Screening of cyanobacterial and microalgal biodiversity in the North Adriatic area (Italy) based on microscopy and the DNA barcoding method

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Abstract

Microalgae are a wide group of photoxygenic microorganisms, spanning different taxa and living in various environments. Commonly speaking, under this term also cyanobacteria are often included, even if, contrarily to all the rest of microalgae, they are prokaryotes. To face the conditions of their habitats, cyanobacteria and microalgae show a vast range of adaptations, including the production of bioactive compounds that can be also exploited in several human fields. With this background, a project was started aiming at isolating different cyanobacterial and microalgal strains from natural environments (with a particular focus on water habitats) and at characterizing these strains to understand their biotechnological potentials. Here we present the first results obtained after samplings carried out in the North Adriatic Italian area, which were followed by the isolation and preliminary characterization of seven photoxygenic strains. The isolated strains encompass five phyla: Bacillariophyta, Chlorophyta, Ochrophyta, Rhodophyta and Cyanobacteria.

Keywords: 16S rRNA, 18S rRNA, cyanobacteria, DNA barcoding, microalgae, microscopy, North Adriatic Sea

Riassunto

Screening della biodiversità cianobatterica e microalgale nel Nord Adriatico (Italia) mediante microscopia e metodo del DNA barcoding
Le microalghe sono un ampio gruppo di organismi fotossigenici, comprendenti diversi taxa e presenti in svariati ambienti. Comunemente parlando, sotto questo termine sono spesso inclusi anche i cianobatteri, anche se, contrariamente a tutto il resto delle microalghe, sono procarioti. Per affrontare le condizioni dei loro habitat, cianobatteri e microalghe mostrano un vasto range di adattamenti, inclusa la produzione di composti bioattivi che possono essere anche sfruttati in diversi settori di interesse antropico. Con queste premesse, è stato avviato un progetto allo scopo di isolare differenti ceppi cianobatterici e microalgali da ambienti naturali (con una particolare attenzione per gli habitat acquatici) e di caratterizzare questi ceppi per comprendere le loro potenzialità biotecnologiche. Sono qui presentati i primi risultati ottenuti in seguito a campionamenti condotti nell'area Nord Adriatica Italiana, che sono stati seguiti dall'isolamento e dalla preliminare caratterizzazione di sette ceppi fotossigenici. I ceppi isolati abbracciano cinque phyla: Bacillariophyta, Chlorophyta, Ochrophyta, Rhodophyta and Cyanobacteria.

Parole chiave: 16S rRNA, 18S rRNA, cianobatteri, DNA barcoding, microalghe, microscopia, Nord Adriatico

Introduction

The term "microalgae" includes a wide group of photoxygenic microorganisms, belonging to different taxa, able to colonize several habitats, and showing different adaptations to the environment where they live (Andersen, 1992; Malcata *et al.*, 2018). Practically speaking, also cyanobacteria are often regarded as microalgae, even if these microorganisms are prokaryotes contrarily to all the rest of microalgae.
To cope with their habitats and often also to face sudden environmental changes, cyanobacteria and microalgae put in place several adaptations, including the production of bioactive compounds. Many of these compounds find application in human fields, such as agriculture, nutraceutics and cosmeceutics (e.g., Lauritano *et al.*, 2016; Renuka *et al.*, 2018; Gaignard *et al.*, 2019; Levasseur *et al.*, 2020; Alvarez *et al.*, 2021; Fernandes & Cordeiro, 2021; Kiran & Venkata Mohan, 2021).
Since the bioactive compound production capability of cyanobacteria and microalgae can vary not just among high taxonomic ranks (e.g., phyla, classes, orders, families), but even within genera and species (e.g., Lauritano *et al.*, 2016; Mo-

LINO *et al.*, 2018; LEVASSEUR *et al.*, 2020; FIGUEROA-TORRES *et al.* 2021; KRIVINA *et al.*, 2023), the correct identification of cyanobacterial and microalgal strains is at the base of every more practical study. However, the identification of these photoxygenic microorganisms is often complicated by the simple morphologies of several groups (with none or very few morphologically diagnostic characters), by the high phenotypic plasticity observed for some taxa (whose morphology is greatly influenced by the environmental conditions) and by the existence of cryptic or semi-cryptic species (i.e., distinct species whose morphologies are totally or almost totally overlapping) (e.g., DARIENKO *et al.*, 2015; MALAVASI *et al.*, 2016; SCIUTO *et al.*, 2015; 2017; 2019; 2021; 2023; ZAMMIT, 2018; KRIVINA *et al.*, 2023). For this reasons, besides the classical taxonomic studies based on microscope observations, molecular analyses are generally required to precisely identify cyanobacteria and microalgae. Among molecular techniques, the DNA barcoding method is often used as the first step, often followed by more elaborate analyses, since it is a fast approach to identify even photoxygenic microorganisms (e.g., HALL *et al.*, 2010; DARIENKO *et al.*, 2015).

In this context, the research project "Biotechnological potentials of microalgae for environmental sustainability" has been recently founded by the European Social Fund (ESF) - Italian National Operational Programme (NOP) on Research and Innovation 2014-2020. The project aims at characterizing several cyanobacterial and microalgal strains to understand their possible use in human fields, considering both strains already available in public/private collections, but only partially characterized, and new strains isolated from natural environments, with a focus on marine and transitional water habitats. The exploitation of terrestrial and aquatic biodiversity for the sustainable production of bioproducts and, in particular, the attention for the biomarine resources are highlighted by the Research Priority 2a of the Area 5.6.3 of the Italian PNR (i.e. "Programma Nazionale per la Ricerca"; in English "National Program for Research") 2021-2027.

Given this background, with the purpose of better characterizing the North Adriatic photoxygenic microorganism biodiversity, first samplings were carried out both along the coasts and from more internal water basins of the North Adriatic Italian area since summer 2022 to summer 2023. Once in the laboratory, the collected water samples were processed for the isolation of photoxygenic strains, which were then preliminary characterized using both microscopy (i.e., light microscopy and/or electron microscopy) and the DNA barcoding method. Here we present the first results of these characterizations. The present work can help to understand which strains are the most promising ones for the aims of the above research project and, therefore, to address the future research efforts.

MATERIAL AND METHODS

Samplings

Water samples were collected during several sampling campaigns between August 2022 and June 2023 in different sites along North Adriatic Sea Italian coasts: the Venice Lagoon (Venice, Veneto region) and the Comacchio area (Ferrara, Emilia-Romagna region). In particular, in the Comacchio

Fig. 1. Map of the sampling area, with the four sampling sites numbered as follows: 1) Santa Maria del Mare, Venice Lagoon; 2) Lido of Spina coast, Comacchio; 3) Fattibello, Comacchio lagoons; 4) Lido of Spina internal basins, Comacchio.

area water samples were taken both along the coast (at Lido of Spina) and from more internal transitional water environments (at Fattibello and Lido of Spina) (Fig. 1). During each sampling, water temperature and salinity were measured. In one case, the sampling site was represented by an aquarium tank, filled in with water taken from Santa Maria del Mare (Venice Lagoon) and used to grow the seagrass *Cymodocea nodosa* (Ucria) Ascherson, collected from the same site (Fig. 2A). The aquarium was kept at room temperature, under natural light filtering from a window, and equipped with an air pump. Samples of the microbial mats growing on the aquarium walls were collected using a scalpel (Fig. 2B).

