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Isolation, identification, and chemical composition analysis of nine microalgal and cyanobacterial species isolated in lagoons of Western Greece

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ABSTRACT

In the present study, nine halotolerant microorganisms were collected from lagoons of Western Greece, successfully isolated, and maintained in laboratory cultures. Five species of Chlorophyta (*Tetraselmis marina*, *Tetraselmis* sp., *Nephroselmis* sp., *Dunaliella* sp., and *Asteromonas gracilis*), three cyanobacteria (*Phormidium* sp., *Anabaena* sp., and *Cyanothece* sp.) and one dinoflagellate (*Amphidinium carterae*) were identified based on morphological observations and DNA sequencing data. All the above-mentioned species have the potential for future exploitation. Their physicochemical composition analysis on dry biomass exhibited 30–50 % protein, and sufficient fatty acids accumulation, with *A. carterae* and *Nephroselmis* sp. reaching 20 and 15 % respectively. The cellular pigments of all the examined species did not exceed 5 % w/w. Interestingly, chlorophyll *b* accounted for 30 % of the content of the total pigments for *Tetraselmis* sp., *Nephroselmis* sp., and *T. marina*. Carotenoids on the other hand were found at 35 % in *A. carterae*. The current study paves the way for new species exploitation in terms of several valuable compounds' valorization and recovery.

1. Introduction

The on-growing global demand for energy (around 50 % increase expectations up to 2050) [1], intensified by the continuous increase of the human population (estimated growth rate of 0.1 % up to 2100) [2], the challenge of exploiting new areas for crops, and the need to mitigate the carbon emissions, constantly challenge scientists to find environmental-friendly and sustainable resources with a minimum carbon footprint. Microalgae are the best candidates to support human needs as special food products, bio-chemicals, and pharmaceuticals. Algae are already exploited for some decades in various sectors, such as aquaculture, but their production potential is very promising, as their capacities to fulfill the needs of humanity are still far from being settled [2,3]. In this respect, screening for the isolation of new un-exploitable species and optimization of production are important steps for the exploitation of the full potential of microalgae [4].

Microalgae culture presents several advantages over terrestrial plants in terms of yield. Both are photosynthetic, but many algae species can shift over to heterotrophy in certain circumstances, an advantage to be used as a backup to photosynthesis [5]. Microalgae are many times more efficient than terrestrial plants in converting solar energy to biomass, by use of less space, and without irrigation needs, weed control, barnyards, storm concern, soil restoration, and deforestation catastrophes. Even if more than 200.000 microalgae species exist, a limited number of them have been studied in terms of their morphological features and growth characteristics [6]. The research community is still far from establishing a final list of the ideal species in terms of exploitation among existing microalgae species, as, depending on the target, each one of them can offer opportunities for various products [7].

The marine environment can supply a lot of species that grow in saline water, an inexhaustible medium for culture per se, or in coastal areas often remote and unsuitable for other uses [8]. Seawater could be characterized as an inhibitor for land crops [9], but it could be beneficial for algae, indicating cultivation efficiency [10]. Most microalgae can cope with a wide range of salinity and temperature. As the most efficient growth occurs in temperatures >20 °C [11,12], attempts are preferable

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to focus first on tropical and subtropical regions where the mean annual temperatures permit outdoor facilities. In addition, species with high growth potential and adaptation to high nutrient concentrations are often isolated from lagoon areas. Nevertheless, the rapid expansion of innovative equipment for eco-friendly energy production creates a tremendous potential for heating and lighting microalgae cultures in temperate and sub-polar areas [13,14].

In this respect, screening for algae in local areas is a much more rewarding practice in terms of science and economy. In science, due to the bank of preserved species enrichment, and in the economy due to the creation of many local enterprises and/or communities [15], able to use indigenous species that can be proved more efficient than their counterparts in other regions. Microalgae isolated from a particular area may possess special attributes among the vast number of their kingdom and their relative phyla where they belong, which can be suited for growth in the microclimate of their original locality. In this respect, their growth dynamics may be coupled with and benefit the most from the environmental conditions prevailing in the locality from where they have been isolated. It is highly logical first to screen intensively for local species, analyze them for their biochemical profile and initiate culture trials to see if they are promising in culture response and economic feasibility [16–18].

A survey was initiated in the salt waters (of sea and lagoons) of Western Greece screening for microalgae and their characteristics. Greece has a long coastline (around 18.000 km) [19], many lagoons, estuaries, saltworks, and many rivers all around its territory [20]. Western Greece, particularly in Etoloakarnania, Achaia, and Ilia prefectures, is particularly rich in water habitats [21].

Since the 90s, microalgae have been cultured in Greece in marine fish hatcheries and used for the rearing of marine fish larvae [22]. These microalgae are mainly *Nannochloropsis*, *Isochrysis*, *Tetraselmis*, and *Chlorella* initially originating from imported strains. Another use for microalgae in Greece is the commercially produced cyanobacterium *Arthrospira platensis* in farms in Northern Greece using naturally alkaline freshwater focusing exclusively on the production of healthy food supplements for human consumption. A few companies have been also established, targeting microalgae cultivation and aquaculture projects. More specifically, "Microphykos" is a company, which manufactures tailor-made microalgae culture systems for marine hatcheries and both indoor and outdoor farms for Spirulina and other species. Except for their specialization in algae farms, they have developed protocols regarding b-carotene, natural products, cosmetics, and phycocyanin extracts. Another company named "Plankton First", was established in 2019 and targets innovative research, development, production, and commercialization of microalgae and microalgae-derived high-value products. Even if their infrastructures are still under construction, they are developing a pilot production starting from 5 ha (2022), reaching a plot area of 40 ha by 2024, while their biorefinery plant for commercial production of bioactive compounds will be ready by the end of 2023. Furthermore, there is a hybrid AgriBioTech company, named Solmeyea, where functional proteins and high value biobased products are produced for several applications (feed, food, and pharma), through hybrid vertical microalgae farming.

The field of mass microalgal production in Greece is thus wide open for new enterprises that will take advantage of local species to produce various value-added compounds for aquaculture, food additives, antioxidants, pharmaceuticals, and biofuels. Only limited work exists in the field of screening for marine microalgae in Greece [23–26]. Such microalgal and/or cyanobacterial species are usually considered as sturdy, due to their mode of life in the lagoonal unique and nutrient-rich environment. Additionally, lagoonal microorganisms are tailored to harsh environmental conditions, including fluctuations in salinity and temperature levels. The current work focused on local microalgal and/or cyanobacterial species with potential high tolerance, fast growth, and easy adaptation [27,28], aiming at creating a database of biochemical profiles for each of them, serving as a guide for everyone interested in their exploitation.

2. Materials and methods

2.1. Sampling and maintenance

Water samples were collected monthly throughout 2019 and the first half of 2020 from the lagoons of Messolonghi and its saltworks (Pref. of Etoloakarnania), the lagoon of Kalogria and Pappas (Pref. of Achaia), and the lagoon of Kotychi (Pref. of Ilia) (Fig. 1). Care was taken to avoid days of heavy rain that silt the coastal areas of the shallow lagoons. The samples were taken from a water depth of at least 20 cm which is approximately the middle depth of the water column. Capped 500-mL transparent plastic Erlenmeyer flasks were used, which were transported (the same day) to the plankton culture laboratory in Messolonghi to enter the isolation procedure. An amount of 200 mL of each flask was poured into glass 1-L conical Erlenmeyer flasks containing 800 mL of 40 ppt sterilized water enriched with Walne's nutrient formula [29], in triplicate. The so-called maintenance flasks were left for one week to mature, supplied through a 1-mL pipette with filtered air (~1 flask volume/min) fed by a central blower, and exposed to continuous light of 3000 lx emitted by white light LED tubes, in an air-conditioned room (19–22 °C). The maintenance flasks were left for 2–3 weeks to develop the microalgae population evidenced by the coloration of the water and microscopic examination. Those with no sign of coloration after 3 weeks were discarded. By this practice, the research team deliberately focused on algae species that can either solely or in companion with other species be fully adapted to seawater salinity and dominate the culture. The diatoms were excluded due to their peculiar characteristics that ensue in highly unstable and easily collapsing cultures.

