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Biomass productivity enhancement and extraction of high values products of an extremophile microalga from SCCA culture collection

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Thesis
submitted in fulfillment of the requirements for the degree of doctor
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LIST OF ABBREVIATIONS AND DEFINITION

3PG 3-phosphoglycerate

AA Arachidonic acid

ACCs Acetyl-CoA carboxylase

ACPs Acyl carrier proteins

ADP Adenosine diphosphate;

ALA Alpha-linolenic acid

AMD Acid mine drainage

ARA Arachidonic acid

ATP Adenosine triphosphate

BBM Bold Basal Medium

Cb Biomass concentration

CCS Carbon capture coupled with geological storage

cpDNA Chloroplast DNA

DHA Docosahexaenoic acid

EPA Eicosapentaenoic acid

ER Endoplasmic reticulum

FAME Fatty Acid Methyl Ester

G3P Glycerate-3-phosphate

GAP Glyceraldehyde 3-phosphate

GC-FID Gas chromatography-flame ionization detector

GHG Green House Gas

GLA Gamma-linolenic acid

LD Light/Dark

mtDNA Mitochondrial DNA

NADPH Nicotinamide adenine dinucleotide phosphate

NMD **neutral mine drainage**

OD **Optical density**

P **Phosphate**

PAR **Photosynthetic Active Radiation**

PBR **Photobioreactor**

PDC **Pyruvate dehydrogenase complex**

PS I **Photosystem I**

PS II **Photosystem II**

PSBR **Porous Substrate Bioreactor**

PUFAs **Polyunsaturated Fatty Acids**

ROS **Reactive oxygen species**

rRNA **Ribosomal ribonucleic acid**

SCCA **Sardinian Culture Collection of Algae**

SFR **Surface to Footprint Ratio**

SPV **Sulpho-Phospho-Vanillin**

STA **Stearidonic acid**

SVR **Surface area to Volume Ratio**

TAG **Triacylglycerols**

Summary

Coccomyxa melkonianii SCCA 048, from Sardinian Culture Collection of Algae (SCCA), is a green extremophile microalga found in a mine drainage habitat and hence characterized by the ability to live in highly selective environment with extreme conditions such as high concentrations of heavy metals and tolerate a range of different pH values. These characteristics confer the microalga a competitive advantage over other organisms that cannot survive at similar conditions. This strain is able to grow well in a wide pH range and to achieve a quite interesting lipid content, showing the presence of suitable amounts of fatty acids which can be profitably exploited in the food, nutraceutical, and cosmetic industries.

These features, in addition to its potential as lutein producer and the possibility of cultivating it under extreme pH conditions in economic open ponds, makes *Coccomyxa melkonianii* SCCA 048 a very promising strain for high-value lipids accumulation if cultivated under a suitable cultivation strategy.

Aims

The aim of this **Thesis** is the characterization of the growth of a microalgal strain from the Sardinian Culture Collection of Algae (SCCA) for the production of high value bio-products such as lipids through the investigation of its growth and lipids production kinetics; the optimization of its biomass production and the evaluation of the content and extraction of valuable lipids.
These are the first **Thesis** attempts to investigate the effects of nitrogen concentration and pH on the growth and lipids accumulation, as well as the morphological responses of the extremophile microalga *Coccomyxa melkonianii* SCCA 048 under those cultivation conditions. Therefore, the obtained results might provide useful information about a strain which represents a lineage associated to a mine drainage habitat, since no data are available in the literature in this regard. Furthermore, the outcomes might also represent a helpful tool for the identification of the best strategies for the mass cultivation and highly valuable compounds production using this extremophile strain in a potential large-scale plant.

To achieve this objective the following partial steps were accomplished:

- Characterization, for the first time, of the microalga nitrate and pH-dependent growth kinetics and evaluation of the corresponding kinetic parameters. Identification of the best-operating conditions for maximizing biomass productivity, boosting lipid accumulation and improving fatty acid quality. To do that the following activities were performed:
    - Preliminary assessment of the optimal nitrogen concentration for the maximization of the alga growth rate.
    - Preliminary assessment of the capability of the alga to grow under different pH values, and identification of the optimum pH range.
    - Evaluation of the effects of different initial nitrogen concentrations in the medium on the growth rate, nitrate uptake, as well as the synthesis of lipids and their corresponding quality on prolonged growth experiments.
    - Identification of the better culture medium composition in terms of nitrate concentration and pH for the growth and lipid synthesis of *Coccomyxa melkonianii* SCCA 048.
- Evaluation of the possibility of cultivating *Coccomyxa melkonianii* SCCA 048 under extreme pH conditions and assessment of its capability to produce valuable lipids for several biotechnological applications. To do that the following activities were performed:

- Assessment of the growth rate of *Coccomyxa melkonianii* SCCA 048 cells exposed to different pH values.
- Assessment of the morphological plasticity responses of the strain cells exposed to different pH values through light microscope observations.
- Evaluation of the optimum pH for prolonged growth and lipids production by cultivating the alga in growth media where the pH was kept fixed.
- Evaluation, for the first time, of the effect of different pH values on the alga growth rate, biomass and lipid productivity as well as on the lipids composition on prolonged growth experiments.

Thesis outline

The thesis is organized in three parts: **Chapter I** provides an introductive review on the current state of art and challenges of microalgae biotechnology, the cultivation strategies, the production of high-value products and its integration in the biorefinery. **Chapter II** puts into context the findings of the Ph.D. in an introductive overview which encompasses the general description of the extreme environment, particularly the mine drainage habitat, from where the strain investigated was recovered, the economic potential of extremophile organisms as well as the potential high-value products that can be extracted. Then, moves on to the taxonomical description of the stain selected and the methodologies employed for the extraction of lipids in this research. The second part consists of the experimental activities reported in the chapters listed below. These will be referred to in the text by the number written with the Roman numerals III and IV.

This thesis is focused on the study of the effect of different cultivation conditions on the growth of the extremophile microalga *Coccomyxa melkonianii* SCCA 048, testing for the first time its performance in producing marketable products, such as lipids.

Chapter III intends to identify the best strategy for cultivating this alga in large-scale systems and to boost, at the same time, the lipid synthesis. To this purpose, the nitrate and pH-dependent growth kinetics were first evaluated in multiwell devices along with the corresponding kinetic parameters. Subsequently, a cultivation strategy based on the induction of nitrogen starvation phenomena has been applied in prolonged growth experiments. Nitrogen manipulation leads to the unbalancing of carbon and nitrogen content within the cell, which activates specific metabolic processes aimed to store the excess carbon into high energy molecules such as lipids. The effects of three different initial nitrogen concentrations on the growth rate, nitrate uptake, as well as the synthesis of lipids and their corresponding quality, were investigated in two-liter batch photobioreactors operated in batch mode. The assessment of the pH-dependent growth, allowed to demonstrate for the first time that *Coccomyxa melkonianii* SCCA 048 is extremophile as far as pH conditions are concerned. By considering the experimental results, the best nitrates concentration was identified to the aim of maximizing lipid productivity in batch photobioreactors. The obtained growth rates and lipid productivities were high enough to permit the sustainable cultivation at the large scale The composition of the extracted FAMEs was very promising in view of their exploitation in the food and/or lubricant industries. Finally, the experimental data represent a first step towards the

development of a useful tool for the setup and optimization of open raceways systems where *Coccomyxa melkonianii* SCCA 048 might be cultivated.

In **Chapter IV**, for the first time in literature, the effect of pH on the growth and lipids productivity of the extremophile microalga *Coccomyxa melkonianii* SCCA 048 was analyzed. Additionally, the morphological effects of pH, as well as its influence on fatty acids composition, have been investigated. *Coccomyxa melkonianii* SCCA 048 showed the capability to actively grow and operate the lipid biosynthesis at different pH values. The optimal pH value for the growth and the maximum lipid content was identified. During the investigation, a significant phenotypic plasticity of this strain was observed. Such a strong adaptation response may enable this extremophile microalga to reach biomass and lipids productivities similar to those of control cultures. This ability might become a selective competitive advantage for this strain in continuous production processes, compared to non-extremophile microalgae. Finally, the analysis of fatty acid methyl esters (FAMEs) showed that the composition varies depending on the pH value and highlighted the presence of suitable amounts of compounds which can be profitably exploited in the food, nutraceutical, and cosmetic industry. These aspects, suggest that *Coccomyxa melkonianii* SCCA 048 is an interesting candidate that might be viably exploited for performing cultivation in economic open raceways for several biotechnological applications.

CHAPTER I

Theory and literature review on microalgae biotechnology

1 MICROALGAE

Microalgae are a group of photosynthetic organisms characterized by very simple structural organization, which use light energy, carbon dioxide (CO_2) and ions dissolved in the water for the synthesis of complex molecules and for the production of biomass. The term microalgae, in applied phycology, include the microscopic algae "*sensu stricto"* and the photosynthetic bacteria, formerly known as Cyanobacteria (Tomaselli 2004; Bordignon and Cabrini 2015). Microalgae are an extremely diverse group of primary producers present in almost all ecosystems on Earth, ranging from marine, freshwater, desert sands, and hot springs, to snow and ice (Guschina and Harwood 2006; Rajkuma and Yaakob 2013). Only a certain number of species live in symbiotic association with different organisms while microalgae are usually free-living as single cells or in colonies (Tomaselli 2004; Bordignon and Cabrini 2015). The green lineage (Viridiplantae) comprises the green algae and their descendants the land plants and is one of the major groups of oxygenic photosynthetic eukaryotes (Leliaert *et al.* 2012). Viridiplantae (ve-re-de-PLAN-te) is derived from two Latin roots that mean "green" (virida) and "shoot" (planta) (Fig. 1).

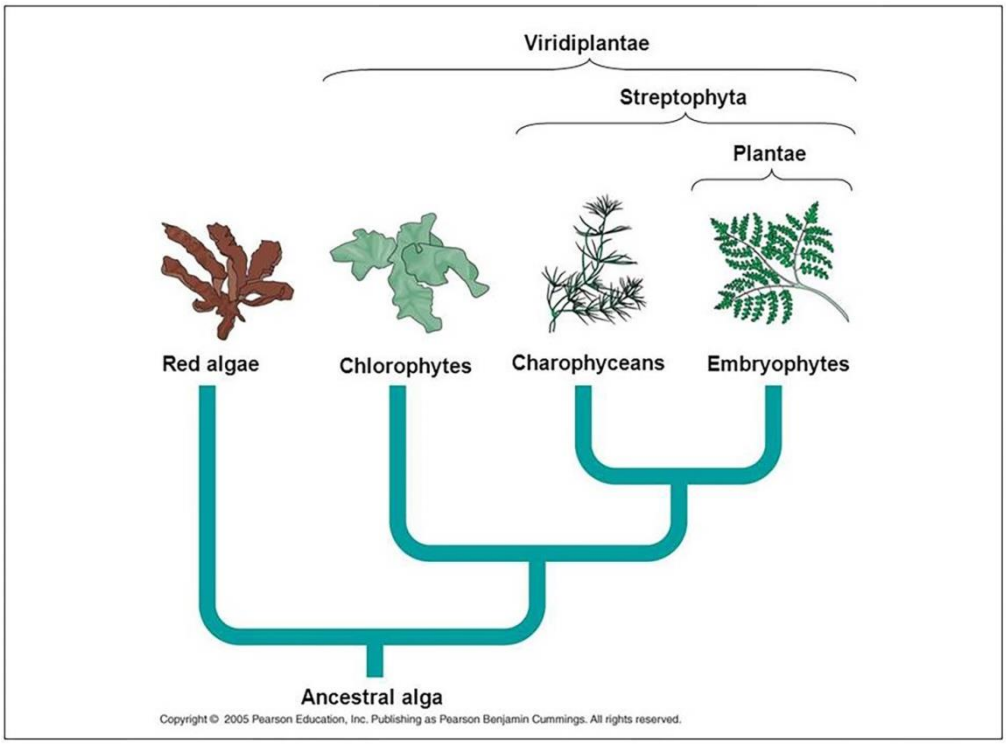

Fig. 1 Phylogenetic tree of the algal lineages. Source Campbell *et al.* (2005).

The green algae are photosynthetic eukaryotes characterized by the presence of chloroplasts with two envelope membranes, stacked thylakoids and chlorophyll a and b (Pröschold and Leliaert 2007). All green algae produce starch as the main reserve polysaccharide, which is deposited inside

the plastids. They and can be found in almost every habitat from Arctic and Antarctic regions to oceans and freshwater lakes as well as in soil from temperate and arid areas. Green algae are also found in different symbioses including lichens, protozoa, and foraminifers, or as parasites on tropical plants (Pröschold and Leliaert 2007). Several green algae have adapted to highly specialized or extreme environments, such as hot or cold deserts (Lewis and Lewis 2005; De Wever *et al.* 2009; Schmidt *et al.* 2011), hypersaline habitats (Vinogradova and Darienko 2008), acidic waters with extreme concentrations of heavy metals (Amaral Zettler et al. 2002), marine deep waters (Zechman *et al.* 2010) and deep-sea hydrothermal vents (Edgcomb *et al.* 2002; Leliaert *et al.* 2012). There are estimated to be at least 600 genera with 10,000 species within the green algae (Norton *et al.* 1996; Pröschold and Leliaert 2007).

This very diversity makes microalgae, as a group, a potentially rich source of a wide array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries (Olaizola 2003). The advantages of microalgae are that they do not require arable land (Chaudry *et al.* 2015), and offer higher biomass yields than terrestrial crops per unit area (Correa *et al.* 2017). Furthermore, their cultivation can be coupled with wastewater systems and industrial CO_2 sources, facilitating water remediation and decreasing CO_2 emissions (Correa *et al.* 2017). In this chapter, a brief analysis of the main biological, chemical and physical phenomena involved in the photosynthetic conversion of CO_2 into valuable bioproducts, the current technologies for CO_2 capture, microalgae growth, will be discussed with a particular focus on the potential exploitation of recent research results at the industrial scale.

1.1 Ultrastructure

• Prokaryotes: The DNA of prokaryotic Cyanobacteria and Prochlorophytes is not organized in chromosomes, lies free in the cytoplasm together with the photosynthetic membranes, and is not surrounded by a membrane. Moreover, the prokaryotes have no membrane-bounded organelles. Cyanobacteria and Prochlorophytes have a four-layered cell wall which is of the Gram-negative type. Mucilaginous envelopes may surround the cell wall (sheaths, glycocalyx, capsule or slime). Thylakoids are the most evident membrane system which lie free in the cytoplasm and contain the photosynthetic apparatus. The most common cell inclusions of cyanobacteria are the glycogen granules, cyanophycin granules, carboxysomes, polyphosphate granules, lipid droplets, gas vacuoles, and ribosomes. In some planktonic forms there are gas vacuoles. Cell division may occur

through binary fission and multiple fission. Some filamentous genera produce akinetes. Heterocysts are cells where nitrogen fixation takes place (Tomaselli 2004).