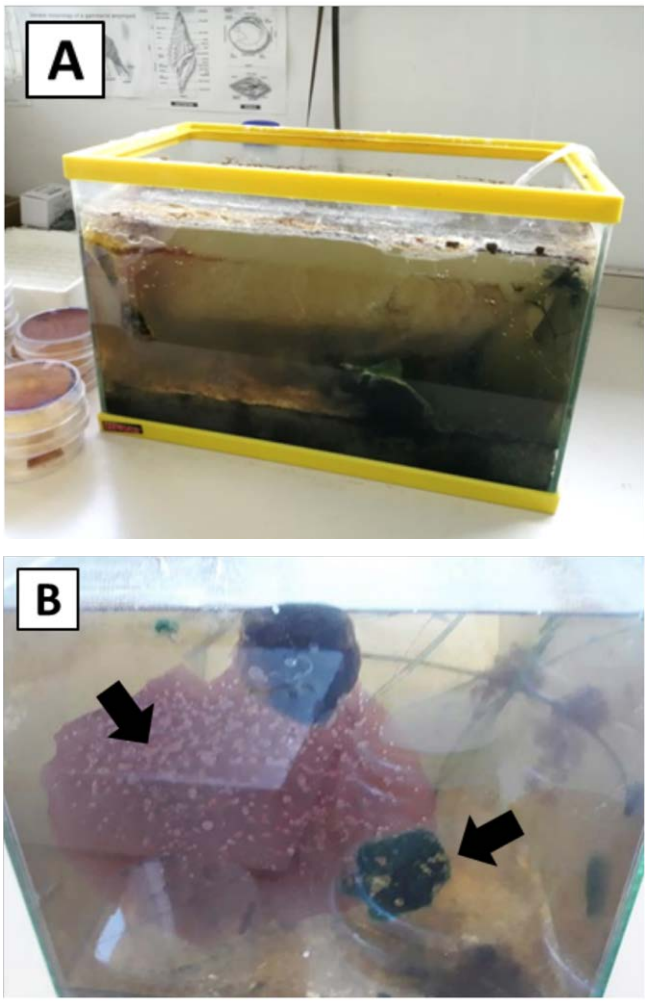

Fig. 2. The aquarium tank, set up to grow *Cymodocea nodosa*, from which microbial mats were collected (black arrows).

Strain isolation and culture set-up

Aliquots of the collected water samples and of the microbial mats growing in the aquarium tank were plated on different agarized media, made with 1.5 % (w/v) agar dissolved in F/2 medium (Guillard, 1975) or in BG11 medium (Rippka *et al.*, 1979), each one prepared with filtered and sterilized 35 ‰ seawater. The solid media plates were incubated either at 16°C or 24°C, under a continuous light intensity of 40 µmol m^{-2} s^{-1}, produced by cool white fluorescent lamps. After some days, the plates were observed and single colonies were picked and put, each one, in a flask with the same liquid medium used to prepare the agarized plate. Light Microscope (LM) observations of the liquid cultures were performed after 1-2 weeks and, if more than one species was still present, a culture aliquot was re-plated in a new plate containing the same agarized medium. These steps were repeated until unicyanobacterial/unialgal cultures were obtained. For each isolated photoxygenic strain, it was assigned a strain identifier represented by the acronym "KS" (= "Katia Sciuto") and a consecutive number starting from "1" (Fig. 3).

Fig. 3. Plates used to isolate the photoxygenic strains and flasks with the liquid cultures of each isolated strain.

Microscopy

LM observations of each isolated strain were carried out on culture aliquots with a light microscope Leitz Dialux 22 (Wetzlar, Germany), equipped with a digital image acquisition system. For some strains, culture aliquots were also fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and brought to the Electron Microscopy Centre of the University of Ferrara, where they were processed furtherly and, successively, observed with a Zeiss Evo 40 Scanning Electron Microscope (SEM) and/or with a Hitachi H800 Transmission Electron Microscope (TEM). Final publishable images of the isolated strains were created with Inkscape version 0.92 and GIMP version 2.8.22.

Molecular analyses

For the DNA extraction, a cell pellet of each isolated strain was obtained after brief centrifugation of a liquid culture aliquot and, successively, it was grounded in a mortar with a pestle and quartz sand (Fluka), to better disrupt the plant cell walls and produce an homogenate. Total DNA was extracted

from each of the obtained homogenates using the Genomic DNA purification kit (Thermo Scientific™, Waltham, MA, USA) according to the manufacturer's instructions.
For eukaryotic microalgae, a ≈1200 bp-portion of the 18S rRNA gene was amplified using the primers Euk528F (EDGCOMB *et al.*, 2002) and EukB (MEDLIN *et al.*, 1988), following the PCR protocol reported by SCIUTO and collaborators (SCIUTO *et al.*, 2019).
For cyanobacteria, the 16S rRNA gene was amplified: alternatively, a ≈1600 bp-fragment was obtained with the primers 16S1 and 16S2 (MORO *et al.*, 2007) or a ≈800 bp-fragment was produced with the cyanobacteria-specific primers CYA106F and CYA781R (NÜBEL *et al.*, 1997), using the PCR protocols suggested in the corresponding papers.
After verification with agarose gel electrophoresis, the obtained PCR products were purified using the HT ExoSAP-IT (Applied Biosystems™, Waltham, MA, USA) and sequenced at the Eurofins Genomics Sequencing Service (Ebersberg, Germany), with the same primers employed in the PCR reactions. Moreover, the following additional internal sequencing primers were necessary: Euk1209F (GIOVANNONI *et al.*, 1988) and U1391R (DAWSON & PACE, 2002), for the 18S rRNA amplicons; 16S3, 16S4, 16S5 and 16S6 (MORO *et al.*, 2007), for the 16S rRNA amplicons obtained with the primer pair 16S1-16S2.
The GeneStudio sequence analysis software (http://genestudio.com) was used to assemble final consensus sequences. The obtained sequences were compared with those available in the INSDC (International Nucleotide Sequence Database Collaboration) archives, using the BLAST tool (ALTSCHUL *et al.*, 1990) available at the NCBI website (www.ncbi.nlm.nih.gov).

RESULTS

During this research work, seven photoxygenic strains were isolated and subjected to a preliminary characterization based on microscopy and the DNA barcoding method. The strains were named KS1, KS2, KS3, KS4, KS5, KS6 and KS7.

Microscope observations

Based on the light microscope observations, strains KS1, KS2, KS3 and KS7 were eukaryotic microalgae (Fig. 4), while strains KS4, KS5 and KS6 were filamentous cyanobacteria (Fig. 5). For some strains electron microscope observations were also carried out (Fig. 6).