2.2. Isolation of desirable species

After the confirmation of one or more species predominance in the maintenance flasks, serial dilutions were performed in successive steps using 20-mL glass Erlenmeyer flasks filled with sterilized and fertilized water of the same salinity (40 ppt, as abovementioned). The inoculated flasks were left to mature for 20 days in a special thermo-regulated chamber (19 °C) in low ambient continuous illumination of 1300 lx and daily mildly hand agitation. After 20 days they were examined microscopically and if a monoculture was observed the content of this flask was transferred to 500-mL flasks prepared with a new fertilized medium and left to mature (19-22 °C, continuous 3000 lx) until used for inoculation of various bigger flasks.

Apart from the serial dilution method above, cultivation in solid media was also accomplished using inocula from the original mature maintenance flasks. For the preparation of 1 L of culture medium, 1 L of seawater (salinity 36 ppt), 15 g of agar, and 0.2 g of Cell-Hi F2P medium (Varicon Aqua, U.K.) were added in a conical flask. The mixture was heated to boiling with stirring and thereafter autoclaved. As cooling proceeded, and before solidification, a supplemental mixture of vitamins was added (0.1 mg L^{-1} thiamine HCl, 0.005 mg L^{-1} biotin, and 0.005 mg L^{-1} B_{12}). The medium was thereafter transferred to sterile 92 mm Petri dishes, which were stored at 4–7 °C until their use. Fifty μL of microalgae culture was spread on each dish and the pure-culture technique was used together with observation of the cells in the microscope to isolate the different species of microalgae.

2.3. Molecular identification of cyanobacteria

DNA extraction was performed (in triplicate) by centrifugation of 1 mL of microalgae culture at 13,000*g* for 5 min. The supernatant was discarded, and the cells were suspended in distilled water. A 50 μL sample of each suspension was heated in a SimpliApp thermal cycler (Applied Biosystems, USA) at 98 °C for 15 min to break the cell walls according to Jensen et al. [30]. The universal primer 27f [31] and a

Fig. 1. Geographical illustration of sampling points from the lagoons of Western Greece.

primer specific for cyanobacteria 23S30r [32] were used to amplify the 16S ribosomal genes. PCR was performed in a 50 μL reaction using a KAPA taq ready mix PCR kit (Sigma), 0.5 μM of each primer, and 1 μL of diluted cell suspension. Reactions were carried out in a SimpliApp thermal cycler with an initial denaturation step of 95 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 2 min; the final extension step was performed at 72 °C for 10 min. The PCR products were visualized by 1 % agarose/TBE gel electrophoresis. DNA purification and Sanger sequencing were performed by Macrogen Europe (Netherlands).

2.4. Molecular identification of eukaryotic microalgae

Extraction of DNA was performed by use of Wizard Genomic DNA purification kit (Promega, USA) following the protocol for gram-negative bacteria. For amplification of a partial sequence of the 18S rDNA, we used forward primer A (5′AACCTGGTTGATCCTGCCAGT 3′) [33] and reverse primer SSU-inR1 (5′-CACCAGACTTGCCCTCCA-3′) [34], as suggested by Lee et al. [35]. PCR was performed in a 50 μL reaction using a KAPA taq ready mix PCR kit (Sigma), 0.5 μM of each primer, and 1 μL of diluted DNA suspension. Reactions were carried out in a SimpliApp thermal cycler with an initial denaturation step of 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; the final extension step was performed at 72 °C for 7 min. The PCR products were visualized by 1 % agarose/TBE gel electrophoresis. DNA purification and Sanger sequencing were performed by Macrogen Europe (Netherlands).

2.5. Phylogenetic analysis

All sequences were checked for similarity against other publicly available sequences using the Basic Local Alignment Search Tool (BLAST) algorithm [36]. DNA sequences were aligned automatically using the ClustalW [37] alignment algorithm under default parameters using Molecular Genetics Analysis (MEGA) 6.06 software [38]. The DNA sequences were compared by BLAST analysis to identify homologous taxa available on the NCBI DNA database.

2.6. Biochemical profile

For the biochemical profile determination, the microalgal biomass was harvested through centrifugation, followed by washing and freeze-drying (Telstar, LyoQuest). The carbohydrate content was measured according to Dubois et al. [39], by a phenol-sulfuric acid method. Concerning the protein content, total Kjeldahl nitrogen (TKN) was determined by the Semi-micro Kjeldahl method [40], while the TKN to proteins conversion was followed by the factor 6.25 [41]. For the determination of intracellular fatty acids (FAs) of microalgae, their conversion to methyl esters (FAMEs) is required, through an in-situ transesterification reaction in the presence of an H_2SO_4 catalyst [42]. Subsequently, the FAMEs were analyzed by gas chromatography (Agilent Technologies, 7890A) with a flame ionization detector (FID) and He as carrier gas. An amount of 10–50 mg of dry biomass was used with 5 mL CH_3O H: $CHCl_3$:H_2SO_4 solution of 100:10:1 ratio. Heptadecanoic acid (C17:0) was used as the internal standard, while the samples were incubated for 2 h at the temperature of 90 °C. After the incubation, 2 mL of 3D water was added to each sample to stop the transesterification, as well as 2 mL of extraction solution (C_6H_{14}:$CHCl_3$ in a ratio of 4:1). Two phases were formed and the oily one was collected for the FAMEs measurement. More details about the process and the equipment (column, time, temperature ranges etc.) are described in detail by Koutra et al. [43]. Pigments (chlorophyll *a* & b, as well as total carotenoids) determination was accomplished after the extraction of 2–5 mg dry biomass sample by *N*, *N*′-dimethylformamide (DMF), while the calculations are described in detail in Ref. [44]. Finally, the ash content was determined gravimetrically at 550 °C, until constant weight [40].

Table 1
Morphological characteristics of the nine isolated species from lagoons of Western Greece. Values are means of 20 replicates.