• Eukaryotes - The eukaryotic microalgae possess a true membrane-bounded nucleus, which contains the major part of the genome distributed on a set of chromosomes, and the nucleolus. They have cytoplasm divided into compartments and membrane-bounded organelles (Golgi body, mitochondria, endoplasmic reticulum, vacuoles, lipid globules, centrioles, and plastids) devoted to specific functions. The chloroplast contains a series of flattened vesicles, or thylakoids, containing the chlorophylls, and a surrounding matrix, or stroma. Pyrenoids can occur within chloroplasts. In many motile forms, there is an orange-red eyespot, or stigma, made of lipid globules. A microfibrillar layer of cellulose, which may be surrounded by an amorphous layer, generally composes the microalgal cell wall. It may be silicified or calcified. Vegetative reproduction by cell division is widespread in the algae. Other types of asexual reproduction occur by fragmentation and by production of spores. Although sexual reproduction occurs in the life-history of most of the species, it is not a universal feature in algae (Tomaselli 2004).

1.2 Microalgal systematics

Over the past 30 years, molecular phylogenetic studies have led to extensive modification of traditional classification schemes for algae; nowadays no easily definable classification system acceptable to all exists for this group of organisms, since taxonomy is under constant and rapid revision at all levels following everyday new genetic and ultrastructural evidence (Barsanti and Gualtieri 2014). The polyphyletic nature of the algal group is somewhat inconsistent with traditional taxonomic groupings, though they are still useful to define the general characteristics and levels of organizations (Barsanti and Gualtieri 2014). Historically, the major groups of algae were classified on the basis of pigmentation, chemical nature of photosynthetic storage product, photosynthetic membrane (thylakoids) organization and other features of the chloroplasts, chemistry and structure of the cell wall, number, arrangement, and ultrastructure of flagella (if any), occurrence of any other special features, and sexual cycles (Barsanti and Gualtieri 2014). The systematic position of the various algal group has changed many times over the years (Tomaselli 2004). Recently revised classifications incorporate advances resulting from the widespread use of phylogenomic-scale, phylogenetic analyses and massively increased taxon sampling in rRNA phylogenies (Fig. 2). All these studies tend to assess the internal genetic coherence of the major phyla such as Cyanobacteria, Glaucophyta, Rhodophyta, Chlorophyta, Charophyta, Haptophyta, Cryptophyta, Ochrophyta,

Cercozoa, Myzozoa, and Euglenozoa, confirming that these divisions are nonartificial (Barsanti and Gualtieri 2014).

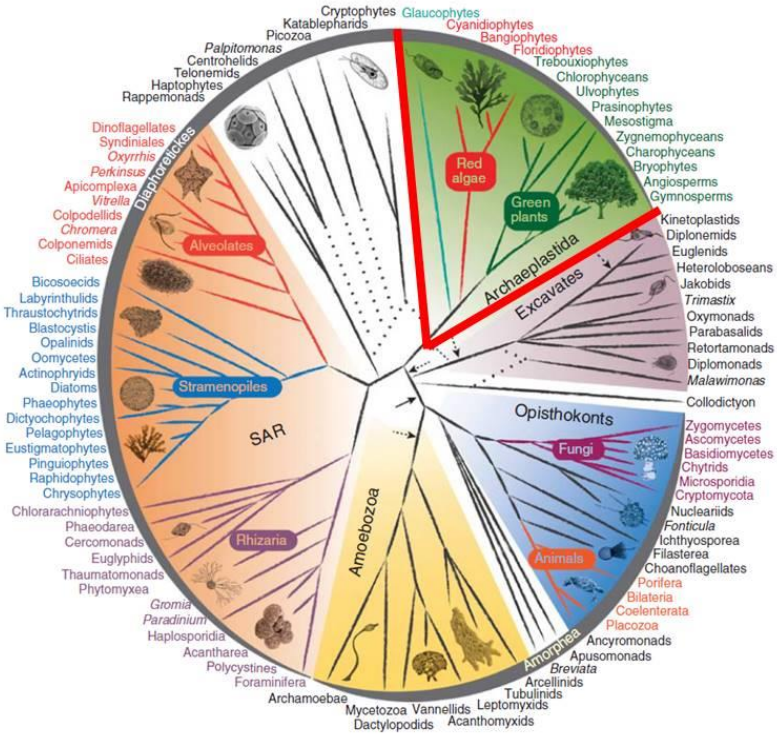

Fig. 2 Global tree of eukaryotes from a consensus of phylogenetic evidence. Red bars highlight the supergroup composed of the three main lineages of primary photosynthetic taxa: the glaucophytes, the red algae (rhodophytes) and the "green" organisms (Viridiplantae), including the green algae. Source Burki (2014).

1.3 Habitats

Microalgae can be found abundantly in a variety of natural and artificial ecosystems:

Freshwater - Internal freshwater environment displays a wide diversity of form of microalgae, although not exhibiting the phenomenal size range of their marine relatives (Barsanti and Gualtieri 2014). Freshwater phytoplankton and the benthonic algae form the base of the aquatic food chain (Barsanti and Gualtieri 2014). Algae are present in various freshwater habitats such as ponds, puddles, lakes, agricultural lands, oxidation ponds, streams, canals, springs, water storage tanks, reservoirs, and rivers (Rajkuma and Yaakob 2013). The information on freshwater algae is vast, yet remains scattered. Blue-green algae, green algae, diatoms, and euglenoid flagellates are the main components of freshwater habitats (Rajkuma and Yaakob 2013).

Marine - The biotic community of marine environments is dominated by microalgae. They are among the largest primary producers of biomass in the marine environment and are common

inhabitants of the tidal and intertidal areas of the marine ecosystem. Phytoplankton have a wide distribution in all habitats of the marine environment, it plays a major role in the food chain of an aquatic ecosystem and produces roughly 50% of the oxygen we inhale (Rajkuma and Yaakob 2013; Barsanti and Gualtieri 2014). The phytoplankton community includes 5,000 marine species of unicellular algae and has a broad diversity of cell size (mostly in the range of 1 to 100 μm), morphology, physiology, and biochemical composition (Margalef 1978; Rajkuma and Yaakob 2013).

Subaerial - A considerable number of sub-aerial algae have adapted to life on land (Barsanti and Gualtieri 2014). They can occur in surprising places such as tree trunks, animal fur, snow banks, hot springs, or even embedded within desert rocks (Barsanti and Gualtieri 2014). The activities of land algae are thought to convert rock into soil, to minimize soil erosion as well as to increase water retention and nutrient availability for plants growing nearby (Barsanti and Gualtieri 2014).

Symbiotic - Algae also form mutually beneficial partnership with other organisms (Barsanti and Gualtieri 2014). They live with fungi to form lichens (Oksanen 2006; Santos and Reis 2014), or inside the cells of reef-building corals (Muller-Parker *et al.* 2015), in both cases providing oxygen and complex nutrients to their partner, and in return receiving protection and simple nutrients (Barsanti and Gualtieri 2014). This arrangement enables both partners to survive in conditions that they could not endure alone (Barsanti and Gualtieri 2014).

1.4 Nutritional strategies

Algae are capable of many kinds of trophism (nourishment) centered on both major forms of nutrition, namely autotrophy (phototrophy) and heterotrophy (phagotrophy and osmotrophy), of which autotrophy is by far the most important.

Phototrophic algae - Most algal groups should be considered photoautotrophs. Autotrophic organisms obtain their energy through the absorption of light energy for the reduction of CO_2 by the oxidation of substrates, mainly water, with the release of O_2. Photoautotrophic organisms only require inorganic mineral ions (Grobbelaar 2004), but they can supplement growth by phagotrophy and/or osmotrophy when light is limiting (e.g., *Dinobryon divergens*, Ochrophyta) (Barsanti and Gualtieri 2014). Obligate photoautotrophs are those that cannot grow in the dark. By far, most algae belong to this category, although many require minimal quantities of organic compounds for growth, such as vitamins and amino acids (Auxotrophy) (Grobbelaar 2004).

Heterotrophic algae - Heterotrophic organisms obtain their material and energy needs from organic compounds produced by other organisms (Grobbelaar 2004), but are capable of sustaining themselves by phototropy when prey concentrations limit heterotrophic growth (e.g., *Gymnodinium gracilentum*, Myzozoa) (Barsanti and Gualtieri 2014). Several algal species can be grown exclusively on organic substrates and this has become a viable option in conventional closed bioreactor production systems for biomass and biocompounds, produced by certain species under specific growth conditions (Grobbelaar 2004). Photoheterotrophic organisms require light as energy source to use organic compounds as nutrients. The organic compounds may also satisfy the energy requirements of the algae (Grobbelaar 2004).

Mixotrophic algae - Mixotrophic or amphitrophic growth is equivalent to autotrophy and heterotrophy, where both organic compounds and CO_2 are necessary for growth (e.g., *Euglena gracilis*, Euglenozoa) (Grobbelaar 2004; Barsanti and Gualtieri 2014). A definite switch between autotrophy and heterotrophy is not manifested and both processes are present, except in total darkness. No clear distinction is possible, except for the obligate trophic types, and some interchange between the various trophic possibilities is likely under most growth conditions (Grobbelaar 2004).

2 PHOTOSYNTHETIC PROCESS

An understanding of photosynthesis is fundamental for microalgal biotechnology (Masojìdek *et al.* 2004). The sun is the universal source of energy in the biosphere. Almost all light in the natural environment originates from the sun. About 99% of the sun's radiation that reaches the surface of the earth has a spectral range from ~300 nm (ultraviolet) to ~4000 nm (infrared) and is called the broadband or total solar radiation. On the broadband radiation, three spectral regions exist the ultraviolet radiation (UV, 100–400 nm), sight (visible light, 400–700 nm), and heat (infrared radiation, 700–4000 nm). The visible range wavelengths are utilized for both vision and photosynthesis (Barsanti and Gualtieri 2014). Photosynthesis represents a unique process of sunlight energy conversion, and virtually, all forms of life on Earth depend directly or indirectly on it as a source of energy for their growth. In this process, inorganic compounds and light energy are converted to organic matter by photoautotrophs (Masojìdek *et al.* 2004). The radiation utilized by oxygenic photosynthetic organisms for the photosynthesis is termed Photosynthetic Active Radiation (PAR) and corresponds to the visible range wavelengths (400–700 nm) (Barsanti and Gualtieri 2014; Concas *et al.* 2014a). It should be noted that shorter wavelengths (<400 nm) carry a

very high energy content that can damage microalgal cells, while at longer wavelengths (>700 nm) the energy carried does not allow photosynthesis to take place (Concas *et al.* 2014a). Photosynthetic activity of algae, which roughly account for more than 50% of global photosynthesis, converts the energy of PAR into biologically usable energy (Barsanti and Gualtieri 2014). Approximately half of incident light intensity impinging on the earth surface (0.42 kW m^{-2}) belongs to PAR. Only 5% of the PAR is used by photosynthetic processes. Despite this high energy waste, photosynthetic energy transformation is the basic energy-supplying process for algae (Barsanti and Gualtieri 2014). Oxygenic photosynthesis can be expressed as a redox reaction driven by light energy (harvested by chlorophyll molecules), in which carbon dioxide and water are converted to carbohydrates and oxygen (Masojìdek *et al.* 2004). As shown in Equation 1 during photosynthesis, carbon is converted from its maximally oxidized state (+4) in CO_2 to 0 in strongly reduced compounds such as carbohydrates $[CH_2O]n$, using the energy of light:

$$n\ CO_2 + n\ H_2O + light \xrightarrow{Chlorophyll\ a} (CH_2O)n + n\ O_2 \qquad (1)$$

In this equation, light is considered as a substrate, **chlorophyll a** is the catalytic agent, and $(CH_2O)n$ represent the reduced organic matter (carbohydrate). These reduced compounds may be re-oxidized to CO_2 during respiration, liberating energy (Barsanti and Gualtieri 2014). Photosynthesis encompasses two major groups of reactions (Barsanti and Gualtieri 2014), the so-called “light reactions” and “dark reactions” (Fig. 3) (Masojìdek *et al.* 2004).

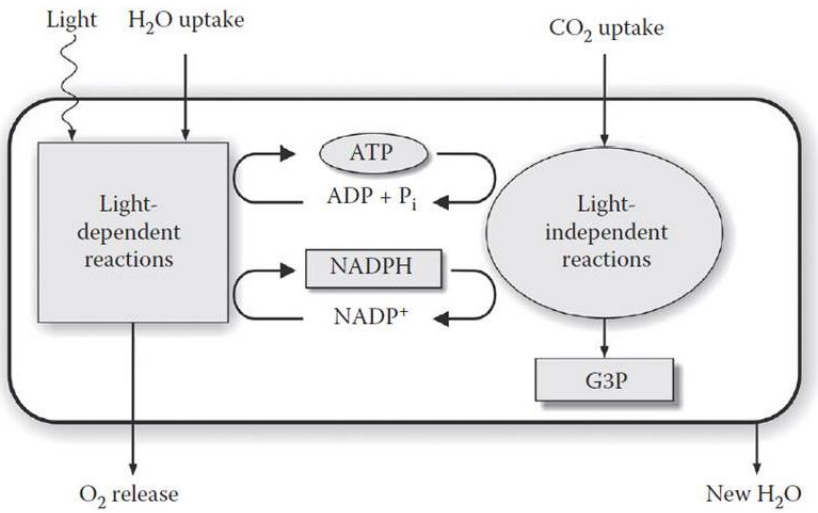

Fig. 3 Schematic drawing of the photosynthetic machinery. ATP: adenosine triphosphate; ADP: adenosine diphosphate; P: phosphate; NADPH: nicotinamide adenine dinucleotide phosphate (reduced form); NADP: nicotinamide adenine dinucleotide; G3P: glyceraldehyde 3-phosphate. Source Barsanti and Gualtieri (2014).

• Light reactions of photosynthesis - The main role of the light reactions is to provide the biochemical reductant ($NADPH_2$) and the chemical energy (ATP) for the assimilation of inorganic carbon (Masojìdek *et al.* 2004). Photosynthetic light reactions take place in thylakoid membranes which contains five major complexes: light-harvesting antennae, photosystem II (PS II) and photosystem I (PS I) (both containing a reaction center), cytochrome b6/f and ATP synthase, which maintain photosynthetic electron transport and photophosphorylation (Masojìdek *et al*. 2004).

Chlorophylls are the essential pigments of photosynthesis, for which they both harvest light and transduce it into chemical energy. There are five chemically distinct chlorophylls known to date, termed chlorophylls a, b, c1, c2, d, and f, and each one has its own absorption spectrum (Barsanti and Gualtieri 2014). Carotenoids are C40 hydrocarbon chains, strongly hydrophobic, functioning as accessory pigments for energy capture and transfer and for also playing protection roles. Xanthophylls are carotenoid derivate with a role in non-radiative dissipation of excess absorbed light energy (Barsanti and Gualtieri 2014). The PSII and PSI photosynthetic complexes are very similar in eukaryotic algae (and plants) and cyanobacteria.

• Dark reactions of photosynthesis - The “light-independent reactions” or “dark-reactions” involve the sequence of reactions by which the chemical potential of the $NADPH_2$ and ATP, produced in the light reaction of photosynthesis, is utilized in the fixation of carbon dioxide into carbohydrates (Masojìdek *et al.* 2004; Barsanti and Gualtieri 2014).

The fixation of CO_2 takes place in the chloroplast stroma (eukaryotic algae) or in the cytoplasm (prokaryotic algae). The light-independent reactions do not occur in the dark; rather they occur simultaneously with the light reactions. However, light is not directly involved.