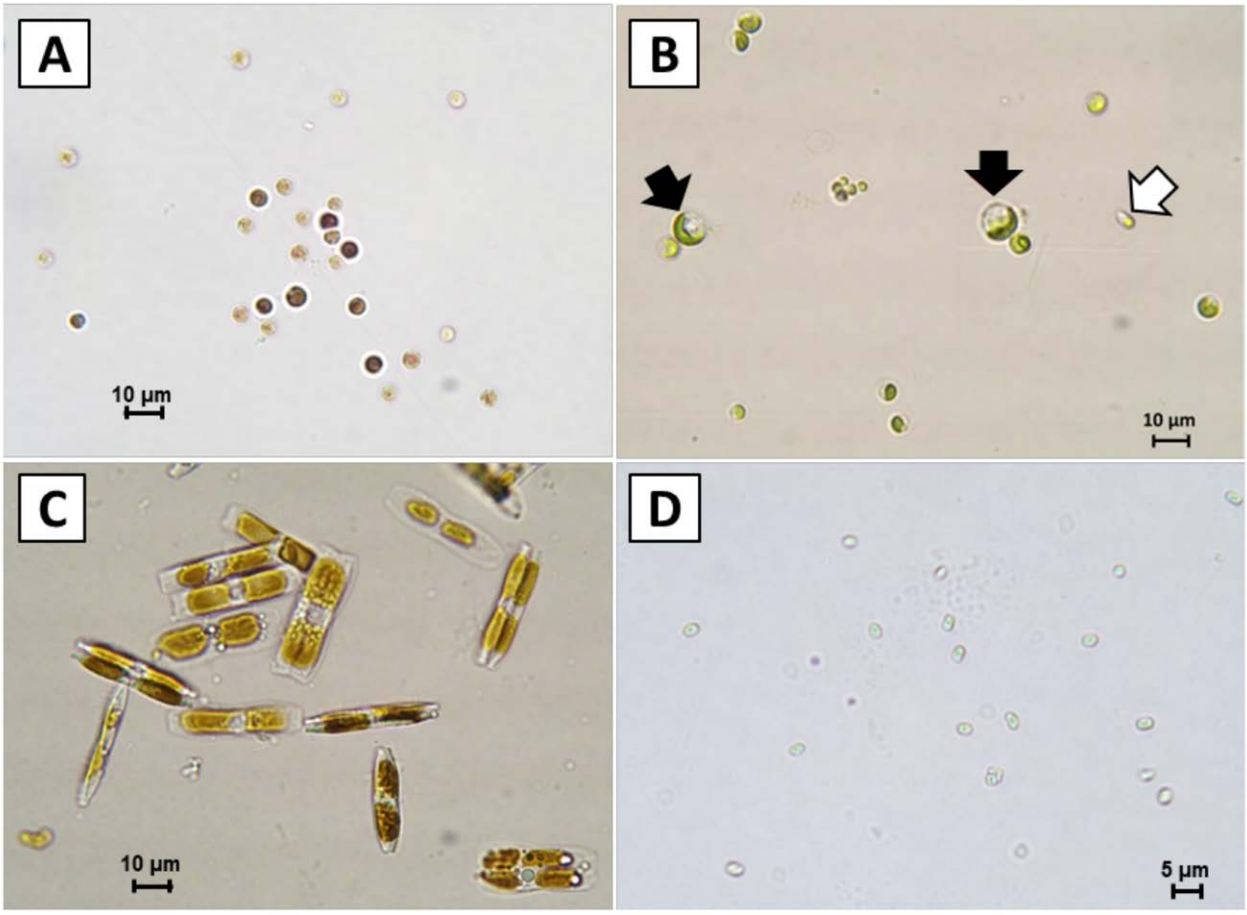

Fig. 4. The isolated eukaryotic microalgae: A) strain KS1; B) strain KS2, with cells showing the parietal chloroplast (black arrows) and a zoospore (white arrow); C) strain KS3; D) strain KS7.

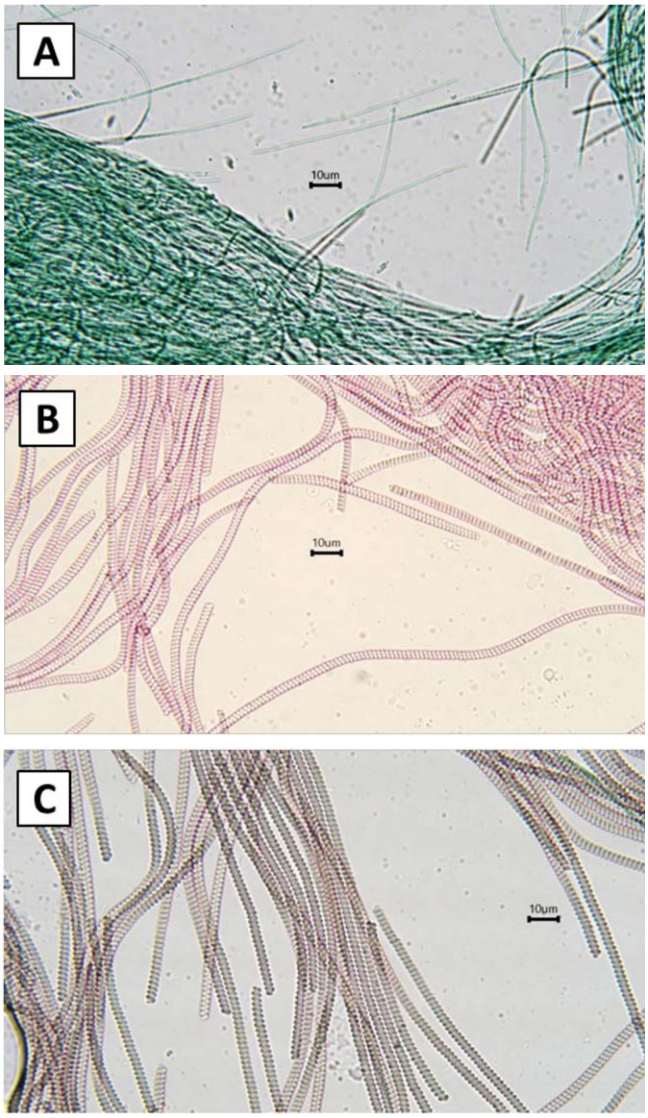

Fig. 5. The isolated cyanobacteria: A) strain KS4; B) strain KS5; C) strain KS6.

Strain KS1 (Fig. 4A, 6A) was a brownish-red coccoid microalga, characterized by spherical cells with a cell diameter variable between about 4.0 and 8.5 µm. With the LM the internal cell details were not distinguishable, but the entire cytoplasm was coloured suggesting that the chloroplast occupied all (or almost all) the cytoplasm (Fig. 4A). This was confirmed by TEM observations (Fig. 6A), which allowed to see a multi-lobed chloroplast, occupying almost all the cytoplasm; starch granules scattered throughout the cell were also present (Fig. 6A). An abundant extracellular polysaccharides production was observed (Fig. 6A), leading to the formation of cell aggregates (data not shown).