Species	Dimension [average ± sd or min-max (μm)]		Shape description	Color range (immature to mature phase)
	Longitudinal axis	Width		
Amphidinium carterae	11.93 ± 1.30	8.64 ± 0.92	Spherical and spindle-shaped	Yellow to orange-brown
Tetraselmis sp. (var. red Pappas)	11.32 ± 0.78	7.95 ± 1.06	Elongated cylindrical and elliptical	Dark green to brown-red
Tetraselmis marina (var. Messolonghi)	18.62 ± 1.79	10.02 ± 0.62	Elongated cylindrical and elliptical, or spherical	Green
Nephroselmis sp. (var. Messolonghi)	5.28 ± 0.35	3.81 ± 0.5	Bean-shaped	Light to dull green
Asteromonas gracilis	18.84 ± 2.88	13.09 ± 2.4	Spindle-shaped, sometimes narrow	Green
Dunaliella sp. (var. Messolonghi)	8–10	4–5	Pear-like	Light green
Phormidium sp. (var. Tourlida)	Not applicable	2.37 ± 0.2	Generally straight thin filaments	Light to oily green
Cyanothece sp. (var. Messolonghi)	8–12		Almost spherical, oval, or bar-shaped with rounded edges	Blue-green to brown-red
Anabaena sp. (var. Kotychi)	Cells: 4.2–4.7	3.8–4.5	Straight filaments comprised of almost spherical cells	Light to oily green
	Filaments: big range depending on the cell number			

3. Results and discussion

3.1. Phylogenetic analysis

Nine species were isolated from the samples taken from Messolonghi lagoon, Messolonghi saltworks, Kalogria lagoon, Pappas lagoon, and Kotychi lagoon (Fig. 1). After a properly designed procedure by serial dilutions and continuous recultivations of cultures, monospecific cultures were attained which were kept in 1-L Erlenmeyer flasks with Walne's nutrient-enriched sterilized seawater of 40 ppt salinity, at 18–20 °C and 4000 lx illumination.

Two cyanobacteria strains were genetically identified using the 16S rDNA marker. The sequence data of *Phormidium* sp. and *Cyanothece* sp. submitted in Genbank have accession numbers ON229416 and ON229417, respectively. Six eucaryotic microalgae strains were genetically identified using the 18S rDNA marker. The sequence data of *Amphidinium_carterae*, *Tetraselmis_*sp., *T. marina*, *Nephroselmis* sp., *Dunaliella_* sp., *Asteromonas_gracilis* submitted in Genbank have accession numbers ON220609, ON220610, ON220611, ON220612, ON220613, and ON220614, respectively.

Five species of them were Chlorophyta (*T. marina*, *Tetraselmis* sp., *Nephroselmis* sp., *Dunaliella* sp., and *A. gracilis*), three were cyanobacteria (*Phormidium* sp., *Anabaena* sp., and *Cyanothece* sp.) and one dinoflagellate (*A. carterae*). Their main morphological characteristics are presented in Table 1 and are discussed in detail in the following subsections, while their biochemical characteristics are shown in Fig. 2.

The biochemical composition of dry biomass was determined in samples from pure cultures for each species. More precisely, the content of proteins, carbohydrates, lipids, and minerals (ash) was measured, and the photosynthesis-affected pigments (chlorophylls a, b, and total carotenoids) of the isolated microorganisms, as well as their fatty acids, were estimated as shown in Fig. 2. After TKN analysis, proteins of all species ranged from 30 to 50 % of the dry biomass weight. Both the biomass of *A. carterae* and *Anabaena* sp. consisted of proteins at 50 % w/w. Contrary to these results, the proteins of *Anabaena* sp. have been recorded only up to 35 % w/w, while in the case of *A. carterae*, the cells consisted mainly of carbohydrates and lipids [45,46]. The above disagreement could be explained by the great influence of the culture's conditions on the characteristics of the biomass produced. Regarding intracellular carbohydrates, the species that stood out were the cyanobacterium *Phormidium* sp. (43 %) and then the microalgae *Dunaliella* sp., *T. marina*, and *Tetraselmis* sp. (around 25 %). Fortunately, the results for biomass sugars are confirmed by previous research related to the distinguished species [47–49]. Strains of *Dunaliella* and *Tetraselmis* exhibited carbohydrates with a concentration from 20 to 25 %, while it has been reported that the *Phormidium* cyanobacteria undergo a significant reduction in their internal carbohydrates when increasing light intensity provided during growth [47–49]. Moreover, the evaluation of biomass samples in terms of FAs accumulation led to the observation that the microalgae *A. carterae* and *Nephroselmis* sp. contained the highest quantities on a dry weight basis, 20 and 15 % respectively.

All the natural isolates were found to contain palmitic acid (C16:0) at a rate of 20 to 35 % of their total fatty acids. However, palmitoleic acid (C16:1) was found in significant levels only in *Nephroselmis* sp. biomass (28 % of total fatty acids). Based on the fact that the local microalgae and cyanobacteria were rich in palmitic acid, their use in the production of cosmetology products could be explored. Palmitic acid is a cleansing agent and helps to moisturize the skin [50]. Especially, *Nephroselmis* sp. did not demonstrate stearic (C18:0) and oleic (C18:1) acids in contrast to all other species, in which these two FAs occupied 5 to 20 % of the FAs content. Additionally, it is tempting to separate the isolated microorganisms according to their unsaturated FAs content. Unsaturated FAs consisting of up to 18 carbon atoms have been reported as suitable for biodiesel, while the high molecular weight polyunsaturated FAs have significant nutritional value and are exploitable in the food industry [51]. The long-chain polyunsaturated FAs, such as eicosapentaenoic

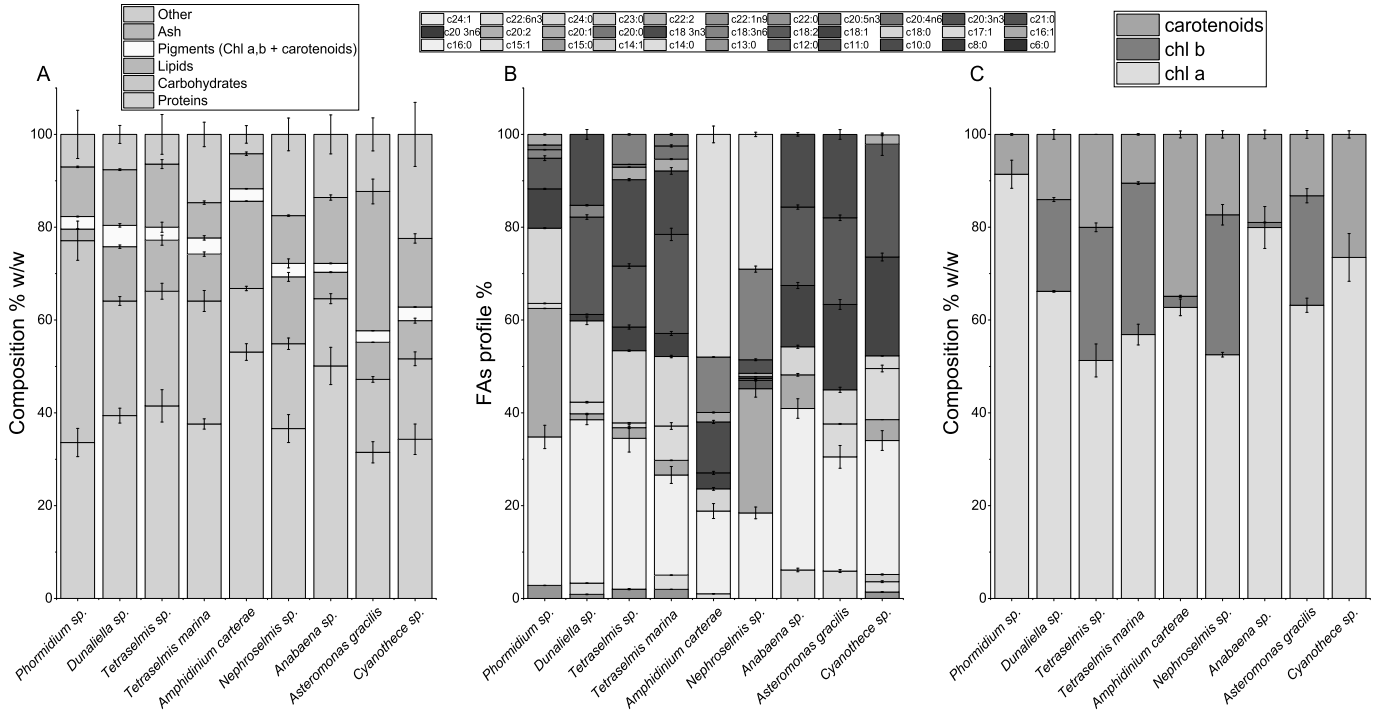

Fig. 2. (a) Biomass composition, (b) fatty acids profile, and (c) pigments profile (chlorophyll *a*, chlorophyll *b*, and total carotenoids). The values correspond to the mean ± standard deviation of measurement analyzed in triplicate.