2.1 Photorespiration

Photorespiration represents a competing process to carboxylation, where the organic carbon is converted into CO_2 without any metabolic gain (Masojìdek *et al.* 2004). Photorespiration can be defined as the light-dependent uptake of O_2 in the chloroplast. It is caused by a fundamental "inefficiency" of RuBisCO (Barsanti and Gualtieri 2014) which functions as an oxygenase (Masojìdek *et al.* 2004). Since the RuBisCO enzyme has low affinity to CO_2 (its half-saturation constant, K_m, is roughly equal to the level of CO_2 in air), the photorespiration depends on the relative concentrations of oxygen and carbon dioxide. A high O_2/CO_2 ratio stimulates this process, whereas a low O_2/CO_2 ratio favors carboxylation. In order to obtain optimal microalgal yields in mass cultures, it is necessary to minimize the effects of photorespiration, for example by stripping the oxygen or by CO_2 enrichment (Masojìdek *et al.* 2004).

2.2 Light and photosynthesis rate

The classical description of photosynthetic activity is based on measurements of oxygen evolution in proportion to light intensity, the so-called light-response (P/I) curve (Fig. 4). The initial slope of such curve is described as $\alpha = P_{max}/I_k$, where I_k represents the saturation irradiance and P_{max} is the maximum rate of photosynthesis (Masojìdek *et al.* 2004). The light response curve (P/I) of microalgae can be divided into three distinct regions (Vonshak and Torzillo 2004):

- a light-limited region, in which photosynthesis increases with increasing irradiance,
- a light-saturated region in which photosynthesis is independent of irradiance,
- a photoinhibited region in which photosynthesis decreases with further increase in irradiance.

At low irradiance (light-limited region), the rate of photosynthesis depends linearly on light intensity (Masojìdek *et al.* 2004). The initial slope of the P/I curve (α) (Jassby and Platt 1976) can be taken as a direct measure of the maximum quantum yield of photosynthesis (Vonshak and Torzillo 2004). At higher irradiances the relationship between absorbed light and rate of photosynthesis is not linear anymore, consequently the quantum efficiency decreases (Fig. 4).

Eventually, photosynthesis becomes light saturated and the photosynthesis rate reaches the maximum (P_{max}) and remains constant with increasing irradiance (Vonshak and Torzillo 2004). Typically the concentration of microalgae in solution increases with the intensity of light up to a certain level (saturation intensity). With further increase of light intensity, the photosynthesis becomes progressively less efficient and does not provoke the increase of algal growth rate which remains almost constant (light-saturated value) (Masojìdek *et al.* 2004; Concas *et al.* 2014a). Under prolonged supra-optimal irradiance, photosynthetic rates usually decline from the light-saturated value. This phenomenon is commonly known as photoinhibition of photosynthesis (Masojìdek *et al.* 2004).

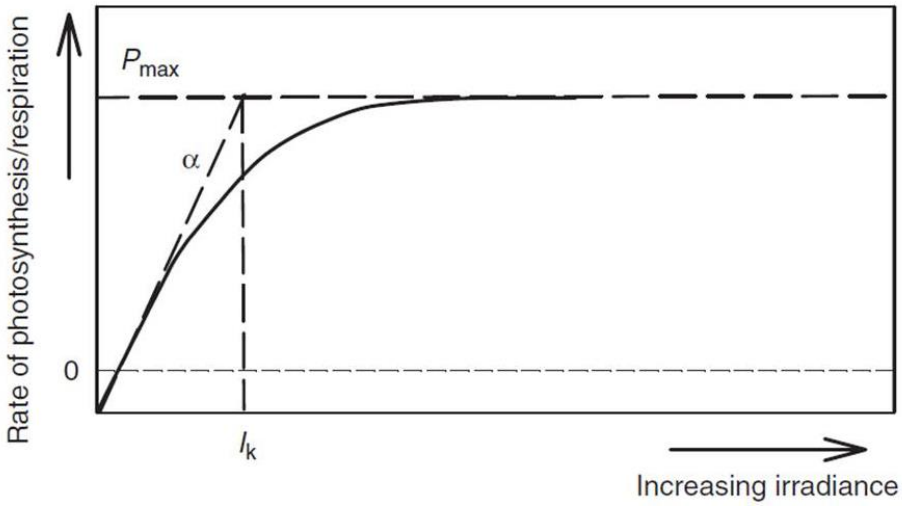

Fig. 4 A schematic representation of photosynthetic light-response curves, i.e. the dependency of photosynthesis vs irradiance. The initial slope of the curve (a) is the maximum light utilization efficiency. The intersection between the maximum rate of photosynthesis Pmax and α is the light saturation (optimum) irradiance. At supra-optimum irradiance, photosynthesis declines, which is commonly called down-regulation or photoinhibition. Source Masojìdek *et al.* (2004).

2.3 Photoinhibition

The photoinhibition has been defined as a light-dependent reduction in photosynthetic efficiency (Kok 1956; Vonshak and Torzillo 2004). The term photoinhibition has also been used to mean damage to PS II (Demmig-Adams and Adams 1992; Vonshak and Torzillo 2004). The over-excitation of PS II occurs when photosynthetic organisms are grown under suboptimal conditions. Environmental conditions that reduce carbon metabolism, such as chilling and freezing temperatures (Long *et al.* 1983; Torzillo *et al.* 1996; Vonshak *et al.* 2001; Vonshak and Torzillo 2004), high temperature (Bongi and Long 1987; Vonshak and Torzillo 2004), and nitrogen deficiency (Herzig and Falkowski 1989; Vonshak and Torzillo 2004), may strongly increase the

possibility of over-excitation of the PS II. Such conditions lead to an increase in the dissipation of absorbed energy through non-radiative processes, eventually reducing the photosynthetic rate (Vonshak and Torzillo 2004). Since algal productivity depends primarily on light energy conversion efficiency, hence, the photoinhibition due to excessive light absorption will result in a decrease in the biomass yield. Dense microalgae cultures may experience large daily variations in light intensity due to changes in irradiance and mixing, and the effect of photoinhibition on their productivity may increase if additional stress, e.g. sub-optimal temperatures or high oxygen concentration are imposed. Various approaches have been proposed to face this problem, for example, the increase of cell density and mixing rate of the cultures in order to prevent the saturation effect (Hu *et al.* 1996), the use of special photobioreactors with an improved light distribution in the culture (Torzillo *et al.* 1993; Tredici and Chini Zittelli 1997) and ultimately the search for strains having small antenna size and thus higher photosynthesis saturation levels (Nakajima and Ueda 1997, 2000; Neidhardt *et al.* 1998; Melis *et al.* 1999; Vonshak and Torzillo 2004).

3 ALGAL CULTURING

Mass cultures of unicellular microalgae represent a special environment, where rather dense suspensions of cells are usually cultivated under conditions of low irradiance per cell, high concentration of dissolved oxygen and limited supplies of inorganic carbon (carbon dioxide or bicarbonate) (Masojìdek *et al.* 2004). Ideally, the theoretical maximum growth rate of an algal culture should be equal to the maximum rate of photosynthesis (Masojìdek *et al.* 2004). Actually, the growth critically depends on the interplay of several parameters. The most important parameters regulating algal growth are light, nutrient quantity and quality, pH, temperature, turbulence, and salinity. Optimal parameters, as well as the tolerated ranges, are species-specific; the different parameters may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another (Barsanti and Gualtieri 2014).

3.1 Parameters affecting microalgae growth

Effect of Light - Light is the principal limiting factor in the culture of photosynthetic organisms (Pulz 2001; Griffiths 2013) so it needs to be considered in terms of intensity, spectral quality, and photoperiod. The Light intensity requirements greatly vary with the density of the algal culture (Barsanti and Gualtieri 2014). At high cell concentrations, the light intensity must be increased to penetrate through the culture, however too high light intensity may result in photo-inhibition (Barsanti and Gualtieri 2014). The most often employed light intensities range between 100 and 200 $\mu E\ s^{-1}\ m^{-2}$ and are usually supplied by natural source or by fluorescent tubes. Constant illumination or light/dark (LD) cycles (maximum 16:8 LD, usually 14:10 or 12:12) can be used in microalgae cultivation (Barsanti and Gualtieri 2014).

pH - The time evolution of a medium's pH during algal growth is a significant indicator of how well are evolving photosynthetic processes. In fact, as algae grow, dissolved CO_2 is consumed by photosynthesis and, consequently, pH increases (Concas *et al.* 2014a). However, pH variation can also, in turn, strongly affect the growth kinetics of microalgae influencing the distribution of carbon dioxide species and carbon availability causing direct physiological effects (Chen and Durbin 1994; Cornet *et al.* 1995; Concas *et al.* 2014a). It has been recognized that pH variation strongly affects the growth kinetics of microalgae in several ways:

- it influences the distribution of carbon dioxide species and carbon availability causing direct physiological effects (Chen and Durbin 1994; Cornet *et al.* 1995; Concas *et al.* 2014a),
- it can affect the enzymatic activity of intra and extra-cellular carbonic anhydrase, thus influencing the carbon capture mechanism of some microalgal strains (Concas *et al.* 2012, 2014a),
- hydrogen ions can limit photosynthetic growth and substrate utilization rates at very low or very high levels (Mayo 1997; Concas *et al.* 2014a),
- the control of pH in culture media is important since certain algae grow only within narrowly defined pH ranges and also to prevent the formation of precipitates (Barsanti e Gualtieri 2014).

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7, though, there are species that dwell in more acid/basic environments (Barsanti and Gualtieri 2014). Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. In the case of high-density algal culture, the

control of pH may be performed by the addition of carbon dioxide that allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth (Barsanti and Gualtieri 2014). Certain media formulations include additions of extra buffer, either as bicarbonate, Tris (Tris-hydroxymethyl-aminomethane), or glycylglycine. Glycylglycine is rapidly metabolized by bacteria and hence can only be used with axenic cultures. The problem of CO_2 depletion in dense cultures may be reduced by having a large surface area of media exposed to the atmosphere relative to the volume of the culture, or by bubbling with either air (CO_2 concentration 0.03%) or air with increased CO_2 concentrations (0.5–5%). Unless there is a large amount of biomass taking up the CO_2, the higher concentrations could actually cause a significant decline in pH (Barsanti and Gualtieri 2014).

Temperature - Temperature is one of the main factors which regulate cellular, morphological and physiological responses of microalgae (Mayo 1997; Durmaz *et al.* 2007; Concas *et al.* 2014a). The optimal growth temperature for microalgae is species-specific, but often in the region of 20°C to 30°C (Chisti 2008; Griffiths 2013). Many algal species can tolerate temperatures of up to 15°C lower than their optimum, with reduced growth rates, but a temperature of only a few degrees higher than optimal can lead to cell death (Mata *et al.* 2010; Griffiths 2013). Under optimal temperature condition, the enzymes of microalgal cells show the highest activity (Concas *et al.* 2014a). Most commonly cultured species of microalgae tolerate temperatures between 16°C and 27°C (Barsanti and Gualtieri 2014). High temperatures generally accelerate the metabolic rates of microalgae, whereas low ones lead to inhibition of microalgal growth (Munoz and Guieysse 2006; Concas *et al.* 2014a). However, the net efficiency of photosynthesis declines at high temperature as the rate of respiration rises significantly, while the increased flux through the Calvin cycle is moderate (Griffiths 2013). This effect is worsened by the fact that CO_2 becomes less soluble at elevated temperatures, more rapidly than O_2 (Pulz 2001; Griffiths 2013). Low temperatures can lead to significant losses in productivity, although they might potentially be advantageous due to a reduction in the respiration rate (Griffiths 2013). Since as much as 25% of the biomass produced during daylight hours can be lost at night due to respiration (Chisti 2007; Griffiths 2013), cool nighttime temperatures can minimize this loss (Griffiths 2013). It has been demonstrated that the saturation light intensity is highly temperature dependent (Shelef 1968; Goldman and Carpenter 1974), and the half-saturation coefficient for nutrient uptake is very sensitive to changes in temperature (Shelef *et al*. 1970; Goldman and Carpenter 1974). Furthermore, temperature influences the so-called temperature coefficient (Q10) where enzymatic reaction approximately

doubles with a 10°C temperature rise (Goldman and Carpenter 1974; Grobbelaar 2004). For the mass cultivation of algae, this would mean an approximate doubling in the uptake of nutrients with every 10°C increase in culture temperature (Grobbelaar 2004). The control of temperature is a key factor for cultivating microalgae outdoors. Temperature can vary depending upon the geographic region of cultivation. Seasonal and even daily fluctuations in temperature can interfere with algae production. The internal temperature in photobioreactors can reach values that are 30°C higher than ambient one if suitable temperature control equipment is not used. To overcome this problem evaporation, cooling or shading techniques are successfully employed (Concas *et al.* 2014a).

Mixing - Good mixing keeps the cells in suspension, eliminates thermal stratification, determines the light-dark regime by moving cells through an optical gradient, ensures efficient distribution of nutrients, improves gas exchange, reduces mutual shading at the center of the reactor, and decreases photo-inhibition at the surface (Ugwu *et al.* 2008; Griffiths 2013). For very dense cultures, the CO_2 originating from the air (containing 0.03% CO_2) bubbled through the culture is limiting and pure carbon dioxide may be supplemented to the air supply (e.g., at a rate of 1% of the volume of air). CO_2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3^- balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, Erlenmeyer's), aerating (bags, tanks), or using paddle wheels and jet pumps (ponds) (Barsanti and Gualtieri 2014). When the nutritional requirements of mass cultured algae are satisfied and the environmental conditions are not growth-limiting, the turbulent flow constitutes the most important requisite for consistently obtaining high yields of algal mass (Richmond and Becker 1986; Grobbelaar 2004). Such turbulence is important not only for enhancing exchange rates of nutrients and metabolites between the cultured cells and their growth medium but the increased light/dark frequencies result in increased productivity and photosynthetic efficiencies (Grobbelaar 1994, 2004).

Salinity - Marine algae are extremely tolerant to changes in salinity. Most species grow best at salinity slightly lower than that of their native habitat, which is obtained by diluting seawater with tap water. Salinities of 20–24 g L^{-1} are found to be optimal (Barsanti and Gualtieri 2014). The fundamental aspects of salt adaptation were intensively reviewed: Kirst (1990) investigated the tolerance of marine macroalgae and phytoplankton species to salinity; Oren (1999) reviewed the energetic costs of salt adaptation. Salt acclimation of cyanobacteria was reviewed by Reed and Stewart (1988) and by Erdmann and Hageman (2001). In many of the algal systems studied, a

decline in productivity is observed once adapted to excessive salinity and clearly associated with a decrease in their photosynthetic capacity (Vonshak and Torzillo 2004).

3.2 Culture media

For the choice of the culture medium, the natural habitat of the species should be considered in order to determine its requirements. It is important to know whether the environment is eutrophic, hence nutrient-rich, or oligotrophic, hence nutrient-poor, and whether the algae belong to an r-selected or a k-selected species. The r-Selected species are characterized by a rapid growth rate, autotrophic metabolism, and a wide environmental plasticity, whereas k-selected species show a slow growth rate, mixotrophic, or photoheterotrophic metabolism, and a low environmental tolerance (Barsanti and Gualtieri 2014). In addition, it is also important to consider the purpose for which the algae will be cultured. Maintenance of cultures in a culture collection, versus growth for optimal biomass yields. The imposition of stress conditions for optimal biosynthesis of valued bio-compounds would require very different formulations of the nutrient recipes. For example, in *Dunaliella salina,* the carotenogenesis is initiated mainly by nitrogen and salt stress (Ben-Amotz and Avron 1989; Grobbelaar 2004). Normally stress would not be applied when maintaining algae in culture collections. Refinement of media composition for laboratory-maintained algal cultures have been the object of research for several decades, resulting in many different media recipes reported in the literature and used in different laboratories (Barsanti and Gualtieri 2014). Media can be classified as defined or undefined. Defined media, which are often essential for nutritional studies, have known constituents all assigned to a chemical formula. Undefined media, on the other hand, contain one or more natural or complex ingredients, for example, agar, or liver extract and seawater, whose composition is unknown and may vary (Barsanti and Gualtieri 2014). Defined and undefined media may further be subdivided into freshwater or marine media.