Strain KS2 (Fig. 4B, 6B) was a light green coccoid microalga, characterized by different cell forms. Indeed, cells had different morphologies (from more spherical to more elongated to some fig-shaped vegetative cells) and sizes (3.0 to 10.0 µm in diameter). In the bigger cells a single parietal cup-shaped chloroplast could be observed (Fig. 4B) and motile elongate zoospores were detected (Fig. 4B). Sporangia, up to 30 µm in diameter, were also present (data not shown). At TEM, a parietal chloroplast, with a single pyrenoid surrounded by a fragmented starch plate, was visible (Fig. 6B). Abundant extracellular polysaccharides were produced by the microalga (data not shown).

Strain KS3 (Fig. 4C, 6C) was a nitzschioid diatom, with about 30.0-40.0 µm long and 3.0-4.7 wide cells. The cell apices were slightly capitate (Figs. 4C, 6C). Two brownish-yellow chloroplasts were visible in each cell (Fig. 4C). An eccentric raphe was present and extracellular polysaccharide production was also observed (Fig. 6C).

Strain KS7 (Fig. 4D) was a yellow-green coccoid microalga, characterized by small elongated cells, 3.0-4.0 µm long and about 2 µm wide. Due to the very small sizes, with the LM the cell details were not distinguishable.

Strain KS4 (Fig. 5A) was a blue-green non-heterocytous filamentous cyanobacterium, characterized by long and very thin isopolar trichomes. The trichomes were unbranched, less than 1 µm in diameter and were composed by longer than wide cells; the filament apical cells were rounded, without a calyptra (Fig. 5A). Due to the small sizes, at the LM no further cell details could be distinguished. In older cultures, strain KS4 was able to form a thick polysaccharide capsule embedding the intertwined trichomes (data not shown).

Strain KS5 (Figs. 5B, 6D) was a pinkish-red non-heterocytous filamentous cyanobacterium, characterized by tightly coiled isopolar trichomes with a spiral/helix shape. Trichomes had a diameter of about 2.8-3.6 µm and the trichome apical cells were without a calyptra. Trichomes were unbranched (Figs. 5B, 6D). In older cultures, abundant extracellular polysaccharides embedded the intertwined trichomes (Fig. 6D).

Strain KS6 (Fig. 5C, 6E) was a brown non-heterocytous filamentous cyanobacterium, characterized by tightly coiled isopolar trichomes with a spiral/helix shape, as well. The trichome were unbranched and had a diameter of about 2.8-3.6 µm; the trichome apical cells were without a calyptra (Figs. 5C, 6E). In older cultures, abundant extracellular polysaccharides embedded the intertwined trichomes (data not shown).

DNA barcoding results

For strain KS1, a fragment of the 18S rRNA gene was obtained and it was 1132 bp long. The main results (with identities ≥ 97%), found using the BLAST tool to compare the 18S rRNA sequence of strain KS1 with those available in the INSDC archives, are reported in Tab. 1. Another interesting BLAST hit, not included in Table 1 since the percent identity between its 18S rRNA sequence and that of strain KS1 was only 96.43% (query cover of 58%), was a *Porphyridium* sp., isolated from Mahdia, Tunisia (Mediterranean Sea).

For strain KS2, a fragment of the 18S rRNA gene was obtained and it was 1163 bp long. The main results (with identities ≥ 97%), found using the BLAST tool to compare the 18S rRNA sequence of strain KS2 with those available in the INSDC archives, are reported in Tab. 2.