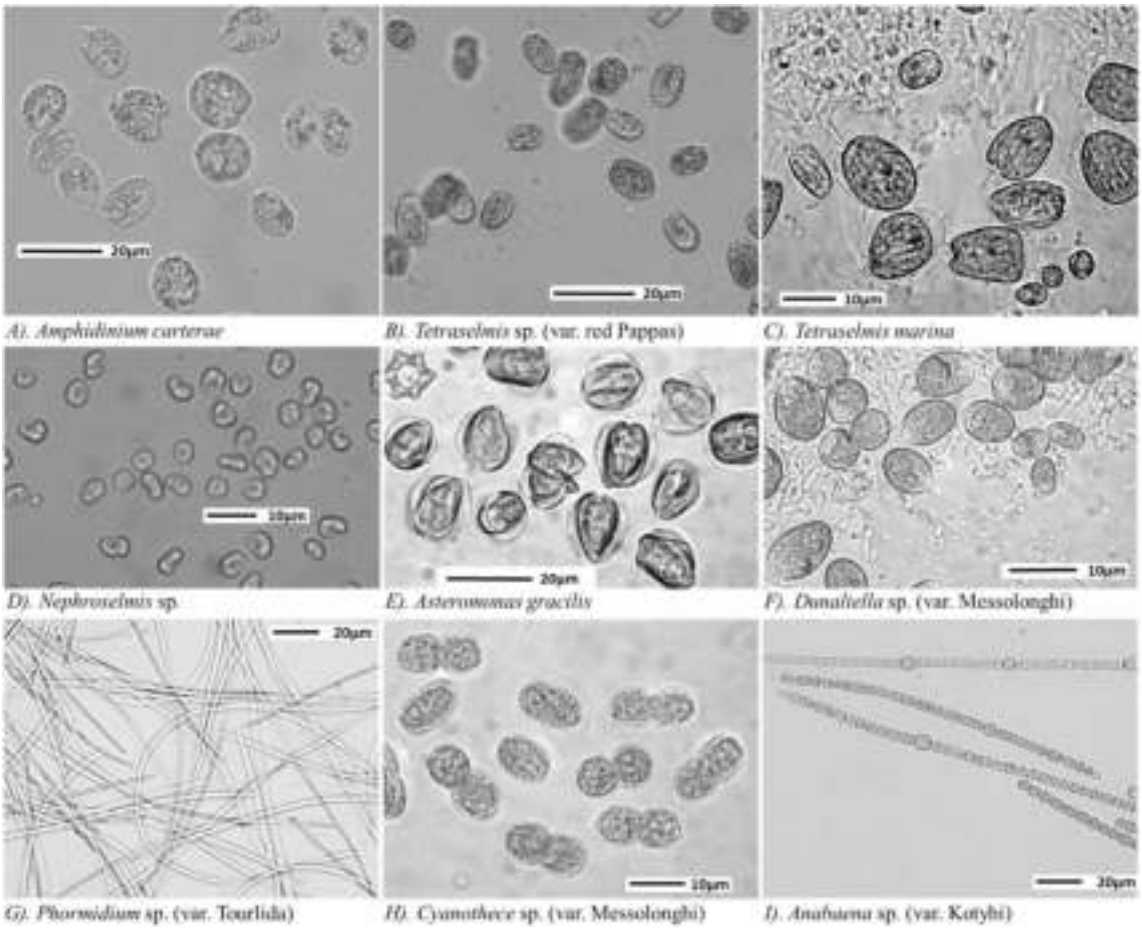

Fig. 3. Cells images (at 10 or 20 μm) of the nine species isolated from the lagoons of Western Greece.

acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), come with greater interest due to their nutritional value. Among the natural isolates, only *A. carterae* and *Nephroselmis* sp. contained DHA, 50 % and 30 % of the total detected FAs respectively, while EPA ranged between 10 and 20 %. For comparison's sake, according to an evaluation study, although *Nephroselmis* sp. yielded neither EPA nor DHA, significant amounts of C16:0 and γ-linolenic (C18:3n6) acids were detected, as they occupied 22 and 56 % of total FAs respectively [52]. Furthermore, apart from C16:0, which constituted 20 % of the total FAs, *Dunaliella* sp. also presented C18:0, which corresponded to approximately 10 % [48]. In contrast to the case of *Dunaliella* sp., which originated from Messolonghi and contained significant amounts of α-linolenic acid (C18:3n3), *Dunaliella salina* of Chen et al., 2015 exhibited the unsaturated fatty acid C18:3n6 close to 20 % of the whole FAs [48]. When *Tetraselmis* cells were cultivated under light irradiation equal to 60 μmol photons m^{-2} s^{-1}, they accumulated mainly saturated acids, C16:0 and arachidic (C20:0) acid, in percentages of 36 % and 10.8 % [53]. The unsaturated fatty acids found in *Tetraselmis* species were close to 12 % each [53]. For *A. carterae*, an increase in lipid accumulation was observed under high light intensity. The FAs profile agreed with that of the natural isolate and more specifically for both strains the polyunsaturated EPA and DHA dominated a large part of the biomass [46].

In contrast to the generally high levels of FAs, the cellular pigments of all the examined species did not exceed 5 % w/w. The species *Dunaliella* sp. (4.6 %), *T. marina* (3.5 %), *Nephroselmis* sp. (2.9 %), and *A. carterae* (2.7 %) emerged as the most promising sources of pigments, regarding chlorophyll *a*, chlorophyll *b*, and carotenoids [54–56]. It is worth mentioning that most of the cyanobacteria isolated for the present study contain another important and valuable pigment, called phycocyanin, although it was not identified and estimated in detail [57]. Chlorophyll a was the most common pigment for all natural isolates and this outcome in combination with the absence of chlorophyll *b* in isolated cyanobacteria was the most interesting observation in the qualitative determination of cellular pigments. The total pigments *in Dunaliella* sp. consisted of 20 % chlorophyll *b*, while in *Tetraselmis* sp., *T. marina*, and *Nephroselmis* sp. chlorophyll *b* reached 30 %. Finally, carotenoids were an important part of the cellular pigments in *A. carterae* and *Cyanothece* sp. at percentages of 35 and 26 % respectively.