3.3 Effect of Nutrients

Typically, microalgae require sunlight and some essential elements such as nitrogen (N), phosphorus (P), iron (Fe) and, in some cases, silicon (Si) for growth (Ubando *et al.* 2014; Duran *et al.* 2018). However, the increased consumption of nutrients could be overcome by utilization of N and P contained in wastewater (Zhou et al. 2014; Duran et al. 2018). Optimal supply of nutrients, mainly carbon, nitrogen, phosphorous, macro- and micronutrients required for algal growth, is a prerequisite for high growth rates (Griffiths 2013). Deficiencies in any nutrient may cause

disturbances in metabolism, physiological changes, and decreased productivity (Pulz 2001; Griffiths 2013).

Carbon - For high rates of autotrophic production, supply of CO_2 and HCO_3^- is most important (Grobbelaar 2004). However, atmospheric CO_2 cannot satisfy the C-requirements of high yielding autotrophic algal production systems, since the diffusion rates for CO_2 from the atmosphere into an open pond can at most sustain productivities around 10 g (dw) m^{-2} d^{-1} (Grobbelaar 2004).

Nitrogen - Beyond carbon, nitrogen is the most important nutrient contributing to the biomass production. The biomass nitrogen content can range from 1% to more than 10% and varies either between different groups (e.g. low in diatoms) and within species, depending on the supply and availability (Grobbelaar 2004). Typical responses to nitrogen limitation is discoloration of the cells (decrease in chlorophylls and increase in the carotenoids) and accumulation of organic carbon compounds such as polysaccharides and certain oils (PUFAs) (Becker 1994; Grobbelaar 2004). Hence, the optimal concentration of nitrogen to be assured in the growth medium depends upon two counteracting effects: high availability of nitrogen typically leads to a high biomass productivity, while a decrease of nitrogen concentration in the cultivation broth typically results in higher lipid contents but lower growth rates (Concas *et al.* 2014a).

Phosphorus - Phosphorus is essential for growth and many cellular processes such as energy transfer, biosynthesis of nucleic acids, DNA, etc. The preferred form in which it is supplied to algae is as orthophosphate (PO_4^{2-}) (Grobbelaar 2004; Barsanti and Gualtieri 2014) and its uptake is energy dependent. Sometimes, organic (glycerol) phosphate is also used (Barsanti and Gualtieri 2014). Although algal biomass contains less than 1% P, it is often one of the most important growth limiting factors in algal biotechnology. In fact, it is easily bound to other ions (e.g. CO_3^{2-} and iron) which can precipitate and consequently hinder the uptake of this essential nutrient by algae. Algae are also able to store excess P in polyphosphate bodies during the so-called luxury uptake (Grobbelaar 2004; Powell *et al*. 2008, 2009; Brown and Shilton 2014). It is known that the supply of P also influences the composition of the produced biomass, especially in the lipid (Liu *et al*. 2007; Concas *et al.* 2014a) and carbohydrates content (Borowitzka 1988; Grobbelaar 2004; Zhu *et al.* 2016). Furthermore, the ratio of N:P in the growth media is also important, as it determines both the potential productivity and the dominance of the candidate species in culture (Grobbelaar 2004).

Other macro- and micronutrients and chelants - Inorganic elements of importance for algae nutrition are also S, K, Na, Fe, Mg, Ca and trace elements such as B, Cu, Mn, Zn, Mo, Co, V, and Se. Most

of the trace elements are incorporated into essential organic molecules, particularly a variety of coenzyme factors that enter into photosynthetic reactions. It is important to monitor their supply and availability as they are prone to binding with other growth media constituents resulting in precipitation and consequently unavailability. It is thought that molecules complexing with metals (chelators) influence the availability of these elements. Chelators act as trace metal buffers, maintaining constant concentrations of free ionic metal, which is the form that primarily influences microalgae growth. The most widely used chelator in culture media additions is EDTA, which must be present at high concentrations since it mostly complexes with Ca^{2+} and Mg^{2+}. As an alternative, the organic chelator citrate is sometimes utilized, having the advantage of being less influenced by Ca^{2+} and Mg^{2+} In some media solutions also nitrilotriacetic acid is used for its chelating potential, however, both citric acid and nitrilotriacetic acid are less effective than EDTA (Grobbelaar 2004). The molar ratio of chelator/metal in culture media may range from 1:1 to 10:1. It should be taken into account that high chelator/metal ratios may result in metal deficiencies for algae (Barsanti and Gualtieri 2014).

4 CULTURING METHODS

Algae can be produced according to a great variety of methods. Indoor culture allows for control over illumination, temperature, nutrient level, contamination with predators and competing algae. Outdoor algal systems, such as uncovered ponds and tanks, though cheaper are more readily contaminated than closed culture vessels. Axenic cultivation free of any foreign organisms such as bacteria, is costly and difficult since it requires a strict sterilization of culture media, and vessels to avoid contamination. These constraints make it impractical and very expensive for commercial operations. On the other hand, non-axenic cultivation, though cheaper and less laborious, are more prone to crash, less predictable, and often of inconsistent quality (Barsanti and Gualtieri 2014). The most routinely types of algal cultures adopted are batch, operated in continuous and semi-continuous mode, ponds, and photobioreactors.

Continuous cultures - The key to the success of algal production is maintaining all cultures in the exponential growth phase. This aim is achieved by the regulated addition of fresh culture medium at a rate proportional to the growth rate of the alga, while an equal volume of culture is removed. This culturing method permits the maintenance of cultures very close to the maximum growth rate since the algae never run out of nutrients. Air is also supplied into the culture vessel through a pump, passes down a long glass tube to the bottom of the culture, and bubbles up. This assures a good

suspension of the cells in the culture as well as high gas exchange levels (oxygen is removed and CO_2 is provided). The flow rate of medium into a continuous culture system is known as the "dilution rate" The principal advantage of continuous culture is that the dilution rate controls the rate of microbial growth via the concentration of the growth-limiting nutrient in the medium. As long as the dilution rate is lower than the maximum growth rate attainable by the algal species, the cell density will increase to a point at which the cell division rate (birth rate) exactly balances the cell washout rate (death rate). This steady-state cell density is also characterized by a constancy of all metabolic and growth parameters. Furthermore, continuous cultures have the advantages of producing algae of more predictable quality and are suitable to technological control and automation, which in turn increases the reliability of the system and reduces the need for labor. The disadvantages of the continuous system are its relatively high cost and complexity. Indeed, the constant illumination and temperature requirements mostly restrict such systems to indoors small production scales (Barsanti and Gualtieri 2014).

Semi-continuous cultures - In a semi-continuous system, the fresh medium is delivered to the culture all at once, by simply opening a valve in the medium delivery line. Fresh medium flows into the culture vessel and spent culture flows out into a collecting vessel. Once the required medium has entered the culture, the valve is closed, and the culture is allowed to grow for 24 h when the procedure is repeated. The semi-continuous technique prolongs the use of large tank cultures by partial periodic harvesting followed immediately by topping up to the original volume and supplementing with nutrients. Such culture systems may be indoors or outdoors, but usually, their duration is unpredictable. Since the culture is not completely harvested, the semi-continuous method yields more biomass than the batch method for a given tank size (Barsanti and Gualtieri 2014).

The production of algal biomass is more expensive and technologically complex than that of other crops (Duran *et al*. 2018). Cultivation systems for microalgae are broadly divided into two categories, i.e. open systems (open ponds or open raceways) and closed systems (photobioreactors).

4.1 Batch cultures

The most common culture system is the batch culture, due to its simplicity and low cost. This is a closed system, volume-limited, in which there is no input or output of materials, that is, resources are finite. A significant advantage of batch culture systems is their operational simplicity. At laboratory scale, the culture vessels most often consist of an Erlenmeyer flask or Pyrex bottle with a

cotton/gauze bung and a sample-to-flask volume ratio of about 0.2-0.5, in order to prevent carbon dioxide limitation (Fig. 5). The flasks may be shaken during the culturing either by hand once a day or by a rotating shaker table. Although batch culture is often considered as the most reliable method, it is not necessarily the most efficient one. Batch cultures are usually harvested just prior to the initiation of the stationary phase and thus must always be maintained for a substantial period of time past the maximum specific growth rate. Another disadvantage is the need to prevent contamination during the early growth period. Because the density of the desired phytoplankton is low and the concentration of nutrients is high, any contaminant with a faster growth rate is capable of outgrowing the culture (Barsanti and Gualtieri 2014).

Fig. 5 Batch cultures Pyrex bottles at the CINSA laboratory (University of Cagliari).

4.2 Open ponds

Different designs have been proposed for open ponds, natural or artificial ones, operating at large scale. Typical examples are the unstirred ponds (lakes and natural ponds), the inclined ones, central pivot, and the raceway ponds. Among the others, the most widespread typology of open pond is the so-called "raceway pond". It basically consists of open channels where a paddlewheel is used to drive the flow, while algae are kept suspended in water around a racetrack. Baffles in the channels guide the flow in order to minimize space. Raceways are typically made by concrete but can also simply dug into the soil and waterproofed with a plastic liner to prevent the liquid filtration through the ground. These systems are usually operated in a continuous mode, where the fresh medium (containing macro and micronutrients) is fed in front of the paddlewheel and algal broth is harvested behind it after being circulated through the loop (Singh and Sharma 2012; Concas *et al.* 2014a). The raceways (cf. Fig. 6) are characterized by low water depths of about 15-20 cm in order to assure a suitable light penetration along the hydraulic section thus avoiding dark zones where microalgae can't grow. At such depths, biomass concentrations of 1 g L^{-1} can be achieved and productivities ranging from 15 to 25 g m^{-2} day^{-1} are possible (Schenk *et al.* 2008; Concas *et al.* 2014a).

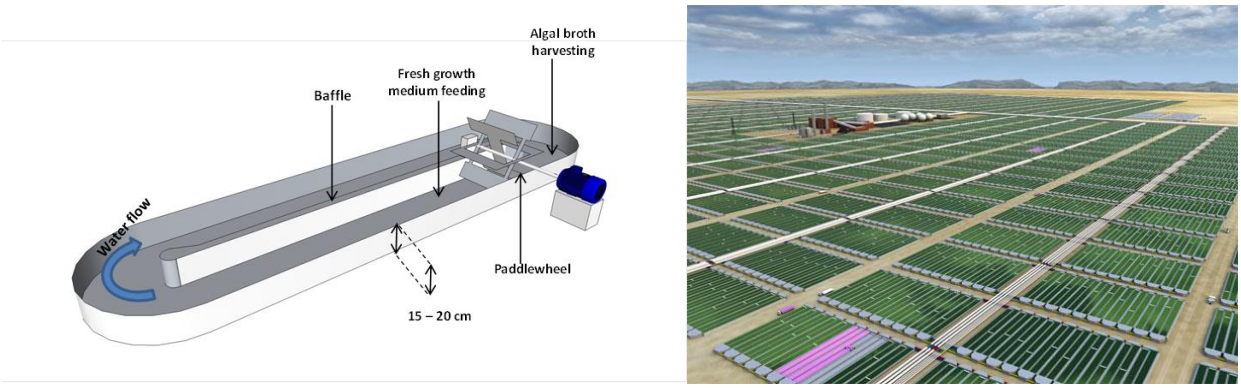

Fig. 6 Scheme of a single raceway pond and photography of raceway pond farm. Source Concas *et al.* (2014a).

In general, these cultivation systems are less expensive to build and simpler to operate than closed ones. For this reason, they are currently considered as the most cost-effective way for the massive production of microalgae at a large scale. However, open ponds display several limitations. In particular, when compared to closed systems, open raceways are characterized by a lower productivity that is the result of several factors. Evaporative losses can lead to changes in the ionic composition of the growth medium thus potentially provoking negative effects on culture growth such as iper-salinity, nutrient precipitation etc. Changes in temperature and photoperiod deriving

from seasonal variation cannot be suitably controlled in open ponds (Rawat *et al.* 2013; Concas *et al.* 2014a). These systems are more susceptible to contaminations by competing organisms such as fungi, bacteria, and protozoa. Furthermore, since atmospheric carbon dioxide is used as carbon source, its transfer rate is very low and consequently, carbon starvation phenomena could take place. Finally, sunlight is available only at the surface of the pond and hence, in the deeper strata of the liquid bulk, light limitation phenomena can arise. Improved mixing and bubbling the air at the bottom of the ponds by means of suitable spargers can minimize impacts of both CO_2 and light limitation but in general the productivity of these systems is very low whereby large areas of land may be required to meet the desired output of cultivation (Rawat *et al.* 2013; Concas *et al*. 2014a). To overcome limitations related to open system although keeping their low operating cost, the potential use of closed raceway ponds are currently under study (Fig. 7). These systems consist essentially of an open pond covered by a transparent or translucent barrier which turns it into a greenhouse (Singh and Sharma 2012; Concas *et al.* 2014a). This configuration prevents the microalgae to be contaminated by competing bacteria and allows a better control of crucial operating parameters such as temperature, evaporation etc. Moreover, by using closed raceways the amount of CO_2 provided can be increased since the gas bubbled at the bottom cannot escape to the atmosphere.

Fig. 7 Closed raceway photobioreactor at the Department of Mechanical, Chemical and Materials Engineering (DIMCM) laboratory (University of Cagliari).

4.3 Closed systems (photobioreactors)

Photobioreactors (PBR) are closed systems with no direct exchange of gases and contaminants with the environment where culture broth and microalgae are exposed to a photonic energy flux which triggers photosynthetic phenomena hence allowing biomass growth. Since they are closed reactors the crucial operating parameters such as temperature, pH, nutrient concentration, light intensity distribution, mixing, gas mass transfer rate can be suitably controlled and optimized. As a result, photobioreactors typically have higher biomass productivities than open ponds. On the contrary, photobioreactors are more expensive and complicated to operate than open ponds. Ideally, a photobioreactor for production of biomass should catch all sunlight available, dilute and distribute it uniformly in the growth medium where algae are suspended in such a way that all the caught light energy can be suitably exploited by algae for biomass formation. For this reason, a critical design parameter of photobioreactors is the illumination surface area per unit volume. Typically, a high illuminated surface area to volume ratio (SVR) results in a higher light availability in the liquid bulk and consequently in higher volumetric productivities of the systems. The surface to footprint ratio (SFR) is another critical design parameter. Higher values of SFR correspond to a larger areal productivity of the photobioreactor and consequently, the lesser is the land's area needed for producing the required output of microalgal biomass. Different types of photobioreactors are currently under study and development with the aim of reaching the more suitable configuration where SVR and SFR are maximized (Concas *et al.* 2014a).