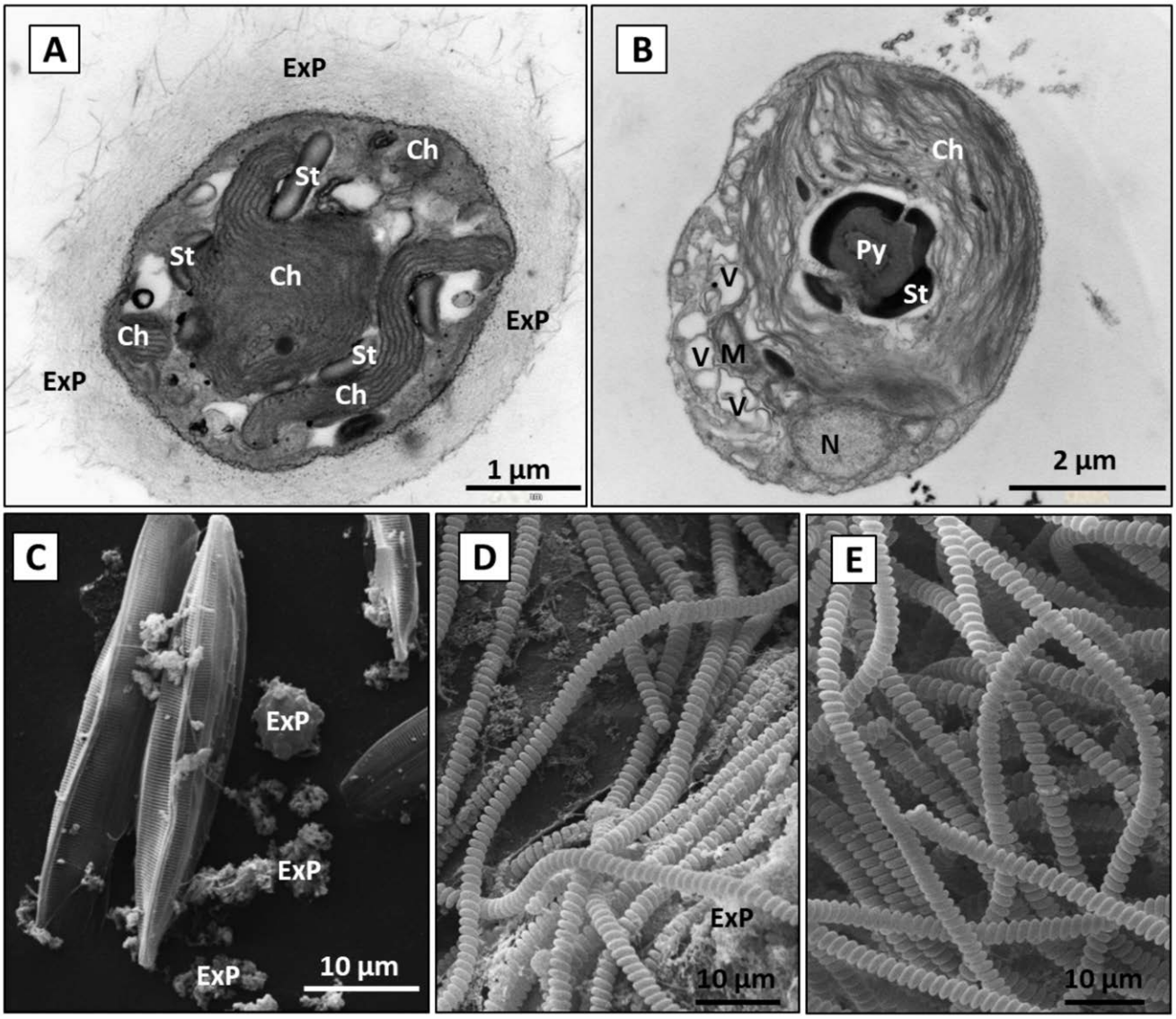

Fig. 6. The isolated microalgae seen with electron microscopy: A) strain KS1 at TEM; B) strain KS2 at TEM; C) strain KS3 at SEM; D) strain KS5 at SEM; E) strain KS6 at SEM. Ch = Chloroplast, St = Starch, Py = Pyrenoid, ExP= Extracellular Polysaccharides, N = Nucleus, M = Mitochondrion, V = Vacuole.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
Porphyridium sp. ex foraminifera (16 isolates)	99.20-99.87%	66%	Pacific Ocean, Japan, probably associated to foraminifera
Porphyridium sp. MBIC10451	99.29%	100%	not reported; probably Pacific Ocean, Japan
Porphyridium sp. RCC2962	99.06%	65%	Pacific Ocean, Japan, Iki Island
Porphyridium sordidum CCAP 1380/6 (2 deposited sequences)	97.01-98.36%	75-88%	USA, Kansas, greenhouse Lawrence, moist soil

Tab. 1. BLAST hits found for the 18S rRNA sequence of strain KS1. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 18S rRNA sequence obtained for KS1 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

For strain KS3, a fragment of the 18S rRNA gene was obtained and it was 1199 bp long. The main results, found using the BLAST tool to compare the 18S rRNA sequence of strain KS3 with those available in the INSDC archives, are reported in Tab. 3. Since, in this case, several INSDC 18S rRNA sequences had percent identities above 97% with that of strain KS3, only the BLAST hits with percent identities ≥ 99% are included in Tab. 3.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
Sykidion dyeri CCMP 257	99.91%	100%	Atlantic Ocean, USA, Connecticut, Milford, from tank
Pseudoneochloris marina UTEX 1445	99.48%	99%	not available
Sykidion droebakense CCMP 258	99.31%	100%	Pacific Ocean, Canada, British Columbia, Vancouver Island, from South Long Beach
Sykidion droebakense CCMP 438	99.31%	100%	Antarctic Ocean Antarctica, from Palmer Station
Pseudoneochloris sp. NKY372003	99.21%	98%	Pacific Ocean, Japan, Yakushima island

Tab. 2. BLAST hits found for the 18S rRNA sequence of strain KS2. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 18S rRNA sequence obtained for KS2 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
Nitzschia sp. (2 isolates)	99.56-99.73%	93%	Mediterranean Sea, Spain, Catalonia, Ebro Delta, Trabucador Beach
Nitzschia sp. AnM0026	99.66%	96%	Antarctic Ocean, Antarctica, King George Island, Marian Cove, near shore
Nitzschia traheaformis (3 isolates)	99.50-99.57%	96-99%	Pacific Ocean, Yellow Sea, China, two different sites: 2 from Jiaozhou Bay and 1 from Laizhou Bay
Nitzschia sp. SZCZCH658	99.57%	96%	Pacific Ocean, Yellow Sea, China, Yantai, holoturioidean aquaculture sediment
uncultured marine eukaryote clone I-7-MC660-OTU-28	99.50%	99%	Pacific Ocean, Bering Sea, ice algae sample collected from U.S. ice breaker Polar Sea cruise
uncultured marine eukaryote clone ME_Euk_FW16	99.48%	96%	Atlantic Ocean, USA, Maine, on the thallus of the seaweed *Ascophyllum nodosum* wrapped on a live bait
Nitzschia dubiiformis s0311	99.24%	99%	not available; probably Germany
Nitzschia sp. RCC 2276	99.10%	92%	Arctic Ocean, Beaufort Sea
Nitzschia dubia TA37	99.04%	95%	Pacific Ocean, Yellow Sea, Korea, Taean, Geunso Bay

Tab. 3. BLAST hits found for the 18S rRNA sequence of strain KS3. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 18S rRNA sequence obtained for KS3 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

For strain KS4, a fragment of the 16S rRNA gene was obtained and it was 1283 bp long. The main results (with identities ≥ 97%), found using the BLAST tool to compare the 16S rRNA sequence of strain KS4 with those available in the INSDC archives, are reported in Tab. 4.

For strain KS5, a fragment of the 16S rRNA gene was obtained and it was 633 bp long. The main results (with identities ≥ 97%), found using the BLAST tool to compare the 16S rRNA sequence of strain KS5 with those available in the INSDC archives, are reported in Tab. 5.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
unidentified marine cyanobacterium (2 isolates)	99.30-99.53%	100%	Pacific Ocean, Yellow Sea, China, Bohai Bay
unidentified marine cyanobacterium (4 isolates)	98.99-99.53%	100%	Pacific Ocean, Tasman Sea, New Zealand, Hokianga Harbour, from marine surface and marine sediment water
Phormidium sp. MBIC10210 (now = NBRC 102751)	99.29%	98%	Pacific Ocean; high sea
unidentified cyanobacterium BP2013-8	98.61%	89%	Argentina, Laguna Negra lake, from black pustular mats
unidentified cyanobacterium CAWBG124	97.82%	100%	New Zealand, Waihau Stream
unidentified prokaryote Reef_H09	97.19%	100%	Atlantic Ocean, Caribbean Sea, Panamá, Bocas del Toro, from reef seawater
unidentified prokaryote CG13	97.12%	91%	USA, Utah, Utha, Crystal geyser, from travertine rock
Leptolyngbya sp. PCC 7376	97.04%	100%	Atlantic area, Bermuda,Crystal Cave, from limestone
unidentified prokaryote 5M47	97.04%	100%	Pacific Ocean, South China Sea, China, Hong Kong Port Shelter, from marine biofilm

Tab. 4. BLAST hits found for the 16S rRNA sequence of strain KS4. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 16S rRNA sequence obtained for KS4 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
Spirulina sp. LEGE 11439	99.68%	100%	Atlantic Ocean, Portugal, near Leixoes Harbour, less than 1 km off the shore
Spirulina subsalsa SABC051501	99.68%	100%	Atlantic Ocean, Ireland, Cork, coastal water
Spirulina sp. MPI S2	98.14%	84%	Mediterranean Sea, Spain, Catalonia, Ebro Delta, La Trinitat salt marshes
unidentified marine prokaryote I09-E5-S7	98.10%	100%	Atlantic Ocean, USA, New Jersey, Tuckerton, boat basin of the Rutgers University Marine Field Station, from seawater-saturated sediment samples
Spirulina sp. MPI S4	98.10%	100%	Mediterranean Sea, Spain, Catalonia, Ebro Delta, Alfacs Bay
Spirulina sp. P7 (2 sequences)	97.31-97.60%	98-100%	Mediterranean Sea, France, Corsica, Harbour of Calvi
Spirulina subsalsa CCAP 1475/1	97.48%	100%	North Sea, United Kingdom, England, Norfolk, Hunstanton
uncultured marine bacterium (3 isolates)	97.16-97.31%	100%	North Sea, Netherlands, Schiermonnikoog, from marine microbial mats in a sandy intertidal beach
Spirulina subsalsa 06S082	97.16%	100%	Baltic Sea, south-west coast of Finland, from surface water
Spirulina sp. TIOX113	97.12%	87%	South China Sea, China, Weizhou Island, from seawater near coral reefs

Tab. 5. BLAST hits found for the 16S rRNA sequence of strain KS5. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 16S rRNA sequence obtained for KS5 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

For strain KS6, a fragment of the 16S rRNA gene was obtained and it was 1315 bp long. The main results, found using the BLAST tool to compare the 16S rRNA sequence of strain KS6 with those available in the INSDC archives, are reported in Tab. 6. Since, in this case, only few INSDC 16S rRNA sequences had percent identities above 97% with that of strain KS6, also the BLAST hits with percent identities ≥ 96.5% were included in Tab. 6.