3.2. A. carterae

Single-celled planktonic dinoflagellate with solitary cells 11.93 ± 1.30 μm along the longitudinal axis and 8.64 ± 0.92 μm across the width. Motile, with characteristic straight-line motion and frequent abrupt changes of direction without swirling. Sometimes the cells appeared completely immobile in a vitreous cyst. The cells (Fig. 3A) were spherical or spindle-shaped and along the large axis compressed. Their color ranged between shades of oil yellow and yellow-orange, sometimes with a slight greenish tinge. Epicone is much smaller than hypocone and unique in its shape among the dinoflagellates as it looked like a curved "proboscis". There was variation in the size of the epicone, as in some cells it was bulky and in others, smaller. The transverse groove (cingulum) was not as obvious as in other classes of dinoflagellates, as the epicone, small as it was in relation to the hypocone (that occupies most of the cell's volume), was not separated from it by a large groove. Both the transverse and the vertical flagella were visible with the transverse being partially inside the transverse groove and a part of it protruding with a characteristic wavy motion, and the elongated one projecting enough from the vertical groove and moving less

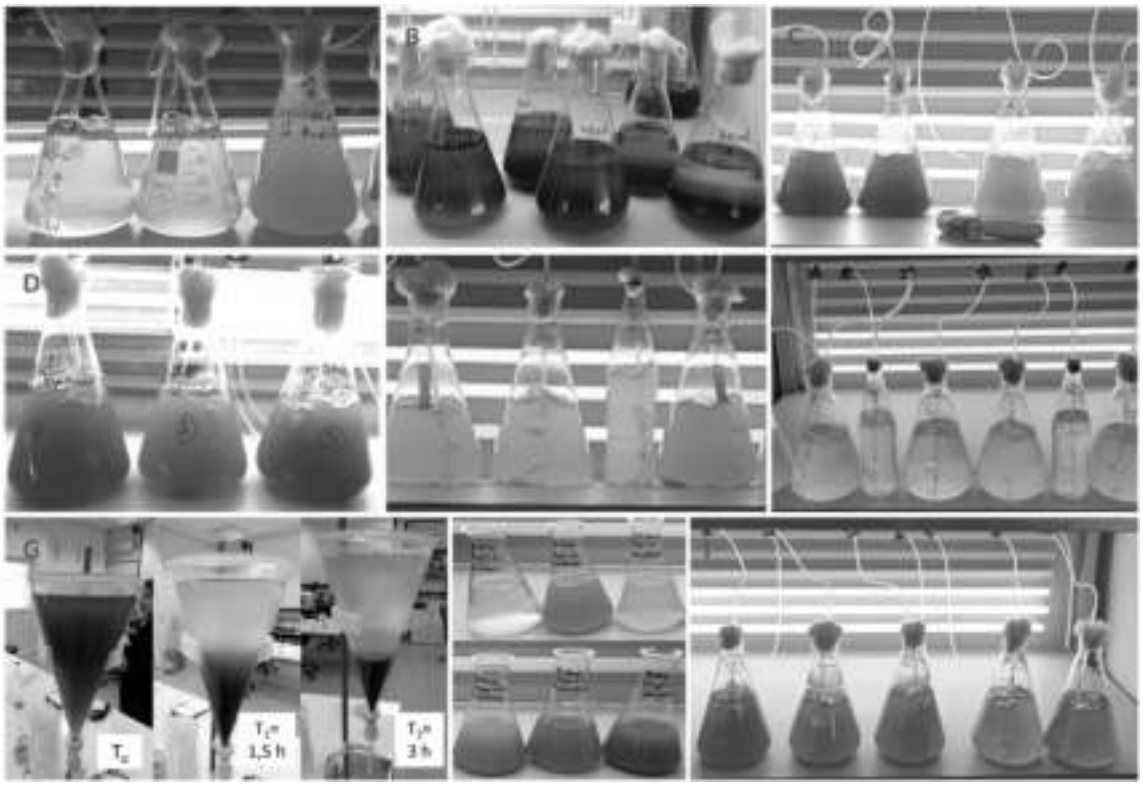

Fig. 4. Images of various stages of cultivation of the nine species isolated from the lagoons of Western Greece. A) Immature to mature (left to the right) cultures of *A. carterae*, B) Immature (dark-green) to mature (red) *Tetraselmis* sp. (var. red Pappas), C) Cultures of *T. marina* (var. Messolonghi), D) The characteristic pale green color of *Nephroselmis* sp. (var. Messolonghi) cultures at the middle of exponential phase, E) The typical grass-green color of *A. gracilis*, F) The bright green color of *Dunaliella* sp. (var. Messolonghi) culture in an early exponential phase, G) Time lapse of *Phormidium* sp. (var. Tourlida) filaments sedimentation, H) *Cyanothece* sp. cultures at various stages of maturation and I) Matured *Anabaena* sp. cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

wavy than the transverse. The nucleus was at the bottom of the hypocone. The oil-green chloroplast occupied most of the protoplasm and was lobed. The cytoplasm was vitreous with a variety of encapsulated particles and a variety of shades depending on the growth phase of the cultured cells.

Proliferation occurred by simple cell division and although amphigenic reproduction [58–60] and cyst production [59–62] are reported in the literature, this has not been observed in this species. *A. carterae* grew well in salinities of 20–50 ppt [27] and the typical color of its culture was orange-yellow at the beginning to brown-orange in the mature phase. This isolate was characterized by a high content of protein and lipids. Specifically, 50 % of its biomass consisted of proteins, while FAs occupied 18.8 % in terms of dry weight. However, *A. carterae* has been reported to accumulate inner sugars at levels up to 36.5 % on a dry biomass weight basis [46]. Almost half of the detectable FAs consisted of DHA and 12 % of them were comprised of EPA. After photometric analysis of the biomass extract from *A. carterae*, it was found that the main pigment of the cells was chlorophyll *a*, and the carotenoids made up 35 % of the sum of cellular pigments (chlorophyll *a*, chlorophyll *b*, and total carotenoids).

3.3. Tetraselmis sp. (var. red Pappas)

Tetraselmis sp. (var. red Pappas) is a single-celled planktonic chlorophyte similar in morphology to *T. marina* (var. Messolonghi), with cell dimensions of 10.52 ± 1.2 μm along the longitudinal axis and 8.11 ± 1.08 μm in width for the species isolated from the Kotychi lagoon and 11.32 ± 0.78 μm along the longitudinal axis and 7.95 ± 1.06 μm in width for the species isolated from the Pappas Achaia lagoon. The extremely interesting feature of this microalgae was the dark red color, that the water acquired in its mature culture. Thus, while in its initial phase (low cell concentration) the culture was dark green, as time passes, a brown-red color was acquired (Fig. 4B). The reddish color of the culture was naturally due to the corresponding reddish color of its cells (Fig. 3B), in which granules of various diameters enclosed throughout the mass of the cell, mixed with similar others of a green hue were observed microscopically.

Two varieties of this microalgae were isolated, one from the Kotychi lagoon which showed the green normal color of the cells when in the initial exponential growth phase, and then when the culture matured (static phase) they turned slightly red, acquiring finally a dark burgundy color. Concerning the second variety, (from the Pappas Achaia lagoon) its cells were reddish from the beginning. The first variety was named *Tetraselmis* sp. (var. red kotychi) and the second *Tetraselmis* sp. (var. red Pappas). Both varieties, when centrifuged, created reddish supernatant with green precipitate (cells) in *Tetraselmis* sp. (var. red kotychi) and dark red in *Tetraselmis* sp. (var. red Pappas). According to our knowledge, there are no similar findings in the literature. The red color of the water in the supernatant was due to unspecified substances produced by the cells and excreted in the water with varying intensity.