Vertical tubular photobioreactors - The classical configuration of vertical tubular photobioreactor is the bubble column. It is basically a cylinder with radius of up to 0.2 m and height of up to 4 m. The height to diameter ratio is typically kept greater than 2 in order to maximize the SVR ratio. The CO_2 is provided to the algae by bubbling the gas from the bottom upwards through suitable spargers. While allowing a better CO_2 mass transfer, the bubbles flow provides also the suitable mixing degree without provoking significant shear stresses on microalgae. Moreover, the gas flow enables the effective removal of photosynthetic O_2 produced by algae which, if accumulated in the liquid, can inhibit the growth. The height constraint of these columns (< 4m) depend upon the gas transfer limitations and the strength of the transparent materials used to construct the columns. Since CO_2 supply and O_2 removal is optimized, in such type of reactors algal growth is often limited by other parameters such as light (Wang *et al.* 2012; Concas *et al.* 2014a). A schematic representation of different types of vertical tubular photobioreactors is shown in Fig. 8.

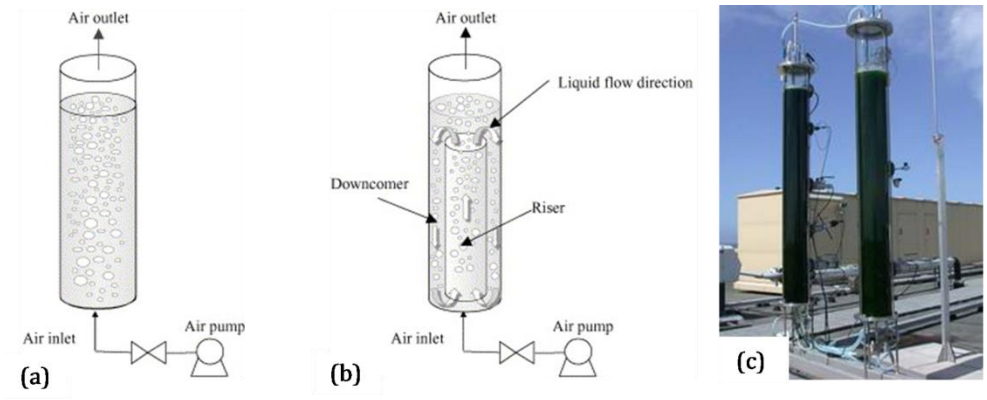

Fig. 8 Schematic representation of bubble column (a) and airlift (b) photobioreactors and picture (c) of an industrial bubble column photobioreactor. Source Concas *et al.* (2014a).

A specific configuration of vertical tubular photobioreactors is the so-called airlift reactor. It consists of a vessel with two interconnecting zones (i.e. the riser and the downcomer). The gas flow is introduced at the bottom of the riser and carries the liquid upward. At the top of the column liquid/gas separation takes place in the freeboard regime thus allowing the removal of accumulated photosynthetic oxygen. Subsequently, the degassed liquid falls downward in the downcomer. Mixing is therefore guaranteed by aeration and liquid circulation. This system allows a better exposure of microalgal cells to light radiation than classical bubble columns as well as an effective mixing and degassing of the liquid. Airlift PBR configurations may include an internal loop airlift, split column airlift and external loop airlift (Concas *et al.* 2014a).

Flat panel photobioreactors - The flat-plate photobioreactors have been developed for decades and a lot of research has been performed. They are made of transparent flat plates in which algae are cultivated. Because of the transparent material used for the plates and the large surface area, a high utilization of solar energy can be reached. Thus, flat photobioreactors can provide higher photosynthetic efficiency compared to tubular versions. Flat panels (cf. Fig. 9) are parallelepiped shaped photobioreactors having a minimal light path and a large illumination surface area (SVR) which can reach values of up to 40 m^{-1} (Singh and Sharma 2012; Concas *et al.* 2014a). The thickness of plate is the crucial parameter in the design of flat panels because it determines the surface area/volume ratio and the length of light path (Wang *et al.* 2012; Concas *et al.* 2014a). They can be made from transparent materials like glass, plexiglass, polycarbonate etc. The CO_2 is provided by bubbling the gas from one side of the panel through suitable perforated tubes. Mixing of the liquid is assured by the gas flow or by rotating the photobioreactor through a motor (Singh and Sharma 2012; Concas *et al.* 2014a).

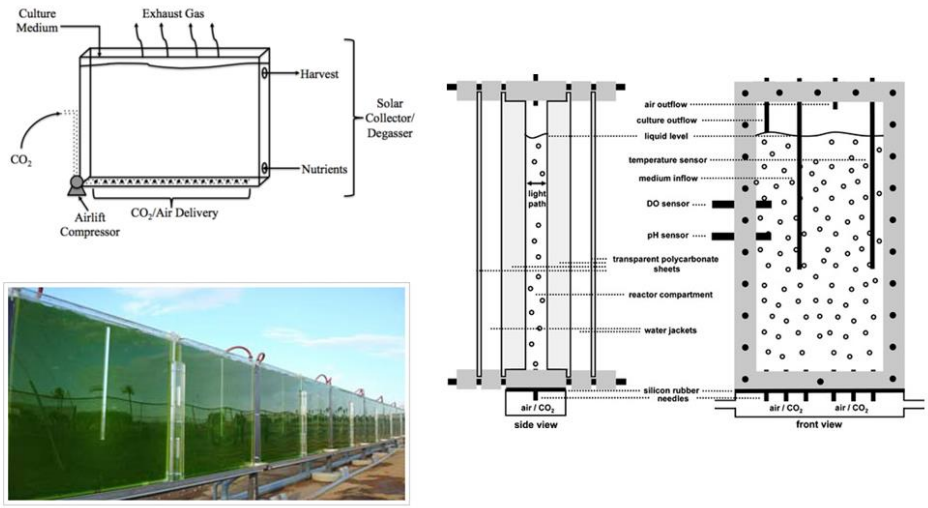

Fig. 9 Schematic representations and picture of flat panel photobioreactors. **Source Concas *et al.* (2014a**).

Major limitations of conventional flat panels are the difficulties of controlling the liquid flow and the relatively high construction costs. To overcome these problems vertical alveolar panels, made in plexiglass, were proposed (Tredici and Materassi 1992; Concas *et al.* 2014a). These systems allows to obtain a high surface-to-volume ratio of about 80 m^{-1}. A good biomass productivity can be achieved by using these alveolar panels as well as a good mixing degree and suitable mass transfer rates. Moreover, the manufacturing costs of these reactors are quite low. However, critical operating parameters such as temperature and light penetration should still be optimized in such a PBR (Wang *et al.* 2012; Concas *et al.* 2014a).

Biofilm photobioreactors - Most microalgae are able to grow in a biofilm. Within a biofilm, microalgae live in densely packed slimy layers of numerous microalgae together with other microorganisms that attach themselves to solid surfaces (e.g. slippery rocks in shorelines). Recent advances in the design of bioreactors for cultivating microalgae immobilized as a phototrophic biofilm that are densely packed layers of microalgae that grow attached to a solid surface (Fig. 10). The phototropic biofilm should be illuminated and should be frequently exposed to water containing nutrients including nitrogen, phosphate and trace elements. One type of algal biofilm design, the immobilized cultivation using porous substrate bioreactor (PSBR), is characterized by the almost complete separation of algal biomass from the bulk of the liquid culture medium during cultivation, which is likely to improve the cost-effectiveness of the overall process. By reducing the water volume by several orders of magnitude, as well as exposing microalgae directly to carbon dioxide and light, PSBR technology offers significant advantages compared with both suspension and submerged biofilm cultivation (Podola *et al.* 2017).

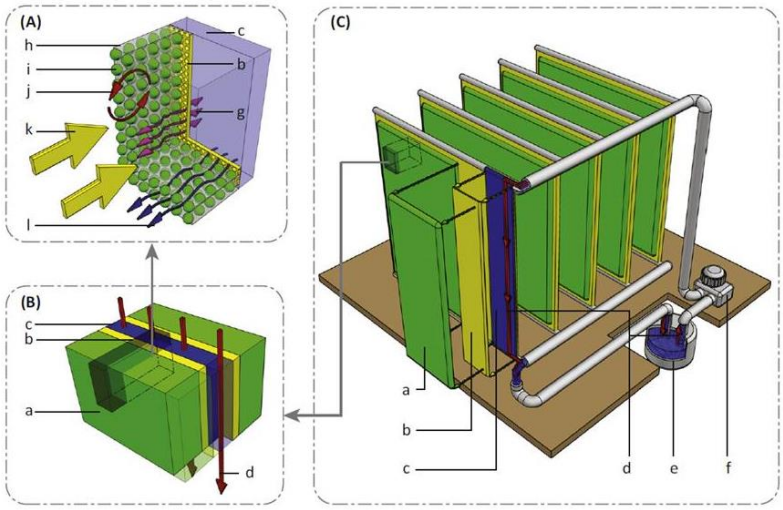

Fig. 10 Schematic Representation of a Porous Substrate Biofilm Photobioreactor (PSBR). (A) Principle of a PSBR illustrated using a small section. (B) Arrangement of layers in a vertical PSBR. (C) Large-scale vertical PSBR with multiple sheets: a, algal biofilm; b, microporous membrane; c, culture medium; d, direction of medium flow; e, medium reservoir; f, medium pump; g, diffusion; h, extracellular matrix; i, algal cells; j, gas exchange; k, irradiance; and l, evaporation. Source Podola *et al.* (2017).

Horizontal tubular photobioreactors - Horizontal tubular reactors typically consist of arrays of transparent thin tubes built in different patterns (i.e. straights, loop or serpentine shaped etc.). The arrays of tubes can be arranged in parallel or in series and then placed horizontally on the ground. Horizontal placement of these tubes results in a better angle for incident light compared to vertical tubular reactors, allowing for more efficient light harvesting (Wang *et al.* 2012; Concas *et al.* 2014a). Moreover, the tubes are preferably oriented towards the sunlight in order to maximize the light capture and the ground under the tubes can be covered with white plastic sheets in order to increase the albedo. In fact, a high albedo determines an increase of the total light received by the tubes. Typically, these tubes are less than 0.1 m in diameter since otherwise, the light does not suitably penetrate in the less exposed zones of dense cultures. However, larger diameters may be used when suitable regimes of turbulence of the fluid are employed in order to assure the movement of algae from the illuminated part of the tube to the dark one and vice-versa. Prolonged exposure to light in the illuminated part of the tube can trigger photo-inhibition phenomena while a long time exposure to darkness can inhibit photosynthesis. Furthermore, the tubes should not be longer than 80 m, in order to avoid the accumulation of photosynthetic oxygen in the culture and a too high increase of pH as algae grow (Concas *et al.* 2014a). Besides the tubes, which act as solar collectors, the horizontal photobioreactors include the following components: the harvesting unit to separate algae from the suspension, a degassing column for gas exchange and cooling (or heating) and a

circulation pump (Wang *et al.* 2012; Concas *et al*. 2014a). In Fig. 11, a specific configuration of horizontal photobioreactors is shown.

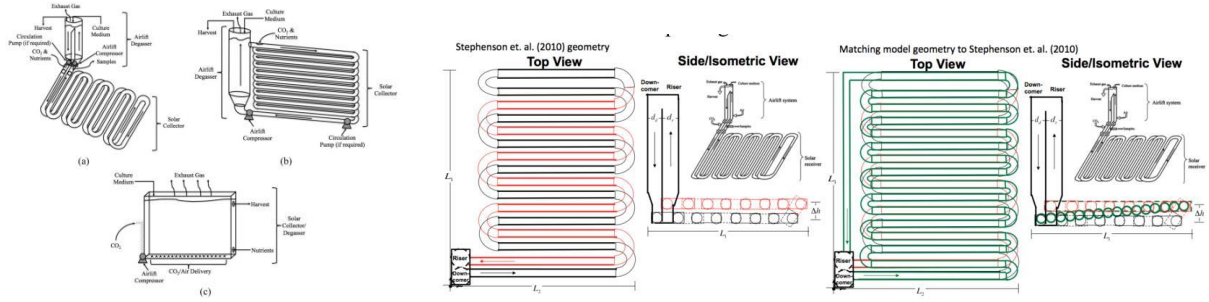

Fig. 11 Schematic representations of horizontal (serpentine type) photobioreactors. **Source Concas *et al.* (2014a).**

In the degassing device, air or CO_2 enriched air is injected in order to strip dissolved oxygen and at the same time provide the CO_2 to algae culture. In the degasser also the feeding of fresh medium can be carried out. Typically horizontal photobioreactors are capable to capture light better than other photobioreactor thus potentially assuring higher productivities. On the other hand, just this characteristic can cause the onset of photo-inhibition phenomena as well as the accumulation of high amounts of heat. Thus, expensive temperature control systems such as heat-exchangers are often required during large-scale cultivation of algae. Furthermore, long tubular PBRs are characterized by gradients of oxygen, CO_2 and pH along the tubes. The increase in pH of the cultures would also lead to frequent re-carbonation of the cultures, which would consequently increase the cost of algal production. Finally, it should be noted that adherence of the cells to the walls of the tubes is common. This results in a progressive fouling of the tubes and a consequent worsening of light penetration in the culture.

5 MICROALGAE UTILIZATION

5.1 Culture collections

It is well known that algal collections are essential for research purposes, to guarantee the reproducibility of the obtained results for the corresponding biotechnological applications (Malavasi and Cao 2015). Moreover, a great variety of disciplines is using algae as research material. The need for defined material has led to several important culture collections of algae around the world. Through the study of microbial cultures maintained in the culture collection, potential properties of microorganisms have been developed, and the future perspective of microbiology will be presumed. Effective research needs adequate and reliable sources of properly preserved cultures (Komagata 1999). With their main functions of preserving and providing algal resources, algal culture collections serve an essential infrastructural function for scientific investigation (Friedl and Lorenz 2012). Culture collections have the crucial role of providing the authenticated biological material upon which high-quality research is based. Importantly, they serve as repositories for strains as part of patent deposits, providers of safe and confidential services to store key organisms for research and industry, and sources of organisms cited in scientific papers that can be used in the confirmation of results and for further study (Smith 2003). In those culture collections, all strains are maintained in active growth in defined media. Cryopreservation in liquid nitrogen is being used for the most important strains. The conservation, management, and knowledge of all forms of biodiversity remain of vital importance to the well-being and functioning of our planet. Currently, little is known on a global scale of the extent of all types of biodiversity and specifically microbial diversity (Davison *et al.* 1999). Until recent times, identification of prokaryotic and eukaryotic microorganisms has been based on morphological features and in a few cases ecological features; the introduction of molecular techniques used for diversity studies has given a significant contribution to integrate the species identification knowledge in the taxonomical context. Through a "polyphasic" approach, some algal collection carry out phylogenetic analyses in order to study biogeography and distribution of different ecotypes of species (D'Elia *et al.* 2018). Thus, culture collections are an untapped resource, both in terms of the unique organisms available and the mostly untapped genetic resource they represent. Hopefully, the future researchers will make use of the collections as a source of new products for energy production. Moreover, culture collections have attempted to rationalize the number of media recipes and to standardize recipes for algal strain maintenance. For a full range of possible culture media, it is possible to refer to the catalog of strains from culture collections present all over the world (Barsanti and Gualtieri 2014).