For strain KS7, a fragment of the 18S rRNA gene was obtained and it was 1128 bp long. The main results, found using the BLAST tool to compare the 18S rRNA sequence of strain KS7 with those available in the INSDC archives, are reported in Table 7. In this case, the 100 found BLAST hits had all identities above 97% with that of strain KS7; moreover, 26 of the found BLAST hits showed identities above 99%. For this reason and since almost all the found BLAST hits indicated the same taxa, only the first 10 different results are included in Tab. 7.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
uncultured marine bacterium (3 isolates)	97.63-98.09%	99%	North Sea, Netherlands, Schiermonnikoog, from marine microbial mats in a sandy intertidal beach
Spirulina sp. LEGE 11439	96.73%	100%	Atlantic Ocean, Portugal, near Leixoes Harbour, less than 1 km off the shore
Spirulina subsalsa SABC051501	96.53%%	96%	Atlantic Ocean, Ireland, Cork, coastal water

Tab. 6. BLAST hits found for the 16S rRNA sequence of strain KS6. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 16S rRNA sequence obtained for KS6 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

BLAST hit description	Percent identities	Query cover	Geographical and habitat information
Nannochloropsis gaditana BEA1917B	99.91%	99%	Spain, seawater sample (probably in the Mediterranean Sea)
Nannochloropsis gaditana (RCC culture collection; 2 isolates)	99.91%	96%	Mediterranean Sea, Spain, Blanès, along the coast
Nannochloropsis gaditana MEJ25102017-5G6_M42	99.82%	100%	Mediterranean Sea, France, Mejean Lagoon
Nannochloropsis gaditana XZ1.5	99.82%	100%	not available; probably China
Nannochloropsis gaditana strain CCMP526	99.82%	100%	Atlantic Ocean, Morocco, Lagoon of Oualidia
Nannochloropsis salina D12	99.82%	100%	Pacific Ocean, Yellow Sea, China, Shandong
Nannochloropsis gaditana IVP	99.82%	100%	Mediterranean Sea, Italy, Apulia, Foggia Lesina
Nannochloropsis gaditana	99.82%	100%	Mediterranean Sea, Italy, Ferrara, Comacchio lagoons
Nannochloropsis gaditana MBIC10118	99.82%	100%	Indian Ocean, Australia, Shell Beach
Nannochloropsis gaditana #B-3	99.82%	100%	Mediterranean Sea, Spain, Cadiz Bay

Tab. 7. BLAST hits found for the 18S rRNA sequence of strain KS7. The BLAST hit description (if more than one isolate/sequence corresponded to the same taxonomic entity, the number of isolates/sequences was reported; if the hit was represented by just one taxonomic entity/sequence, the strain identifier was reported), the percent identities between the 18S rRNA sequence obtained for KS7 strain and those found in the INSDC archives, the query cover (i.e., the percent overlap between the sequence of interest, that is the query, and each of those found by BLAST) and habitat/geographical information on the microorganisms from which the INSDC sequences were obtained (if available/recoverable) are reported.

DISCUSSION AND CONCLUSION

Photoxygenic microorganisms (i.e., cyanobacteria and eukaryotic microalgae) are a vast group that, besides their importance for natural ecosystems, can have several applications in different human fields (e.g., LAURITANO *et al.*, 2016; RENUKA *et al.*, 2018; GAIGNARD *et al.*, 2019; LEVASSEUR *et al.*, 2020; ALVAREZ *et al.*, 2021; FERNANDES & CORDEIRO, 2021; KIRAN & VENKATA MOHAN, 2021). More and more studies point out how the possible exploitation of cyanobacteria and microalgae varies according to the different taxa to which they belong and how some properties are strictly specific not just of genera and species, but even of different strains inside a given species (e.g., LAURITANO *et al.*, 2016; MOLINO *et al.*, 2018; LEVASSEUR *et al.*, 2020; FIGUEROA-TORRES *et al.* 2021; KRIVINA *et al.*, 2023). Moreover, the correct taxonomic assignment of cyanobacterial and microalgal strains and the related studies on their ecology can help to address the cultivation methods in artificial conditions (e.g., research laboratories or industries). Therefore, systematic and ecological studies on photoxygenic microorganisms are at the base of different more practical purposes.

Nevertheless, the identification of cyanobacteria and microalgae is not always an easy task because of the characters typical of several taxa (i.e., simple morphologies, high phenotypic plasticity, presence of cryptic or semi-cryptic species) (e.g., DARIENKO *et al.*, 2015; MALAVASI *et al.*, 2016; SCIUTO *et al.*, 2015; 2017; 2019; 2021; 2023; ZAMMIT, 2018; KRIVINA *et al.*, 2023). For this reason, besides more classical systematic studies based on microscope observations, molecular techniques are generally used for their identification. One of the most used molecular methods, to identify not just photoxygenic microorganisms but every living being, is the DNA barcoding method. The DNA barcoding method was first ideated by Hebert and collaborators (HEBERT *et al.*, 2003) as a simple, fast and reliable method to identify animals and, since then, it has been adapted for almost all the biological groups. However, for some organisms, like the plant ones, it still needs to be perfected and it is continuously improved for the different plant taxa (e.g., CBOL PLANT WORKING GROUP, 2009; HALL *et al.*, 2010; DARIENKO *et al.*, 2015). Among the main principles of the DNA barcoding method, there is the use of relatively short DNA sequences (of about 600-800 bp) as a sort of biological barcodes (HEBERT *et al.*, 2003). However, in this research work, whenever possible, we decided to amplify a longer DNA fragment, which can be potentially used also for future accurate phylogenetic analyses.

This preliminary investigation on cyanobacterial and microalgal strains isolated from the Italian North Adriatic area highlights how the biodiversity of these microorganisms is very high. Indeed, the only seven strains, so far isolated from just four sampling sites, span five different phyla: the phylum Rhodophyta (= strain KS1), the phylum Chlorophyta (= strain KS2), the phylum Bacillariophyta (= strain KS3), the phylum Cyanobacteria (= strains KS4, KS5 and KS6) and the phylum Ochrophyta (= strain KS7). Moreover, inside these phyla, different taxa were represented by the isolated strains.

