 14214 standard it should be higher than 51, however too high CN would lead to incomplete fuel combustion. The estimated CN for the tested cyanobacteria strains varied from 55.93 to 60.80, and are comparable to the CN of some Chlorophyta such as *Chlorella* and *Scenedesmus* [69] indicating good ignition quality of the extracted triglycerides. Because of the high biomass yield and lower proportion of polyunsaturated FAs *Synechococcus* sp. MK568070, *Phormidium lucidum* MK568072 and *Pseudanabaena* sp. MK568071 are considered more suitable candidates for the purpose of biofuel production compared to the other tested strains. The unsaturation degree (ADU) of *Spirulina subsalsa* MK573248 of 1.73 can be considered extremely high compared to all other analyzed species. High ADU is considered unfavorable for biodiesel production because of higher polymerizing tendency of unsaturated components, and is in relation with IV value, that should not be higher than 120 gI_2/100 g fat. In the present study, it is much lower for all analyzed strains in comparison to the threshold value.

Cloud point (CP) is an important cold-flow property indicating the temperature at which the crystallization of biodiesel occurs. Although there are no regulatory standards for cloud point, this is an important parameter, particularly for winter fuel and higher geographic latitudes. In our study the CP values of tested cyanobacteria range from 6.08 °C to 15.84 °C. Results are comparable to most of the Chlorophyta reported by Yu et al. [69] and to the palm biodiesel reported by Sarin et al. [70]. The only exception demonstrating significantly lower CP value of 6.08 °C is *Spirulina subsalsa* MK573248 (Table 3). The values of CP significantly higher than −20–0 °C are common in plant/vegetable based biodiesel [71] and are usually lowered by blending various biodiesel fuels [70]. Higher heating values (HHV) for the analyzed strains are in a range 39.08–40.37 MJ/kg. Although there are no threshold values for HHV in any standards for biodiesel, the HHV of 40.37 MJ/kg of *Spirulina subsalsa* MK573248 corresponds most closely to the range of values for regular diesel (39.8–40.4 MJ/kg) that is normally 10–12% less than the petroleum-derived diesel (46 MJ/kg) [52].

The experiments presented herein represent growth on wide range of NH_4^+, however under constant high salinity (38 psu) corresponding to Northern Adriatic. Previous research [72] demonstrated wide salinity tolerance of *Synechococcus* sp. MK568070. Moreover, its biomass yield was highly dependent on nutrient source and concentration,

Table 3
Biodiesel properties calculated from the FAME profile of five indigenous Northern Adriatic cyanobacteria in stationary phase of growth on refinery wastewater enriched medium.

Biodiesel property (unit)	Biodiesel standard EN 14214	*Phormidium lucidum* MK568072	*Synechococcus* sp. MK568070	*Spirulina subsalsa* MK573248	*Neolyngbya* sp. MK568073	*Pseudanabaena* sp. MK568071
Average degree of unsaturation, ADU		0.52	0.31	1.04	0.40	0.48
Kinematic viscosity, KV (mm^2/s)	3.5–5.0	4.88	5.01	4.55	4.90	4.90
Specific gravity, SG (kg/m^3)	0.86–0.90	0.87	0.87	0.87	0.87	0.87
Cloud point, CP (°C)		13.06	15.84	6.08	14.19	13.56
Cetane number, CN (−)	≥51	59.42	60.80	55.93	59.98	59.66
Iodine number, IV (g I_2/100 g fat)	≤120	51.30	35.85	90.21	45.06	48.54
Higher heating value, HHV (MJ/kg)	NA	39.45	39.08	40.37	39.30	39.38

salinity and CO_2 addition. Highest biomass yield was 767 mg/l of dry weight under 18 psu. The research on *Chlorella vulgaris* (FACHB-31), known of its high lipid content, was conducted by Hu et al. [9]. *C. vulgaris* was grown on NH_4^+ as N-source reaching maximum lipid yield of 16% depending strongly on diurnal cycle, CO_2 bubbling and addition of essential minerals such as Mg^{2+}. Relying on the above-mentioned studies, as well as the results presented herein, we suggest that NH_4^+/NH_3-tolerant marine cyanobacteria should be further researched by applying ecophysiological modeling to the growth conditions such as CO_2 supply, light intensity and regime, salinity, pH and essential micro-constituents [7,73,74]. This approach would be particularly useful for biomass production of *Pseudanabaena* sp. MK568071 and *Spirulina subsalsa* MK573248 that have shown high discrepancy between growth in microwells and in culture medium.

Herein presented results on marine cyanobacteria are in linewith previous research on freshwater species conducted by Sarmah and Rout [75] who have described several freshwater cyanobacteria species (e.g. *Phormidium lucidum* and *Lyngbia diguetii*) as efficient sequesters of sewage water, producing high contents of lipids, proteins and vitamin C. Furthermore, tested cyanobacteria have easily formed biofilms on polyethylene and glass surfaces due to high release of extracellular polysaccharide (EPS) that could provide a favorable environment for simultaneous growth of some other mixotrophic microorganisms. It is demonstrated that the establishment of the microalgal consortia, in particular with the Chlorophyta species, enhance wastewater treatment efficacy and lipid production [76]. Although the positive traits of mixed microalgal consortia (including heterotrophs) are well recognized [24], the mechanisms of controlling the stability of a community in such a complex system are still to be better understood [11].

Cogeneration of biofuel by remediation of wastewater and flue gases has shown to significantly decrease the production cost, yet the whole chain of largescale processing is still economically quite challenging. The exploitation of residual biomass rich in valuable compounds such as carbohydrates (*Phormidium lucidum* MK568072) and proteins (*Spirulina subsalsa* MK573248 and *Pseudanabaena* sp. MKMK568071) is of a great significance for integrated processing into food, feed and biofuels. The hydrothermal liquefaction, although shown as the most efficient and with positive net energy balance, would not be suitable for recovery of carbohydrates and proteins later applied for foodstuffs [77]. Instead, targeted fractionation and subsequent extractions with a careful choice of acceptable solvents according to the EC Directive 2009/32, and other regulations must be applied.

4. Conclusions

Five Northern Adriatic cyanobacteria, isolated on oil refinery wastewater enriched medium, have demonstrated good viability at high concentrations ofNH_4^+, under a redox balance with its toxic form ammonia, as an exclusive source of inorganic nitrogen. *Synechococcus* sp. MK568070 and *Neolyngbia* sp. MK568073 both demonstrated to be equally rich source of lipids with more than 21% of biomass dry weight. However, final biomass and resulting lipid yield (82.4 mg/l) were much higher in *Synechococcus* sp. MK568070 indicating its favorable properties for biodiesel production. The highest protein content (60.9%) was reached in *Spirulina subsalsa* MK573248 suggesting its potential for use in the food industry as high value nutritional product. Biodiesel properties of candidate species showed values comparable to most of the microalgae oils reported in literature and adhering to the EN 14214.

CRediT authorship contribution statement

Ines Haberle: Conceptualization, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Enis Hrustić**: Investigation, Writing - review & editing, Formal analysis, Data curation. **Ines Petrić**: Formal analysis, Data curation. **Ena Pritišanac**: Investigation. **Tina Šilović**: Conceptualization,

Methodology. **Lana Magić**: Resources. **Sunčana Geček**: Conceptualization, Writing - review & editing. **Andrea Budiša**: Validation, Writing - review & editing. **Maria Blažina**: Funding acquisition, Project administration, Supervision, Conceptualization, Data curation, Writing - original draft.

Declaration of competing interest

None.

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