Regarding the biochemical biomass composition, the naturally isolated *Tetraselmis* sp. consisted of proteins close to 40 % and carbohydrates up to 25 %. The FAs that were extracted mostly from the cells of the present strain were the saturated fatty acid suitable for biodiesel production C16:0 and the unsaturated C18:3n3. *Tetraselmis* sp. is also known for its pigments, as in addition to chlorophyll *a*, it demonstrated chlorophyll *b* and a variety of carotenoids [63].

3.4. T. marina (var. Messolonghi)

T. marina (var. Messolonghi) is a single-celled planktonic chlorophyte with intense green color (in cells and culture medium-Fig. 3C). Cells elongated, cylindrical but slightly compressed, and slightly elliptical features generally common among others to this genus [64]. From a characteristic cell invagination emerged 4 isometric flagella distinguished in 2 pairs facing each other. The flagella impart intense mobility to the cell, which followed a straight course with periodic vortexing and frequent changes of direction.

Sometimes the cells took on a spherical shape, expelled the flagella, and immobilized. Very often the cells were also enclosed in a membranous, transparent bubble structure with a watery and sometimes slightly granular content. In this form, they were called palmelloid cells and while they retained their intense green color, they were immobile without flagella and most often filled with distinct spherical inclusions, obviously storage material (starch or lipids). Their metabolism during the palmelloid stage seemed to remain active, as in several of them cell divisions were observed similarly to what was reported for *T. indica* [65].

In "aged" cultures the palmelloid cells lost part or all their green color and appear to be filled with colorless spherical inclusions. A large cup-shaped chloroplast with a pyrenoid in a central position was prominent. A large orange blot was visible in the upward position of the cell (along the longitudinal axis) and the lateral position. In the center of the cell, there was the nucleus adjacent to the orange blot.

Reproduction was observed by simple cell division in both the vegetative mobile and the palmelloid stage. No sexual reproduction involving quasi-gamete fusion was observed but often, thick-walled spherical cysts were formed, and the cell divided into 4 daughter cells, which were then released and increased in size as motile vegetative cells.

This species was named by the current research team as *T. marina* (var. Messolonghi). Its size was very large, compared to the known species of the genus *Tetraselmis* (*T. suecica*, *T. chui*, *T. tetrathele*, etc. [64]), presenting a great variety in size and shape of the nucleus, the chloroplast, and in the various inclusions of the cell. However, the size of *T. marina* (var. Messolonghi) ranging from 18.62 ± 1.79 μm along the longitudinal axis and 10.02 ± 0.62 μm in width was much larger (perhaps the largest of all species of the genus [64]). Additionally, its high resistance to very high salinities of 150 ppt clearly distinguished it from the rest.

This particular species of the genus *Tetraselmis* appeared with increased quantities of carbohydrates, lipids, and pigments inside the cells [53]. The unsaturated fatty acids C18:2 and C18:3n3 were the most abandoned among others, while the saturated C16:0 covered 22 % of all the detected FAs.

3.5. Nephroselmis sp. (var. Messolonghi)

Nephroselmis sp. (var. Messolonghi) is a single-celled planktonic chlorophyte with bean-shaped cells flattened along the longer axis (Fig. 3D), each bearing two unequal flagella which protrude from the middle of the hollow portion of the cell. Cells without cell wall 5.28 ± 0.35 μm along the longitudinal axis and 3.81 ± 0.5 μm across the width. The flagella offer intense mobility to the cell with a characteristic linear movement slightly swirling and tremorous. The shorter flagellum moved in the forward direction and the longer pulsating in the back. Chloroplast large in a lateral position with a prominent pyrenoid. The chloroplast occupied most of the cell volume and had a photosensitive spot in a position corresponding to the lower part of the short flagellum outlet. Overall, its cell characteristics resembled those described for *Nephroselmis viridis* in the study of Yamaguchi et al. [66]. The color of the cells under the microscope was dull green while in the culture vessel was light green (Fig. 4D). Reproduction was observed either by cell division (usually) or amphigenically in a hologamous manner, i.e. by fusion of two identical vegetative cells to form a zygote, but without cyst formation. The cells grew adequately in salinities between 35 and 65 ppt [27].

The *Nephroselmis* sp. biomass stood out along with *A. carterae* for its content of polyunsaturated FAs such as DHA and EPA. Lipids accounted for about 15 % of dry biomass. In contrast to the relatively low percentage of proteins that makes it inadequate for nutrition applications, pigments could appear up to 5 % and give antioxidant properties. Lutein

and β-carotene are some of the valuable carotenoids mentioned after *Nephroselmis* sp. biomass analysis [52].

3.6. A. gracilis

A. gracilis is a single-celled planktonic chlorophyte without a cell wall (naked). The most important morphological characteristics of its normal, mature cells (vegetative) were: (i) spindle shape (sometimes narrow) (Fig. 3E), (ii) occasionally due to its characteristic tremor-like motion it looked asteroid (Fig. 3E), (iii) two equal flagella attached to the apical area, tilted sideways, (iv) 3 to 6 (the most characteristic 6) "notches" (like a keel), running lengthwise along the entire cell, (v) average length 18.84 ± 2.88 μm along the longitudinal axis and 13.09 ± 2.4 μm across the width and with extreme sizes of 12–22 μm along the longitudinal axis and 8.7–16.36 μm in width, (vi) flagellum length equal to 1½ to 2 times the length of the cell, and (vii) outer surface without scales, however, on the surface of the cell and flagella were often visible fine fibrous deposits.

Chloroplast with an almost basin-like form, thin and pale (without intense green color) on its larger surface extended to the upper part of the cell, almost to the base of the flagella. It had an asymmetric pyrenoid, which was directly connected to the posterior part of the large nucleus. In the upper part of the cell and inside the chloroplast, there was an orange eye spot (stigma). The large nucleus with a distinct nucleolus was located almost in the center of the cell with its front part extending almost to the base of the flagella. In the outer layer of the cytoplasm, many small mitochondria could be seen. In the anterior part of the cell more or less symmetrically around the nucleus, several reticulosomes 4–6 in number were distributed, which together form the Golgi system. The large number of micro-vesicles, visible in the anterior part of the cell, between the reticulosomes, probably emerged from them.

Many thin-walled cysts were often accumulated at the bottom of an old culture. No evidence had been found linking cyst formation to the reproductive process. The cells that were going to form cysts enlarged significantly, keeping the nucleus relatively small. The cyst's wall initially appeared to settle, like a discontinuous layer peripherally of the cytoplasm, which then thinned, starting from the inside. Mature cysts had a diameter ranging from 13 to 18 μm and a wall thickness of 2 to 3 μm.

This species reproduced by cell division by separating the daughter cells along the longitudinal axis, i.e. the imaginary line that connected the emerging area of the flagella with the posterior bulge of the cell in a similar manner described by Floyd [67]. No sexual reproduction was observed, therefore neither a zygote of any form (swollen cell, cyst, or palmella) nor, spores (aplanospores or multiple zygote divisions) were observed.

One of the characteristics of *A. gracilis* isolated for the present work was the low levels of proteins and carbohydrates, which were offset by the high content of minerals (30 % on a dry weight basis), measured after biomass incineration. The main FAs of *A. gracilis* were C16:0, C18:1, C18:2, and C18:3n3 which made up 80 % of the accumulated FAs [68]. In addition, the pigment that prevailed after extractions was chlorophyll *a*, while carotenoids corresponded to 13.2 % of the total pigments estimated.