Based on the molecular analyses, strain KS1 unequivocally belongs to the genus *Porphyridium* Nägeli (NÄGELI, 1849) (phylum Rhodophyta, class Porphyridiophyceae, order Porphyridiales), for which only four species are currently accepted: *Porphyridium aerugineum* GEITLER, *Porphyridium sordidum* Geitler, *Porphyridium wittrockii* Richter and *Porphyridium purpureum* (Bory) K.M. Drew & R. Ross (GUIRY & GUIRY, 2023). The first three ones are freshwater and/or moist soil species, while the last one has been found in freshwater, brackish and marine habitats (GUIRY & GUIRY, 2023). Indeed, the morphological features observed for strain KS1, like the cell sizes and the multi-lobed chloroplast occupying almost all the cell cytoplasm, fit well with the description of this genus (NÄGELI, 1849; GUIRY & GUIRY, 2023).

The percent identities between the found BLAST hits and the 18S rRNA sequence of strain KS1 (Tab. 1) suggest that this microalga probably represents a new marine species of *Porphyridium*. To our knowledge, currently there are not proposed identity thresholds for defining species boundaries based on the 18S rRNA gene, as instead for the another widely considered rRNA gene that is the 16S rRNA (generally used for prokaryotes). The percent identity thresholds, for distinguishing genera and species in prokaryotes based on the 16S rRNA, have been traditionally considered to be 95% and 97.5%, respectively (STACKEBRANDT & GOEBEL, 1994). However, currently, several authors consider an identity ≥ 98.5% to be more reliable for indicating the belonging of two prokaryotic strains to the same species (e.g., COLE *et al.* 2010; STACKEBRANDT & EBERS, 2006). Making a parallelism with the above reported 16S rRNA thresholds, if we use the most stringent one, strain KS1 belongs to the same species of the first three BLAST hits, which are all sequences obtained from marine *Porphyridium* sp. If instead we consider the 97.5% threshold, strain KS1 could belong to the species *P. sordidum*. Indeed, only accurate phylogenetic analyses can help to solve this question; however, based on the habitat of *P. sordidum* and of the specific *P. sordidum* BLAST hit (i.e., most soil), we think that it is highly improbable the belonging of strain KS1 to the species *P. sordidum.* In our opinion, strain KS1, collected from the Lido of Spina coast (Comacchio area, Emilia-Romagna region; site 2 in Fig. 1), represents a new species inside the genus *Porphyridium,* but further investigations are required to confirm this hypothesis.

Looking at the geographical information of the BLAST hits found for strain KS1, they are all from extra-Mediterranean areas, in particular the Pacific Ocean near Japan (Tab. 1). However, interestingly, another BLAST hit, not included in Tab. 1 for the percent identity lower than 97%, is a *Porphyridium* sp. recently isolated from Mahdia, Tunisia (Mediterranean Sea). Probably, the lower percent identity (i.e. 96.43%) is due to the low query cover (i.e., 58%) and the two strains are conspecific. Further studies are needed to more precisely identify strain KS1 and to understand if it is a new introduction in the Mediterranean area or if it is a cosmopolite, but so far unnoticed, species; currently, these two hypotheses are equally probable, as it was argued for other microalgal strains isolated from the Mediterranean sea (e.g., SCIUTO *et al.*, 2021).

Based on the molecular analyses, strain KS2 clearly belongs to the genus *Sykidion* E.P. Wright (Wright, 1881) (phylum Chlorophyta, class Ulvophyceae, order Sykidiales), for which five species are currently recognized: *Sykidion dyeri* E.P. Wright, *Sykidion droebakense* Wille, *Sykidion gomphonematis* K.I. Meyer, *Sykidion marinum* (S. Watanabe *et al.*) Darienko *et al.* and *Sykidion praecipitans* (Tschermak-Woess) Komárek (Guiry & Guiry, 2023). As for *Porphyridium*, also in the genus *Sykidion* both freshwater and marine species are reported (Guiry & Guiry, 2023). The morphological traits found for strain KS2, in particular the presence of different cell forms and of motile zoospores, are in agreement with the description of the genus *Sykidion* (Wright, 1881; Darienko *et al.*, 2021; Guiry & Guiry, 2023). Strain KS2 was collected from one of the internal basins in the Lido of Spina (Comacchio area, Emilia-Romagna region; site 4 in Fig. 1).

The percent identities between the found BLAST hits and the 18S rRNA sequence of strain KS2 (Tab. 2) suggest that it can equally belong to the species *S. dyeri*, type species of the genus (Wright, 1881), to the species *S. droebakense*, or to the species *S. marinum*, based on *Pseudoneochloris marina* S. Watanabe *et al.* recently transferred under the genus *Sykidion* (Darienko *et al.*, 2021). Only phylogenetic analyses will help to identify strain KS2 also at the specific level.

Looking at the geographical origin of the BLAST hits found for strain KS2, they are all from extra-Mediterranean areas, including the Atlantic, the Pacific and the Antarctic Oceans (Tab. 2). Considering all the currently recognized species of *Sykidion*, only two ones have been reported from the Mediterranean Sea up to now: *S. droebakense* and *S. gomphonematis* (Cambra-Sánchez *et al.*, 1998), based on morphological observations. Therefore, if strain KS2 will be verified to coincide with the species *S. droebakense,* this will be the first sequenced record of this species from the Mediterranean area; if strain KS2 will be demonstrated to belong to *S. dyeri* or *S. marinum*, this will be the first record of one of these species in the Mediterranean area.

Based on both the microscope observations and the DNA barcoding results, strain KS3 unequivocally belongs to the genus *Nitzschia* Hassall (Hassall, 1845), one of the species-richest diatom genera with 883 accepted species names (Guiry & Guiry, 2023). The percent identities of the found BLAST hits do not allow to identify strain KS3 at the species level, even if, among the results with the highest identities, three isolates represented the species *Nitzschia traheaformis* Ch. Li, Witkowski & Yu, first described from the Yellow Sea along the Chinese coasts (Witkowski *et al.*, 2016). About the geographic origin of the found BLAST hits, they were all from extra-Mediterranean areas, except for two *Nitzschia* sp. isolates that had the highest percent identities with strain KS3 (99.56-99.73%) and that were sampled from Trabucador Beach, in the Ebro Delta, Catalonia, Spain (Mediterranean Sea). Strain KS3 was obtained from a water sample collected along the Lido of Spina coast (Comacchio area, Emilia-Romagna region; site 2 in Fig. 1), near the mouth of Logonovo Canal. Further investigations, based on phylogenetic analyses and more SEM observations, are required to identify strain KS3 also at the species rank.