5.2 Strain selection

Several microalgae species are either not suitable for mass cultivation or living in extreme habitats and cannot be identified well yet (Kose and Oncel 2017), consequently, microalgae are regarded as a hidden reservoir for biotechnology studies (Yu *et al.* 2015; Kose and Oncel 2017). The isolation and selection of microalgae capable of producing high biomass and high amounts valuable compounds, such as lipids, is a prerequisite for the successful industrial production (Thao *et al.* 2017; Duran *et al.* 2018). Typically sources of microalgae include existing collections of microalgae, commercially available either from Universities or other national and international foundations (such as the ones previously mentioned) or from companies specifically devoted to algae growth, or water and soil samples obtained from diverse environments (Mata *et al.* 2010). In particular, algae samples obtained in harsh environments such as thermal springs or industrial wastewaters may represent a viable option better adapted to specific conditions (Mata *et al.* 2010). Microalgae have great potential for oil production, not only for biodiesel but also for edible oils. Some microalgae produce abundant lipids and can be induced to produce even more by changing growth conditions, generally by nitrogen starvation strategies (Spolaore *et al.* 2006; Mata *et al.* 2010; Thao *et al.* 2017). The key in strains selection is to decide the capacity and aim of a commercial production facility, and should meet basic criteria common for all types of production: (*i*) low dark respiration rates; (*ii*) resistance to environmental conditions changes (temperature, nutrients input, light, competition of other microalgae species and/or bacterial tolerance, high O_2 levels); (*iii*) high lipid content; (*iv*) high growth rates and low nutrient requirements; (*v*) high photosynthetic activity; (*vi*) higher survival rates; (*vii*) ease of biomass separation and processing; (*viii*) genetic stability; (*ix*) predictable biochemical composition; and (*x*) well-known growth characteristics in the level of genomics and as a novel trend of metabolomics; (*xi*) possibility of obtaining other valuable chemicals (Mata *et al.* 2010; Kose and Oncel 2017). Thus, in the biofuel industry, for example, the areal productivity, oil production yield, and fatty acid composition should play a major role for the selection of the most-suited strain in order to prevent further losses required for downstream processes (Kose and Oncel 2017). At this regard, microalgae species like *Nannochloropsis* sp., *Chlorella* sp., *Botryococcus braunii*, *Scenedesmus* sp., *Neochloris oleoabundans*, *Crypthecodinium cohnii*, and *Tetraselmis suecica* are considered as a sustainable oil and biomass feedstock for large-scale microalgae cultivations mostly focusing on biodiesel production (Chisti 2007; Isleten-Hosoglu *et al.* 2012; Sun *et al.* 2014; Kose and Oncel 2017).

5.3 Carbon capture by microalgae

Cultivation of microalgae might be coupled with the direct bio-capture of CO_2 emitted by industrial activities that use fossil fuels for energy generation (Usui and Ikenouchi 1997; Francisco *et al.* 2010; Concas *et al.* 2014a). For this reason, microalgae can be seen as a new technology for carbon mitigation. Driven by the increasing concerns related to the effects of CO_2 levels on global warming, several technologies for carbon mitigation are currently being developed around the world. Among those, carbon capture coupled with geological storage (CCS) is considered the most realistically feasible and economically viable method for reducing CO_2 concentration in the atmosphere (Zhang and Sahinidis 2012; Concas *et al.* 2014a). However, CCS techniques are characterized by several limitations that might hinder their application at the real scale, whereby biological carbon capture through microalgae is today becoming a realistic alternative to CCS technologies as will be discussed later on.

5.4 Genomes

The development of genome sequencing technology has provided a powerful tool to investigate the interaction between genotypes and phenotypes of cells. Genome sequencing technology has grown to employ millions scientists worldwide in the passing years (Mardis 2008; Pham 2016) and the advance of high-throughput technologies in many 'omics' fields including transcriptomic, proteomic and metabolomics, research on putting all this genetic information into a system, and using it to model metabolic networks has become more and more common (Oberhardt *et al.* 2009; Triana and *et al.* 2014; Pham 2016). Fewer than 300 green plants and protists have had their genomes sequenced and published. The vast majority of the tree of life remains unexplored at the level of complete genomes. Much of eukaryotic microbial diversity remains unexplored and the number of genera and species is essentially unknown (Fig. 12). Many of the genomes to be sequenced are cornerstones for addressing important and longstanding questions in biology and evolution, while others represent unexplored potential for medicinal compounds and/or the discovery of high-value natural products (Cheng *et al.* 2018).

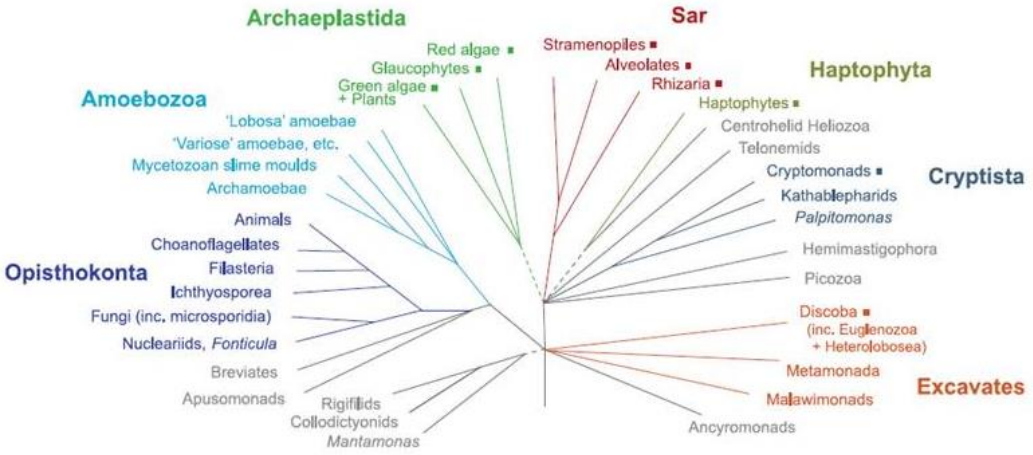

Fig. 12 Summary tree of eukaryotes. Schematic diagram shows the known or predicted relationships among the major eukaryotic groups, based on multi-gene analyses, featuring diverse eukaryotic microbes (algae and protists). Source Cheng *et al.* (2018).

Algae are also currently in the spotlight because of the need for developing a new source for sustainable energy. This is because many algae produce relatively large amounts of fatty acids, which could be extracted and converted to biodiesel (Amin 2009; Demirbas 2010). In this framework, the complete sequencing and characterization of mitochondrial (mtDNA) and chloroplast (cpDNA) genome of species like *Chlorella variabilis* NC64A (Orsini *et al.* 2016a) and *Chlorella sorokiniana* (Orsini *et al.* 2016b, c; Malavasi *et al.* 2017) may be relevant because both strains represent a promising feedstock for a variety of biotechnological applications which might involve the production of biofuels (Schenk *et al.* 2008; Concas *et al*. 2014b; Orsini *et al.* 2016a). In this regard, the knowledge of such genomes represents the first step towards the identification of suitable genetic engineering strategies aimed to increase their lipid productivity as well as a tool to further shed light on the evolution of the green algae lineage (Orsini *et al.* 2016a, b). The chloroplast genome, indeed, contains information that is applicable in many scientific fields, such as plant systematics, phylogenetic reconstruction, and biotechnology, because its features are highly conserved among species (Malavasi *et al.* 2017). Green algae have been used extensively in aquaculture, mainly for the production of secondary metabolites, such as β-carotene and astaxanthin (Hannon *et al.* 2010). The genomes of a number of green algae have been sequenced, and molecular tools are in place to transform both the chloroplast and nuclear genome of *Chlamydomonas reinhardtii* (Kindle *et al.* 1989; Boynton and Gillham 1993, 1996; Hannon *et al.* 2010). Currently, in several laboratories work is underway to transform plastids of additional species, while nuclear transformation has been achieved in a number of other green algae species, such as members of the

Chlorella and *Volvox* genera (Hawkins and Nakamura 1999; Lerche and Hallmann 2009). In terms of oil production, of the published algal species, members of the *Scenedesmus* genus have been identified as potential oil-producing species, with both rapid growth, as well as relatively high lipid content (Ahlgren *et al*. 1990; Rodolfi *et al.* 2009; Xin *et al.* 2009; Hannon *et al.* 2010). In addition, a number of species have been identified that produce interesting metabolites, such as *Botryococcus* spp. that produce the triterpenoid botryoccenes, a potential fuel molecule that requires minimal refining (Metzger and Largeau 2005; Hannon *et al.* 2010). In addition, genomic data from these species can be used as sources of new genes and, combined with screening for natural products, may illuminate new metabolic pathways, which may be useful for engineering other species.

6 MICROALGAE APPLICATION

The impacts of anthropogenic climate change are slow in coming, it is sometimes difficult to see the signal above natural variability, and impacts are coupled to some of the most basic needs of society, such as energy production and utilization, food security, and infrastructure (Ravishankara *et al.* 2015). Global energy demands are expected to increase between 17% and 50% as a consequence of on-going population and economic growth (IEA 2014; Correa *et al.* 2017). With this rapid consumption, the oil resources will be exhausted within 40 years (Shafiee and Topal 2009; Yellapu *et al.* 2018). To meet rising energy demands, the high consumption of fossil fuels is depleting available resources and increasing energy prices (Heinberg 2011; Chaudry *et al.* 2015). Extensive use of fossil fuel has caused high carbon dioxide (CO_2) emissions into the atmosphere and there is an urgent need to reduce its emission to avoid harmful impacts of global warming (Acheampong *et al.* 2017). Use of fossil fuel derived conventional fuels has been considered highly unsustainable due to limited reserves (Singh *et al.* 2017). To meet future energy demand without damaging the environment, fossil fuels should be replaced with some alternative energy sources that are environmentally friendly and sustainable (Chaudry *et al.* 2015). In this context, microalgae are a promising source to produce biomass due to several applications they can offer, such as bioremediation (Gressler *et al.* 2014; Raeesossadati *et al.* 2014; de Souza *et al.* 2018), biofuel production (Özçimen *et al.* 2012; Chernova and Kiseleva 2017; de Souza *et al*. 2018), as well as other valuable products such as carotenoids, phycobiliproteins and polyunsaturated acids (Spolaore *et al.* 2006; de Souza *et al.* 2018), among others (Koller *et al.* 2014; de Souza *et al.* 2018). Microalgae are photosynthetic microorganisms that can be found in all existing ecosystems (Mata *et al.* 2010; Thomassen *et al*. 2017). The thriving biodiversity enables microalgae to be especially applicable in a variety of fields, including aquaculture, food, pharmacy, as well as environmental

engineering (Pulz and Gross 2004; Chen *et al.* 2018). The estimated total amount of algal species is about 72500 (Guiry 2012; Thomassen *et al*. 2017). However, only approximately 15 species of microalgae are currently used on a commercial level, therefore, microalgae are still considered as an untapped resource for a bio-based economy (Thomassen *et al.* 2017).

6.1 Biofuel from microalgae

Global energy demands are expected to increase as a consequence of ongoing population and economic growth (Chaudry *et al*. 2015; Acheampong *et al.* 2017; Correa *et al.* 2017). Meeting these demands under current levels of fossil fuels exploitation is depleting available resources and increasing energy prices (Chaudry *et al.* 2015). Furthermore, a high rate of fossil fuel use is responsible for increasing atmospheric CO_2, which is a major climate change gas and one of the main causes of global warming (IEA. 2012; Chaudry *et al.* 2015; Acheampong *et al.* 2017; Singh *et al.* 2017). Moreover, the estimation of the world crude oil reserves is a difficult mission because it is affected by political, economic and technological factors and finding new energy resources to compensate the decrease of the global petroleum reserves is a significant challenge (Pirog 2005; Faried *et al.* 2017). To meet future energy demand without damaging the environment, fossil fuels should be replaced with some alternative energy sources that are environmentally friendly and sustainable (Chaudry *et al.* 2015). In this context, the use of biofuels presents an attractive potential and has a stronger expansion in comparison to other alternatives (Carneiro *et al.* 2017). Havlík *et al.* (2011) stated that the interest in developing biofuel first arose after the oil crisis in the 1970's and now, the declining cost of production due to subsidies provided by national governments, and the continued swelling up of oil prices are contributing to its competitiveness (Acheampong *et al.* 2017). Biofuels are defined as high-density energy carriers derived from biomass transformation could be a sustainable alternative to replace fossil fuels (Goldemberg 2006; Panwar *et al.* 2011; Correa *et al.* 2017). Biodiesel is the mono-alkyl esters of long chain fatty acids derived from animal fats or waste cooking oil or vegetable oils. In addition, it is readily available, renewable, non-flammable, non-toxic and eco-friendly (Concas *et al.* 2014a). There are some advantages of biodiesel which have been highlighted such as higher flash point, biodegradability, improved certain number and reduced exhaust emissions (Faried *et al.* 2017). Biofuels produced renewable biomass sources, with zero net CO_2 emission, has the potential for mitigating global warming problems since all the CO_2 emitted during their burning can be fixed by plants used as biomass feedstock through photosynthetic mechanisms (Concas *et al.* 2014a; Saber *et al.* 2016). Nonetheless, it has become a major controversy because of the competition between food and fuel

(Carneiro *et al.* 2017; Faried *et al.* 2017). Biofuels can be produced via various renewable feedstocks. Currently, they are driven by biological material from plants, microorganisms, animals, and wastes (Acheampong *et al.* 2017). Based on the source and production technology, biofuels are classified into the first, second, third and the fourth generation biofuels (Acheampong *et al.* 2017) (Fig. 13). However, first and second generation biofuels are characterized by several drawbacks which can limit their exploitation as an alternative source of energy (Concas *et al.* 2014a).

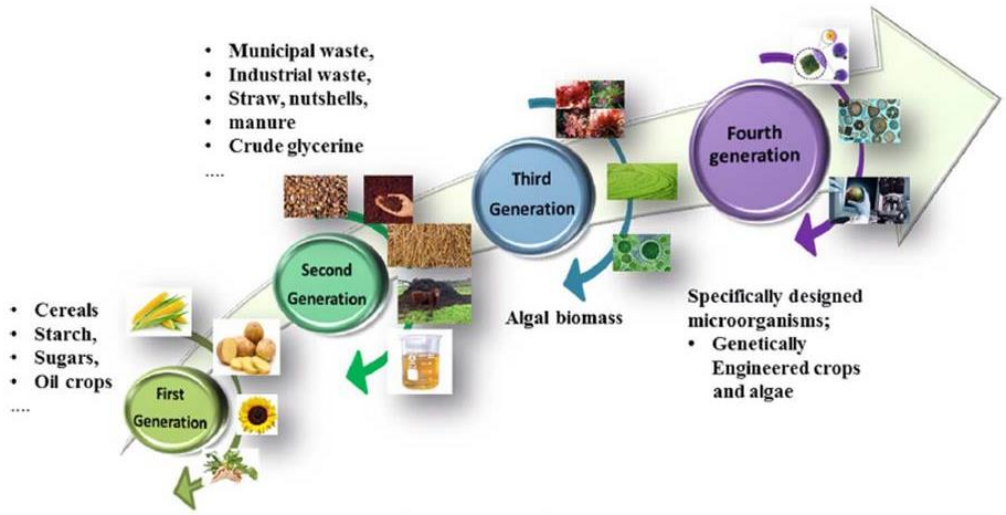

Fig. 13 Generations of biofuel production. Source Acheampong *et al.* (2017).