3.7. Dunaliella sp. (var. Messolonghi)

This species is a single-celled planktonic chlorophyte without a cell wall and with 2 equal flagella 1.5–2 times the length of the thallus. Cells pear-like in their usual appearance with flagella emerging from the pointed edge. The large nucleus in a central position of the upper part (the pointed) of the cell, was surrounded for its most part by the large green chloroplast, which occupied almost the bulk of the protoplasm. At a centric place of the chloroplast, there was a discernable pyrenoid surrounded by starch granules. Additionally, several small vacuoles were observed around the front part of the chloroplast. The absence of a cell wall did not allow the rigidity (stability) of the cell shape which was very malleable. It was easily affected by the osmotic state of the surrounding water and accordingly swelled or shrunk depending on the water salinity without losing viability similarly to what was reported for *D. salina* [69]. Cell size varied greatly depending on the conditions. A typical size was 7–12 μm along its long axis but was also found in bigger sizes (14–18 μm). Very mobile species, and the very fast blows of its flagella pushed it forward in a characteristic course with sharp and twisting movements along the horizontal axis of the cell. The average (typical) size of *Dunaliella* cells, however, was much smaller compared to other flagellated species found in hypersaltability such as *A. gracilis* (17–25 μm) or *T. marina* (18–25 μm).

The shape of the *Dunaliella* cells varied, even if its typical image was pear-shaped; it could become cylindrical, oval, elliptical, spindled, or spherical (Fig. 3F) with changes depending on conditions, i.e. temperature, light, nutrients, and above all salinity. However, as a general rule, under adverse conditions (mainly depletion of nutrients) it turned into a spherical form.

Cell size also varied greatly and in a thriving culture with cells in the exponential phase of population growth, a variety of sizes was observed with the majority at a typical size of 8–10 μm along the longitudinal axis and 4–5 μm in width, and a smaller percentage of smaller cells in the order of 5–8 μm, apparently from a recent zygote or aplanospore proliferation. In conditions of very high salinity where cell proliferation was dramatically reduced, the cells grew in size and could reach 15 μm while at the same time they began to acquire an orange hue due to the accumulation of carotenoids.

It lacked a pulsated vacuole (usually at the front of the cell) that helps other algae to osmoregulate because *Dunaliella* genus had a different "strategy" for dealing with osmotic stresses by producing and accumulating glycerol intracellularly [70].

At the anterior end of the cell and depending on the physiological state, a various number of granules (droplets) of oil (lipids) could be distinguished. The glycerol (glycerin) produced at high salinity levels by this *Dunaliella* strain was sometimes so profound that its excess was excreted from the cell, and the culture water accumulated on the surface a thick white foam of creamy texture.

Normally the cells were very mobile, the flagella pulsated, and the movement was fast with rather irregular movements and "rest" intervals with trembling. Sometimes the cells were concentrated in large numbers in clusters that looked like "bunches" of grapes that were irregular in shape and size. Some were made up of a few cells and others of hundreds. In these bunches, the cells were arranged in a way that seemed to serve an unknown purpose. The pointed sides of the cells, where the flagella emerge, were located inside the bunch, while their opposite side "faced" outwards. The flagella in these agglomerates, no matter how densely packed, seemed to retain some mobility. With constant observation, it was noticed that new cells were added to each bunch periodically, while other times some cells were detached from it, removed, and acquired their usual high mobility. Sometimes the removal of many cells in a short time dissolved the agglomerate. The significance of this phenomenon for the survival of the species remained unexplained in the literature, but of what has already been observed it occurs in vigorous cultures.

Dunaliella multiplied by longitudinal cell division particularly when it was in its kinetic phase (there was also a phase that rounds and does not move). Multiplied also by gamete coupling (isogamy), i.e. the union of two cells with their anterior axial part for the fusion of their haploid nuclei and the formation of the diploid zygote. This was the other mode of reproduction (amphigenic), where each conjugated cell played the role of a "female" or "male" gamete. During coupling, in addition to the nuclei, a common larger cytoplasm was created, and the formed zygote was round, had lost the flagella, was green or red, and was surrounded by a smooth and thick cell wall. The zygote first fell into a phase of inertia (its exact duration is unknown) and then initially with a meiotic

division and then with mitotic, formed up to 32 haploid microscopic cells which were released after rupture of the zygote wall and grew to the normal size of the vegetative cell.

This natural isolate was distinguished for its high levels of protein accumulation and its rich biomass in pigments. Half and more of its pigments were assigned to chlorophyll *a*. In addition to the fact that this strain showed the highest content of pigments (4.6 % of dry biomass weight) among the other isolates, it also exhibited a significant content of saturated FAs, C16:0 and C18:0 (52 % of total FAs). Thus, it can be very promising for biodiesel production [71].

3.8. Phormidium sp. (var. Tourlida)

This species is a filamentous type of cyanobacterium (Fig. 3G), not nitrogen-fixing as it does not possess heterocytes (specific atmospheric N_2-binding cells). Filaments (trichomes) were unbranched, generally straight very long without much twisting and slightly curved when in large masses. Sometimes the filaments were wrapped in a spiral, while with a smooth appearance and exhibiting slow gliding motion. Their color varied depending on the phase of cultivation from light green to green-olive, to olive-yellow. The final parts of the filaments (ends) were not sharp but slightly curved. The mucus sheath that covered the filaments was compact, very thin, and absent in the terminal cells.

No akinetes were formed, and the filaments consisted of cylindrical cells with a length slightly greater than their thickness (2.37 ± 0.2 μm). Adjacent cell walls at the contact points of the cells did not form visible partitions. Aerotopes were not usually seen in cells, but sometimes a small number of small aerotopes appeared. The content of the cytoplasm at high magnification appeared granular with very small granules of green-cyan-green hue.

Propagation was by fragmenting the filaments into pieces of varying lengths which were then elongated by cell division. The cell divisions (fissions) of the cells were made transversely, i.e. perpendicular to the axis of the filament. The cell first grew to the appropriate double size and then was divided. Only the terminal cells of the filament did not have such a capacity. The points of the thread, where the rupture was to take place, developed necridia, i.e. cells that would dissolve to cause rupture and separation of the filament.

Phormidium grew rapidly under normal conditions (20–22 °C, 2000–8000 lx) and could be characterized as very hardy, withstanding a wide salinity range of 15–60 ppt with better growth around 40 ppt [26,72]. Its starting culture (green color) created a mass of filaments, which were visible to the naked eye as they swirled with the aeration in the culture vessel. After a few days, the culture acquired an oil-green color and finally oil-yellow when the growth reached high levels of biomass. Water with *Phormidium* left undisturbed for a long time showed impressive sedimentation of the filaments creating a solid precipitate with almost transparent supernatant (Fig. 4G). Sedimentation was completed in about 2.5 h and occurred at the same rate regardless of the color phase of the culture.

If the precipitate from the green phase was left for a long time (especially in the freezer at −19 °C), then, after thawing, an intense blue color will appear in the water due to the release from the cells of the water soluble pigment phycocyanin, which is used by industry as a powerful antioxidant. Therefore, *Phormidium* offers many advantages for its mass cultivation such as quick growth with dense concentrations, ease of collection due to spontaneous sedimentation or by filtration through a plankton pore net of 50–100 μm and phycocyanin production [26,72].

Despite the low accumulation of FAs, *Phormidium* sp. from Tourlida reached 45 % in carbohydrates. As a cyanobacterium, it did not demonstrate chlorophyll *b* and 90 % of its estimated pigments corresponded to chlorophyll *a*. Microorganisms of the genus *Phormidium* are gaining more and more research interest due to isoprene production and the easy isolation of phycocyanin, a pigment of a strong blue color [73].