Of the seven isolated strains, strain KS4 was the one that had more unidentified microorganisms among its BLAST hits (Tab. 4). Most of the unidentified microorganisms were tagged as "cyanobacteria" or simply "prokaryotes" from both the Pacific and Atlantic areas, including several environments: seawater and freshwater habitats (i.e., a lake and a stream), but also rocks in a cave and in a geyser (Tab. 4). Strain KS4 was one of the microorganisms obtained from the microbial mats growing in the aquarium tank (Fig. 2), used to grow the seagrass *Cymodocea nodosa* and filled in with water taken from Santa Maria del Mare (Venice Lagoon, Veneto region). Just two BLAST hits found for stain KS4 were identified at least at the genus level: *Phormidium* sp. MBIC10210 (now = NBRC 102751), from the Pacific Ocean, and *Leptolyngbya* sp. PCC 7376, from a limestone in the Crystal Cave, Bermuda (Atlantic area). Even if *Phormidium* sp. MBIC10210 has a higher percent identity (99.29%) with strain KS4 than *Leptolyngbya* sp. PCC 7376 (97.04%), the microscope observations clearly indicate that strain KS4 belongs to the genus *Leptolyngbya* Anagnostidis & Komárek and surely not to the genus *Phormidium* Kützing ex Gomont (Komárek & Anagnostidis, 2005). In fact, the long, unbranched and very thin trichomes, less than 3.5 μm in diameter (and, more exactly, less than 1 μm), observed for strain KS4, are features typical of the genus *Leptolyngbya sensu lato* (Komárek & Anagnostidis, 2005; Komárek, 2007). Probably, *Phormidium* sp. MBIC10210 belongs to the genus *Leptolyngbya sensu lato* too and its classification under the genus *Phormidium* was due to a misidentification. The presence of sequences produced by erroneously classified microorganisms in the INSDC archives is a longstanding problem; however, without pictures or information available for *Phormidium* sp. MBIC10210, we cannot surely state that this is the case.

Members of *Leptolyngbya sensu lato* are among the most difficult cyanobacteria to be identified for their very simple morphologies and for several features overlapping with those of other cyanobacterial genera; for these reasons, mainly based on molecular data, this genus has been subjected to several systematic revisions, with the separation of new genera (e.g., Johansen *et al.*, 2011; Zammit *et al.*, 2012; Vaz *et al.*, 2015; Sciuto & Moro, 2016; Sciuto *et al.*, 2017; Zammit, 2018). Phylogenetic analyses, with the use of identified cyanobacterial strains for comparison, and electron microscopy (SEM and TEM) observations are required to gain more data on strain KS4 and, hopefully, to identify it more precisely.

Besides few unidentified prokaryotes as BLAST hits, the BLAST results obtained for strains KS5 and KS6 (Tab. 5 and Tab. 6, respectively) indicate that both these cyanobacteria belong to the genus *Spirulina* Turpin ex Gomont (Gomont, 1892-1893), as unequivocally suggested also by their morphology. More precisely, based on the molecular results, both the strains seem members of the species *Spirulina subsalsa* Oersted ex Gomont. This is confirmed by the strain morphology as well as by their sampling site: indeed, these two cyanobacteria were obtained from the microbial mats of the aquarium tank (Fig. 2) and *S. subsalsa* is one the cyanobacterial species living also in brackish waters and frequently found in aquaria (Komárek & Anagnos-

TIDIS, 2005). Interestingly, even if strains KS5 and KS6 were isolated from the same source and have been, then, kept in the same laboratory culture conditions, they have maintained their different colours, suggesting that this is a fixed feature, not influenced by environmental parameters. Indeed, strains KS5 and KS6 show a certain difference also in the overlapping part of their 16S rRNA gene sequence, sharing a percent identity of just 95.35% (4.65% of divergence, due to 22 nucleotide substitutions and 3 indels on 538 aligned positions), which suggests that they are not genetically identical. Perhaps, genetic differences in other part of their genomes can justify the different colour of these two strains. However, only future accurate phylogenetic investigations, ultrastructural observations, pigment analysis and the study of physiological aspects, like salinity and temperature requirements, can give useful information on the two strains and hopefully can help to solve their systematic position, as it was done for other *Spirulina*-like strains (e.g., NUBEL ET AL, 2000; MORO *et al.*, 2021).

Based on the DNA barcoding method (Tab. 7), strain KS7 belongs to the genus *Nannochloropsis* D.J. Hibberd (HIBBERD, 1981), whose correct taxonomic name is currently *Microchloropsis*, a genus erected by Fawley and collaborators mainly based on molecular analyses (FAWLEY *et al.*, 2015). The simple morphological traits of strain KS7, which was characterized by very small, slightly elongated, yellow-green cells, fit well with the description of this genus (HIBBERD, 1981; ELIÁS *et al.*, 2017). Most of the found BLAST hits indicate that strain KS7 corresponds to the species *Nannochloropsis gaditana* L.M. Lubián and just one to the species *Nannochloropsis salina* D.J. Hibberd, now respectively *Microchloropsis gaditana* (L.M. Lubián) M.W. Fawley, I. Jameson & K.P. Fawley and *Microchloropsis salina* (D.J. Hibbard) M.W. Fawley, I. Jameson & K.P. Fawley. Based on the majority of the BLAST results, we hypothesize that strain KS7 is a member of *M. gaditana*; however only phylogenetic analyses, ultrastructural observations and pigment analysis (it seems that *M. gaditana* is able to produce α-carotene, while *M. salina* not) (LUBIÁN, 1982; FAWLEY *et al.*, 2015) can definitely tell to which of these two species the microalga belongs.

Looking at the geographic origin of the BLAST hits found for strain KS7 (Tab. 7), they were from several world areas, including the Atlantic, the Pacific and the Indian Oceans, and the Mediterranean Sea. In particular, 6 of the 10 BLAST hits reported in Tab. 7 were from different Mediterranean sites, including a specimen from Comacchio (Emilia-Romagna region, Italy). Interestingly, this specimen was collected and characterized by Andreoli and collaborators in 1999 from a bloom in the Comacchio lagoons (ANDREOLI *et al.*, 1999); thus it is probable that *M. gaditana* is present in the Comacchio area at least for 24 years.

In conclusion, the present research work, even if carried out on a limited number of Italian North Adriatic sites and on a limited number of strains, has shown the high photoxygenic microorganism biodiversity of this environment, with the isolates encompassing five phyla (Bacillariophyta, Chlorophyta, Ochrophyta, Rhodophyta and Cyanobacteria). Moreover, with the exception of strain KS4 (which very probably belongs to the genus *Leptolyngbya sensu lato*, but needs much more investigation), all the other isolated strains were identified at least at the genus level and three also at species level (i.e., the two *S. subsalsa* strains and *M. gaditana*). However, as said, the performed characterizations were only the first step for future deepened investigations based on an integrative taxonomy approach (including phylogenetic, ultrastructural, biochemical and physiological analyses). These future characterizations will help to understand also the biotechnological potentials of the isolated microalgae, including the less studied taxa but also the genera and species for which there are already plenty of studies and information in this regard, like the genus *Porphyridium* (e.g., BAYONA *et al.*, 2012; LI *et al.*, 2019) and the widely used species *M. gaditana* (e.g., CAMACHO-RODRÍGUEZ *et al.*, 2014; MITRA *et al.*, 2015; ZANELLA & VIANELLO, 2020).

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