First generation biofuels - are mainly produced in industrial scale by the conversion of terrestrial crops (for example, sugarcane, sugar beet, wheat, corn, rapeseed, sunflower, soybean, palm oil, cereals, etc.) into ethanol and biodiesel (Concas *et al.* 2014a; Acheampong *et al.* 2017; Carneiro *et al*. 2017; Rodionova *et al.* 2017). These forms of biofuels have reached the most advanced forms in biofuel production and are commercially available (Acheampong *et al.* 2017). As a matter of fact, oil palm and sugarcane is said to account for more than 90% of total biofuel feedstock investments in tropical countries since the year 2000 (Popp *et al.* 2014; Acheampong *et al.* 2017). The industrial processes which transform the biomass feedstock into biofuel clearly depend upon the starting feedstock as well as the desired biofuel (Naik *et al.* 2010; Concas *et al.* 2014a). Nowadays, the most widespread processes for converting biomass into first-generation biofuels are fermentation for producing ethanol, anaerobic digestion to obtain biogas, oil extraction followed by trans-esterification for the production of bio-diesel and pyrolysis for converting biomass into useful bio-oil (Naik *et al.* 2010; Concas *et al.* 2014a). Moreover, physical methods are also extensively used for transforming raw biomass into briquettes or pellets to be used for feeding domestic boilers (Concas *et al.* 2014a). Nevertheless, a large debate is associated to their production. The main concerns about first-generation biofuels deal with the impacts on deforestation in the tropics, which

contribute to enhance problems such as soil erosion, loss of habitat and reduction of valuable biodiversity (Concas *et al.* 2014a); the use of arable land and fresh water required for growing food crops (Searchinger *et al.* 2008; Chaudry *et al.* 2015); the competition with food chain that can induce a risk of prices rise which mainly impacts on the most vulnerable regions of the world and make more difficult their social acceptation (Concas *et al.* 2014a; Acheampong *et al.* 2017; Carneiro *et al.* 2017). Moreover, many life-cycle based studies showed a slight decrease of greenhouse gases (GHG) emissions and/or a low net energy output (Benoist *et al.* 2012; Carneiro *et al.* 2017). Other studies stated that instead of an expected decrease of 20% in GHG emissions from biofuels, land use change and fertilizer application that accompanies energy cropping will rather double emissions over the next 30 years (Senauer 2008; Acheampong *et al.* 2017).

Second generation biofuels - The food vs. fuel competition triggered by first-generation biofuels has stimulated a greater interest for the development of biofuels produced from non-food biomass, commonly referred to as second-generation biofuels (Timilsina *et al.* 2010; Concas *et al.* 2014a). Second generation biofuel technologies utilize more flexible and energy-efficient lignocellulosic feedstock such as biomass from agricultural and forest residues and wastes as well as feedstock such as trees, jatropha, straw, bagasse, and purpose energy crops grown on marginal lands (Senauer 2008; Buyx and Tait 2011; Pedroli *et al.* 2013; Acheampong *et al.* 2017), that do not disturb the food supply directly (Antizar-Ladislao and Turrion-Gomez 2008; Chaudry *et al.* 2015). In fact, most targeted energy crops can utilize low quality land, which is not suitable for food crops (Naik *et al.* 2010; Chaudry *et al.* 2015). These feedstocks are converted into ethanol and methanol (Buyx and Tait 2011; Acheampong *et al.* 2017). Unlike the first generation biofuels, this type of biofuels do not only utilize the grains, sugars or fats but the entire plant which means energy yields per hectare of land can be much higher (Senauer 2008; Acheampong *et al.* 2017). Dedicated energy crops for second-generation biofuels are facing many challenges, such as, high cost, low energy density, and high water and nutrient requirements (Sims *et al.* 2006; Chaudry *et al.* 2015), and are still in the research and development stage (Naik *et al.* 2010; Chaudry *et al.* 2015).

Third generation biofuels: Microalgae - microalgae are globally known as the third-generation feedstock and are excellent candidates for renewable energy source due to their ability to produce various bio-products, such as biofuel and bio-hydrogen (Japar *et al.* 2017). Depending on species and cultivation method microalgae can produce biohydrogen, biomethanol, bioethanol, biodiesel, or carbohydrates, proteins or other compounds that are being used in pharmaceutical companies (Carlsson *et al.* 2007; Chisti 2007; Rodionova *et al.* 2017). The algal-derived biofuels production

requires only sunlight, CO_2 and water and generates multiple renewable energy products (Rodionova *et al.* 2017). Microalgae have received much interest as a biofuel feedstock in response to the uprising energy crisis, climate change and depletion of natural sources (Chew *et al.* 2017). The algae are cultured to act as high-energy and entirely renewable feedstock produced at low cost (Chisti 2007; Acheampong *et al.* 2017). Unlike terrestrial feed-stocks such as soybean, rapeseed, jatropha, etc., microalgae have been projected with various advantages as (*i*) algae cultivation does not need arable land and might be coupled with the direct bio-capture of CO_2 emitted by industrial activities, (*ii*) higher photosynthetic rate than terrestrial plants, (*iii*) microalgal oil yield could significantly exceed the yield of the best oilseed, (*iv*) can make use of brackish or seawater (Concas *et al.* 2014a; Chaudry *et al.* 2015; Mallick *et al.* 2016; Correa *et al.* 2017). Algal-based biofuels production is about hundred times higher than that of higher plants (Chisti 2008; Rodionova *et al.* 2017). However, they are limited in commercial availability and are currently under broad research (Acheampong *et al.* 2017). Acheampong et al. (2017) describes a fourth generation of biofuels, that are expected to bring essential advances in the biofuels field (Lu et al. 2011). They are seen as carbon negative rather than just carbon neutral through the capture and storage of carbon. This technology is an emerging field based on direct conversion of solar energy into fuel using raw materials. Revolutionary developments in synthetic and system biology approaches will be vital for further development. Based on current knowledge, the usage of microalgae is being considered as an attractive feedstock for biofuels production (Concas *et al.* 2014a; Rodionova *et al.* 2017) capable of meeting the international request for the fuel of transportation, with the possibility of the complete replacement of the use of fossil fuels and move to the use of biodiesel (Chisti 2007; Patil *et al.* 2008; Faried *et al.* 2017). From a conceptual point of view the process shown in Fig. 14 can be carried out for producing biofuels and capturing CO_2 through microalgae (Concas *et al.* 2014a).

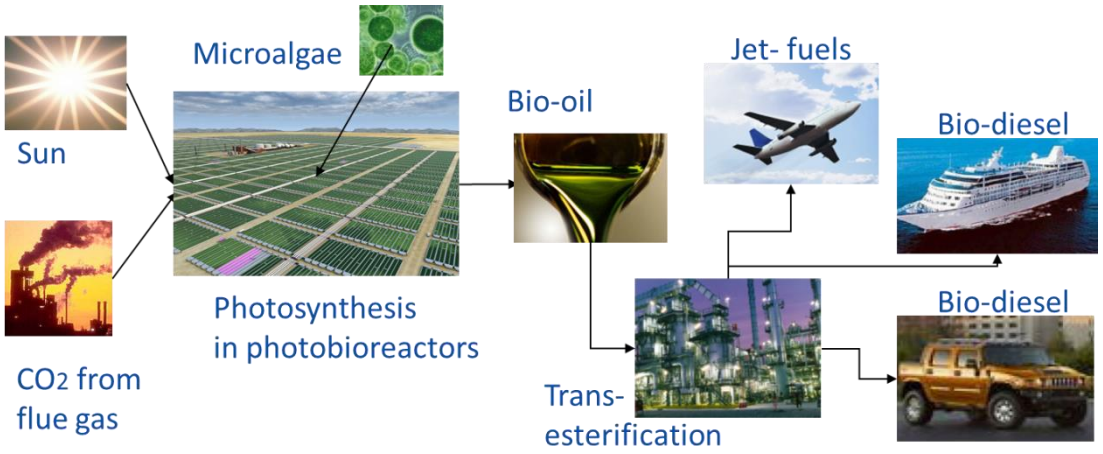

Fig. 14 Conceptual scheme for the production of biofuels and CO_2 capture through microalgae. Source Concas *et al.* (2014a).

Although microalgal biomass is considered as the next generation of feedstock for biofuel production and despite the apparent simplicity of the process, the commercial production of microalgal fuel is still in the research and development stage, and no commercial facility is producing this yet since it is not economically feasible and sustainable (Markou and Nerantzis 2013; Chaudry *et al.* 2015). Its implementation to the industrial scale is still not widespread since it is characterized by technical and economic constraints that might hinder its full scale-up (Concas *et al.* 2014a). Many studies have focused on identifying the bottlenecks and making the process economical and energetically feasible (Chaudry *et al.* 2015). Microalgae are microorganisms living essentially in liquid environments, and thus with particular cultivation, harvesting, and processing techniques that ought to be considered in order to efficiently produce biodiesel. Among the main disadvantages of the process, there is the dependence on light incidence and penetration into the aquatic environment to ensure high biomass production and the difficulty of developing simple and inexpensive procedures to convert lipids into biodiesel. Thus, the cultivation of microalgae for biodiesel production should involve not only the selection and production of species, assessment of types and amount of lipids, but also the potential market for co-products (D'Alessandro and Antoniosi Filho 2016). To promote the sustainability and economic feasibility of this process, an innovative microalgae biorefinery structure might be implemented through the production of multiple products in the form of high-value products and biofuel (Chew *et al.* 2017).

6.2 Human and animal nutrition

The use of microalgae by humans has been reported since ancient times when edible cyanobacteria species such as *Nostoc* and *Arthrospira* (Spirulina) had been used for food (Spolaore *et al.* 2006; Colla *et al.* 2007; Furmaniak *et al*. 2017). However, it is in the early 1950's that the increase in the world's population and predictions of an insufficient protein supply led to the search for new alternative and unconventional protein sources (Spolaore *et al.* 2006). The first commercial large-scale culture of microalgae started in the 1960's-1970's with the cultures of *Chlorella* and *Arthrospira* in Japan and Mexico respectively (Borowitzka 1999; Iwamoto 2004; Spolaore *et al.* 2006). Microalgal biomass is of interest for human nutrition due to its high protein content and progressively applied as dietary or "health food" (Koller *et al.* 2014). One of the main advantages of using microalgae in nutrition is the possibility to offer several compounds of interest simultaneously. In addition to their high protein content, microalgae present fatty acids, a favorable amino acid content, pigments and vitamins that are essential for use in the feed industry and to add value to milk (MacPherson *et al.* 2015; Ibekwe *et al.* 2017; de Souza *et al.* 2018). Furthermore, microalgal bioproducts showed the ability to improve nutrition due to probiotic effects that positively affects human and animal health, and to increase product shelf life acting as a source of natural dyes in foods (de Souza *et al.* 2018). Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs (eg. oysters, scallops, clams and mussels), for the juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food chains for their good nutritional properties as monospecies or within a mixed diet (Sirakov *et al.* 2015).

6.3 Pharmacy and cosmetology

The use of microalgae in pharmacology and cosmetology has also attracted considerable attention due to their beneficial effects on human health (de Souza *et al.* 2018). Among the most important substances in the pharmaceutical industry there is 1,3-β-Glucan obtained from *Chlorella* that acts as an active immunostimulator, eliminates free radicals and reduces blood lipids (de Souza *et al.* 2018). In addition, *Chlorella* promotes health against gastric ulcers, wounds, and constipation, prevents atherosclerosis and showed an antitumoral action (Spolaore *et al.* 2006; de Souza *et al.* 2018). Omega-3 fatty acids and pigments produced by microalgae are the major types of compounds that can be sold in pharmaceutical and cosmetic markets (de Souza *et al.* 2018). A number of review articles have been published that highlight microalgae as effective antimicrobial

producers (Plaza *et al.* 2010; Najdenski *et al.* 2013; Senhorinho *et al.* 2015; Navarro *et al.* 2017). In a few cases, the antibacterial compounds have been identified, and these include fatty acids (Findlay and Patil 1984; Ohta *et al.* 1994; Desboi *et al.* 2009; Jha *et al.* 2017). Bajpai *et al.* (2018) reported that more than 50% of marine cyanobacteria are potentially exploitable for the extraction of bioactive substances, which are effective in killing cancer cells by inducing apoptotic death. The extracts of microalgae show antimicrobial, antiviral, and antifungal properties while the products of *Chlorella* sp. and *Spirulina* sp. are also used as ingredients of different skin care, sun protection, and hair care formulations (Jha *et al.* 2017). The production of cosmetics containing microalgae combined with other antioxidants and/or bioactive compounds for skin protection from sun damage is considered to be a growing field of study (Ariede *et al.* 2017). Microalgae extracts can be also found in face and skin care products such as anti-aging cream, refreshing or regenerant care products, emollient and as an anti-irritant in peelers (Spolaore *et al.* 2006). Other valuable compounds produced by microalgae are polyhydroxyalkanoates (PHAs), semi-crystalline polyesters synthesized and stored in microbial cells, that are considered as good alternative for petroleum-derived synthetic plastics, and may have relevant applications in various fields such as food industry, agriculture, pharmaceuticals, and medicine (Costa *et al.* 2018) and mycosporine-like amino acid (MAA), which act as natural sunscreens (Hartmann *et al.* 2016).

6.4 Direct carbon bio-fixation by microalgae

Current carbon capture and sequestration (CCS) technology are basically characterized by a first step, called capture, where the CO_2 is separated from the other constituents of the flue gas emitted by a point source such as a power station or a generic CO_2-emitting facility. The separated CO_2 is then compressed and transported (usually through pipelines) to the storage site. Finally, in the sequestration step, compressed CO_2 is injected into deep geological formations, such as saline aquifers, depleted oil/gas reservoirs, or deep un-mineable coal seams (Zhang and Sahinidis 2012; Concas *et al.* 2014a). In an alternative version, storage can be carried out by injecting CO_2 in the bottom of oceans. However, both the steps of capture and of sequestration are characterized by specific concerns which can limit the application at the industrial scale of the CCS technologies. A simplified scheme of the CCS technology is shown in Fig. 15.

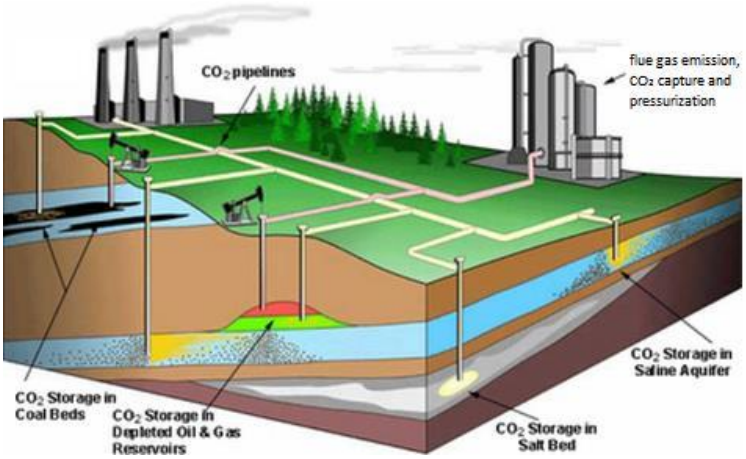

Fig. 15 Conceptual scheme of carbon capture and storage. Source Concas *et al.* (2014a).