3.9. Cyanothece sp. (var. Messolonghi)

Cyanothece sp. (var. Messolonghi) a single-celled planktonic nitrogen-fixing [74] cyanobacterium (Fig. 3H), never formed colonies. Sometimes, however, the cells united to form short straight or curved chains but without heterocytes or akinetes similar to those of *Anabaena* or *Nostoc*.

Cell shape varied from spherical to oval, bar-shaped or slightly swollen, curved or slightly sigmoid, but always rounded at the edges. The size varied greatly for each sample, depending on the maturation phase of the culture. In general, the cells (when solitary and spherical) had a diameter of 8–12 μm, and when they were united during fission ~15 μm. The content of the cytoplasm were homogeneous or with numerous evenly distributed granular inclusions. The color of the cell varied depending on the physicochemical conditions of the water, the age of the culture, and the color of the aforementioned inclusions. This presented a chromatic polymorphism with cells light blue-green (cyan-green), bright cyan-green, oil-green, brown-red to light pink (Fig. 4H). It was not clear whether this variety of coloring was due to the known color adaptation of cyanobacteria [75] or due to different types of metabolic processes.

The cell was covered by a distinct layer of the cell wall. Inside, the dimly visible thylakoids were arranged concentrically following the contour of the wall. The cells could produce mucus, which they sometimes (especially in mature cultures) produced and excreted in large quantities resulting in excessive foam production on the surface and a creamy texture of the water.

It reproduced by cell fission (always 2 daughter cells) in a direction perpendicular to the longest axis of the cell. Absolutely symmetrical daughter cells grew in the original size of the mother cell. Upon completion of the cell division, the resulting cells either separated completely or remained united for a long time in short strands in a kind of yarn (pseudo-filaments). When culture conditions deteriorated (e.g., nutrient depletion), the cells were slightly deformed losing their symmetry and dividing asymmetrically.

This cyanobacterium had low concentrations of proteins, sugars, and lipids in its cells. More precisely, proteins occupied 34.3 % of total dry cell weight, carbohydrates reached only 17.4 % and FAs accounted for 8.2 % of dry biomass. Chlorophyll a was dominant over carotenoids, which were all estimated photometrically. However, compared to the other isolates, *Cyanothece* sp. were cells with high levels of carotenoids (26.5 % of total pigments estimation) that potentially could cause antioxidant properties. Like *Phormidium* sp., *Cyanothece* sp. could be exploited for phycocyanin production [72,76].

3.10. Anabaena sp. (var. Kotychi)

Anabaena sp. (var. Kotychi) is a nitrogen-fixing filamentous cyanobacterium, with unbranched mostly straight filaments, of varying length comprised of a few cells (5–10) ~ 20–30 μm in length, and very long (>250 μm) made up of hundreds of cells (Fig. 3I). The long filaments folded to form large curves but with no filament twisting or tangled cells. In dense cultures the filaments were arranged in some way in parallel lines. The mobility of the filaments was almost absent and only occasionally a very slow sliding movement occurred. It did not have a visible pouch covering the filament, but sometimes there was a thin glassy, and colorless mucus cover.

The cells that made up the filament were clearly distinct from each other by obvious tightening of adjacent cell walls. There were 3 types of cells, the vegetative which were the most numerous and constitute the "thallus" of the algae, the heterocytes which were the nitrogen-fixers found sporadically along the thread, and the akinetes.

The vegetative cells were uniform in size along the filament and their shape was cylindrical like a barrel tending to the spherical with a length slightly greater than their width (~4.2 × 3.8–4.7 × 4.5 μm). In some filaments, the cells were uniform while in others due to cell division they

were distinguished into the normal size and into cells that were half the size of normal due to the division, which was evident from the constriction in the middle.

Their color varied depending on the condition of the filament from light green, to cyan-green or oil-green and olive. However, whatever color they had, it characterized the whole filament, that is, no heterogeneous colored areas could be distinguished in the same filament. In the cytoplasm of vegetative cells, there were granular aggregates and sometimes aerotopes. The terminal cells of the filaments were conical, lighter in color, slightly larger than the others and did not present granular content or aerotopes.

Heterocytes were clearly distinct from vegetative cells, had a spherical shape, and were sometimes slightly or fully cylindrical (Fig. 3I). However, in each case, they were larger (~5.6 μm in the spherical ones, ~6.3 × 5.7 μm in the cylindrical ones) than the vegetative cells. They were characterized by an enhanced thickness of that part of the cell wall that adjoins the adjacent vegetatives. Heterocytes appeared in varying numbers from 1 to 3–9 in each filament and two heterocytes never bordered each other, as vegetative cells were always between them. Sometimes heterocyte neighboring cells were akinetes, apparently because a neighboring vegetative cell had become akinete. Furthermore, a neighboring akinete could also acquire another neighboring akinete.

The akinetes were large cylindrical cells much larger (~14.4 × 6.4 or ~19.7 × 7 μm, two characteristic sizes) than the vegetative or heterocyte cells. They had a thick cell wall and highly granular cytoplasm. They were observed as single cells intervening in the row of vegetative ones or even 2 (rarely 3) together in a row. Several filaments had heterocytes that were adjacent on both sides with akinetes. Very often the akinetes were released from the filaments and existed as solitary cells in the water.

The filaments of *Anabaena* elongated with many divisions of their vegetative cells. In addition, the filaments at some sites ruptured as some vegetative cells became necrotic and disintegrated. Thus, the filament broke into pieces and these fragments were elongated by the proliferation of their cells.

This strain of *Anabaena* sp., had good growth characteristics in its culture [28] as apart from the high concentrations of chlorophyll *a* and carotenoids, exhibited also an increased protein content close to 50 % of the dry biomass. Unfortunately, this cyanobacterium is also characterized by low lipid accumulation. This strain could be more than important for the production of high-value products, as phycocyanin and it has recently been reported for its ability to accumulate biopolymer molecules as potential intracellular energy sources [77].

4. Conclusions

In the search for high-value compounds among the microalgae species examined, those referred in the present study proved very sturdy in establishing monospecific cultures that could support industrial-scale production. Among them, there are certain most prominent candidates. These are, with regard to highly unsaturated fatty acids, *A. carterae* and *Nephroselmis* sp., which exhibited a high content in both DHA and EPA (30–50 % and 10–20 % of their total accumulated fatty acids respectively). *Nephroselmis* sp. showed a higher lipid content (around 15 %), which could mean that the yield of highly unsaturated fatty acids would be higher in cultures of *Nephroselmis* sp. *Phormidium* sp. was another interesting species because it was easily cultured with a profound phycocyanin production. Moreover, agglutination and sedimentation of its filaments facilitates the harvesting procedure on industrial scale.

CRediT authorship contribution statement

George Hotos: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Despoina Avramidou:** Methodology, Validation, Formal analysis, Data curation. **Savvas Giannis Mastropetros:** Methodology, Data curation, Formal analysis, Writing – original draft, Visualization. **Konstantina Tsigkou:** Methodology, Software, Data curation, Visualization, Validation, Writing – review & editing. **Konstantina Kouvara:** Methodology, Data curation, Formal analysis, Writing – original draft. **Pavlos Makridis:** Conceptualization, Methodology, Resources, Data curation, Supervision, Project administration, Funding acquisition. **Michael Kornaros:** Resources, Writing – review & editing, Supervision, Funding acquisition.

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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