The chemical-physical capture technologies fundamentally include the chemical absorption, adsorption onto solid materials, membrane-based technology and cryo-fractionation (cf. Fig. 16). The adsorption techniques are based on the ability of some porous solids (adsorbents) to selectively bind the molecules of CO_2 in the flue gas by exploiting van der Walls forces (physical adsorption) or real chemical bonds (chemical adsorption). The most important adsorbents are activated carbon, zeolite, silica gel, and aluminum oxide etc. The main drawbacks of this technique are the need to remove moisture and contaminants from flue gas, that can reduce the CO_2 recovery from 75% to 60% (Li *et al.* 2008a, b; Concas *et al.* 2014a). The membrane technologies are based on the specific permeability and selectivity of polymeric membranes which, working like a filter, allows the separation of CO_2 from the other constituents of the flue gas. The main limitations of membrane technologies are related to high manufacturing cost, the fouling effect as well as the high membrane surface area that is needed for the treating (Lam *et al.* 2012; Concas *et al.* 2014a). Finally, the cryogenic fractionation technology exploits the difference in boiling points of the various gas species to separate CO_2. However, the cryogenic method is high energy consuming and is strongly affected by moisture of flue gas (Tuinier *et al.* 2010; Concas *et al.* 2014a).

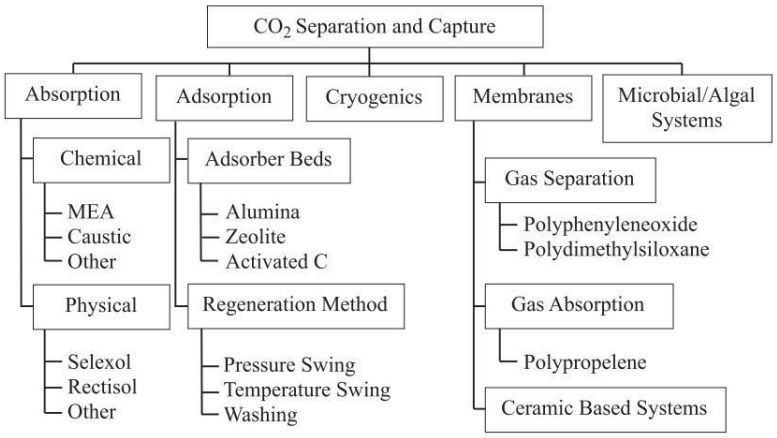

Fig. 16 Chemical-physical methods for carbon capture. Source Concas *et al.* (2014a).

Once captured and concentrated, carbon dioxide must be permanently stored. Geological carbon sequestration, that is currently seen as the only realistic method for CO_2 mitigation, consists in the injection of CO_2 into deep rocks, aquifers or oceans. The major issue related to this approach is the potential triggering of seismicity phenomena owing to the large-scale pressurization resulting from CO_2 injection (Cappa and Rutqvist 2011; Concas *et al.* 2014a). Moreover, when stored into aquifers, CO_2 can cause the acidification of the water body thus rising important risks for the potential users of the acidified groundwater (Wilkin and DiGiulio 2010). Finally, ocean sequestration is based on the fact that at great depths, CO_2 is denser than seawater and hence it may be theoretically stored on the bottom as liquid or deposits of icy hydrates. However, also, in this case, the concerns are related with water acidification that negatively affects all the ecosystems and the biogeochemical cycles hosted by deep sea (Yamada *et al.* 2010; Concas *et al.* 2014a). Since the carbon capture and sequestration (CCS) technologies may give rise to significant concerns which may affect their application to the large scale, the potential exploitation of microalgae as a method for the CO_2 capture and bio-fixation is receiving a rising interest (Olguin 2003; Mulbry *et al.* 2008; Concas *et al.* 2014a). In fact, when compared to CCS technologies, biological carbon mitigation through microalgae shows a number of potential advantages. Among the last ones, there is the possibility of performing CO_2 capture and fixation in well-controlled photobioreactors. Moreover, biological CO_2 fixation techniques doesn't use hazardous chemicals, doesn't affect the environment, can exploit directly flue gases evading the costs of CO_2 separation/capture, and finally leads to the production of valuable products such as biofuels, food and fine chemicals (Farrelly *et al.* 2013). Microalgal photosynthesis is capable to exploit CO_2 in both atmosphere and flue gases. The CO_2 uptake capacity of microalgae is ten times higher than terrestrial plant. Furthermore, they

can accumulate inorganic carbon in their cytoplasm to concentrations several orders of magnitude higher than that on the outside, phenomenon called CO_2-concentrating (Pires *et al.* 2012). The use of flue gases as source of CO_2 can theoretically result in positive effects on microalgae growth. In fact, being equal the gas flow rates, the carbon provided by flue gases to microalgae is much higher than the one supplied by air. This could lead to higher biomass and oil productivities that are critical to ensure a sustainable production of biofuels through microalgae. Several advantages might derive from the exploitation of costless flue gases as carbon source for growing microalgae and producing biofuels. More specifically, reduction of greenhouse gas (GHG) emission, the increase of biomass and lipid productivity as well as the reduction of operating costs are the main advantages which potentially derive from the use of flue gas as carbon source. Moreover, high concentration of CO_2 in the flue gas increases its mass transfer from the gas phase to the medium, being the last one a limiting step of CO_2 fixation by microalgae. For these reasons, the potential exploitation of microalgae for the CO_2 capture from flue gases and the subsequent production of liquid biofuels is currently the subject of a continuously growing number of studies reported in literature. Most of these works shows that using simulated flue gases (15 %v/v of CO_2) has a positive effect towards carbon fixation rate and biomass productivity (Ho *et al.* 2010; Tang *et al.* 2011; Lam *et al.* 2012; Concas *et al.* 2014a). In particular, growth rate of microalgae has been improved due to the feeding of gases having a concentration of CO_2 (1–15%) higher than the typical one of atmospheric air (0.04% CO_2) thus leading to a higher biomass productivity as well as shorter cultivation time (Lam *et a*l. 2012). Moreover, it is noteworthy that, under specific cultivating conditions, the feeding of CO_2 concentrations of 10-15%v/v led also to the increase of the lipid content of microalgal cells even if such increment was limited to 1-6% wt/wt (Tang *et al.* 2011). Finally, it is found that the increase of CO_2 concentration in the gas fed to microalgae can provoke the accumulation of polyunsaturated fatty acids which are a viable form of lipids since they can reduce the pour point of the resulting biodiesel (Tang *et al.* 2011), and can be also commercialized for pharmaceutical and therapeutic applications, yielding much higher prices than after converting the lipids to biofuels.

6.5 Bioremediation

Beside the biological sequestering of CO_2 by living algal cells for abatement of greenhouse gases affecting our planet, microalgae are considered as potential candidates to readily remove various heavy metals (lead, chromium, cadmium and others) from diverse aquatic environments, especially from industrial wastewater, by "bioleaching" (Balaji *et al.* 2014; Koller *et al.* 2014). Microalgae have been proposed as an option for wastewater treatment since the 1960s (Acién *et al.* 2016). Yoshida *et al.* (2009) demonstrated the successful elimination of formaldehyde, an organic pollutant severely toxic for skin, eyes and the respiratory system, by the marine microalga *Nannochloropsis oculata* ST-3, a representative of the Chlorophyta. Phosphorus, nitrogen and emerging micropollutants such as pharmaceuticals and person-care products are causing special concern for the eutrophication of water bodies. Using wastewater as a source of nitrogen and phosphorus represents an attractive option to cultivate microalgae simultaneous with contaminant removal (Cuellar-Bermudez *et al.* 2017). Phycoremediation refers to the assimilation or disintegration of organic and inorganic compounds (carbon, nitrogen, or phosphorus), metals, and emerging contaminants in wastewater by microalgae and cyanobacteria. In addition, added value comes when the microalgae are harvested to become feedstock for biofuels such as biogas (Cuellar-Bermudez *et al.* 2017; Acién *et al.* 2016). A combination of selective acidophilic and non-acidophilic microalgae together with bacteria, all in the form of biofilms, has been proposed as effective treatment for bioremediation of metal-contaminated waters (Abinandan *et al.* 2018). Moreover, a microalgae mediated bioremediation of pharmaceutical contaminants (PCs) such as ibuprofen, caffeine, carbamazepine, has recently gained scientific attention, as microalgal bioremediation is a solar-power driven, ecologically comprehensive, and sustainable reclamation strategy (Xiong *et al.* 2018). Safafar *et al.* (2015) discussed the possibility of combining industrial wastewater treatment and algae cultivation as a feasible, environmentally-friendly approach for sustainable production of algae-based bioactive compounds such as carotenoids, phenols, and tocopherols. By combining the application of available wastewater bodies or hydrolysis products of organic rejects with the utilization of CO_2 stemming from industrial effluent gases, ample amounts of suitable raw materials would be available for cultivation of microalgae and formation of high-value products. This resulting abatement of CO_2 might contribute to meet the agreed global goals for climate protection (Koller *et al.* 2014).

6.6 Market potential of microalgal products

Currently, microalgae are recognized by several authors as promising "bio-catalysts" or "cell-factories" for the production of a variety of highly valuable products and fine chemicals with application in numerous industries such as foods, feeds, cosmetic, etc. in the framework of the "White Biotechnology" (Fig. 17) (Olaizola 2003; Spolaore *et al.* 2006; Chisti 2007; Koller *et al.* 2014). In general, products from microalgae can be classified as pigments (e.g. β-carotene, chlorophyll, astaxanthin, lutein), protein, carbohydrate (such as agar, carrageenan, alginate, fucoidan), as nutritional supplements and lipid (e.g. polyunsaturated fatty acids, omega-3). Recently, microalgae have also been identified as potential producers of biopolymers, bio-plastics and natural sunscreens (Llewellyn and Airs 2010; Kavitha *et al.* 2018; Khanra *et al.* 2018). In what follows, the numerous high valuable products extractable from microalgae as well as their commercial applications are described.

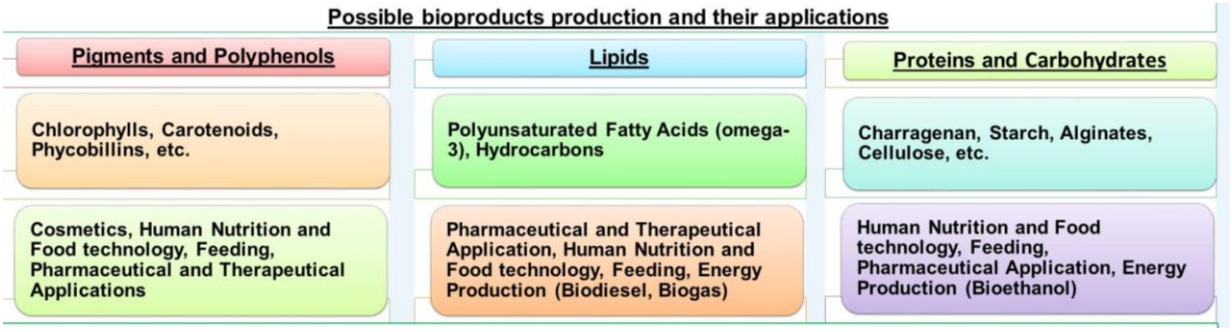

Fig. 17 Main high-value products obtained from microalgal biomass, and major application fields.

Pigments - According to their high market values, pigments are considered as the algal products of highest potential for commercial success in a not too distant future (Koller *et al.* 2014). Natural pigments have an important role in the photosynthetic metabolism and pigmentation in algae, and may also have beneficial biological activities, such as antioxidant, anticancer, anti-inflammatory, anti-obesity, antiangiogenic, and neuroprotective (D'Alessandro and Antoniosi Filho 2016).

Three major groups of pigments are found in microalgae, namely carotenoids (among them, carotenes provide an orange coloration, whereas xanthophylls are responsible for yellowish shade), phycobilins (red or blue coloration), and chlorophylls (green coloration). The pigment fraction of algae can be applied as nutrient supply due to their high contents of pro-vitamin A and vitamin E and for other pharmaceutical, veterinary and medical purposes (anti-inflammatory effects, anti-

oxidative effect, cancer prevention), as well as in cosmetic industry and food technology. Additionally, ß-carotene and lutein are needed for poultry feeding because of its importance for the yellow-orange coloration of egg yolk (Koller *et al.* 2014). Carotenoids (Fig. 18) are lipophilic compounds, usually colored yellow, orange or red and are the most diverse and widespread pigments found in nature (Sasso *et al.* 2012; Varela *et al.* 2015; Gong and Bassi 2016). Most carotenoids share a common C40 backbone structure of isoprene units (termed terpenoid) (Fig. 18) and are divided into two groups: carotenes and xanthophylls, which are relatively hydrophilic oxygenated derivatives of carotenes (Gong and Bassi 2016). Carotenoids perform two roles: absorbing light and quenching excess energy in photosynthetic metabolism (D'Alessandro and Antoniosi Filho 2016; Gong and Bassi 2016). Primary carotenoids like lutein serve as accessory pigments which can transfer absorbed energy to chlorophylls (Gong and Bassi 2016). These pigments absorb electromagnetic energy in spectral ranges where chlorophylls are not able to absorb light energy, (400 - 500 nm) (Koller *et al.* 2014). Secondary carotenoids like astaxanthin and canthaxanthin play a role in cell protective mechanisms (Gong and Bassi 2016). Carotenoids display high antioxidants potential, protecting algal cells against excessive solar radiation and free radicals (Koller *et al.* 2014; Gong and Bassi 2016). These antioxidants properties may also help human metabolism to prevent the negative impacts caused by free radicals (Koller *et al.* 2014). Furthermore, carotenoids are important nutraceuticals because of their known beneficial effects including anti-oxidant, anti-aging, anti-inflammatory, anti-angiogenic, cardioprotective and hepato-protective properties (Hu *et al.* 2018). The main carotenoids of microalgae are β-carotene, lycopene, astaxanthin, zeaxanthin, violaxanthin, and lutein. Among the latter ones, β-carotene, lutein, and astaxanthin are the most studied due to their relevance as "functional food" products (D'Alessandro and Antoniosi Filho 2016). The primary industrial source of β-carotene is the microalgae *Dunaliella salina*, which produces β-carotene above14%dry weight (Spolaore *et al.* 2006; D'Alessandro and Antoniosi Filho 2016). The anti-oxidant property of carotenoids, in general, may protect humans from compromised immune response, premature aging, some kind of cancers, cardiovascular diseases, and/or arthritis. Anti-oxidant pigments are also frequently reported to reduce the risks of AIDS, diabetes, cataract, macular degeneration, and neuro-degeneration (Varela *et al.* 2015; Gong and Bassi 2016). In particular, β-carotene is a orange-yellowish pigment with growing demand, being used as colorant for food or nutritional supple-ment, because it is a precursor of vitamin A (retinol) (Edge *et al.* 1997; Grune *et al.* 2010; D'Alessandro and Antoniosi Filho 2016), astaxanthin produced in large concentrations (8% per dry weight) by *Haematococcus pluvialis* (D'Alessandro and Antoniosi Filho 2016), is considered the strongest anti-oxidant in carotenoids, with primary

applications in aquaculture and dietary supplements it has effects of anti-aging, anti-inflammatory, sun proofing, and immune system boosting (Li *et al.* 2011; Koller *et al.* 2014; Gong and Bassi 2016). Two other bio-products, i.e., lutein and zeaxanthin are, becoming increasingly important in the nutraceutical market for their significant role in eye health (Manayi *et al.*2015). In particular, lutein and zeaxanthin demonstrated antioxidant and anti-inflammatory capabilities, reducing stress, cortisol, and symptoms of sub-optimal emotional and physical health (Stringham *et al.* 2018), while lycopene was marketed as an anti-oxidant and was proposed for treatment of cardiovascular diseases and prostate cancer (Gong and Bassi 2016).

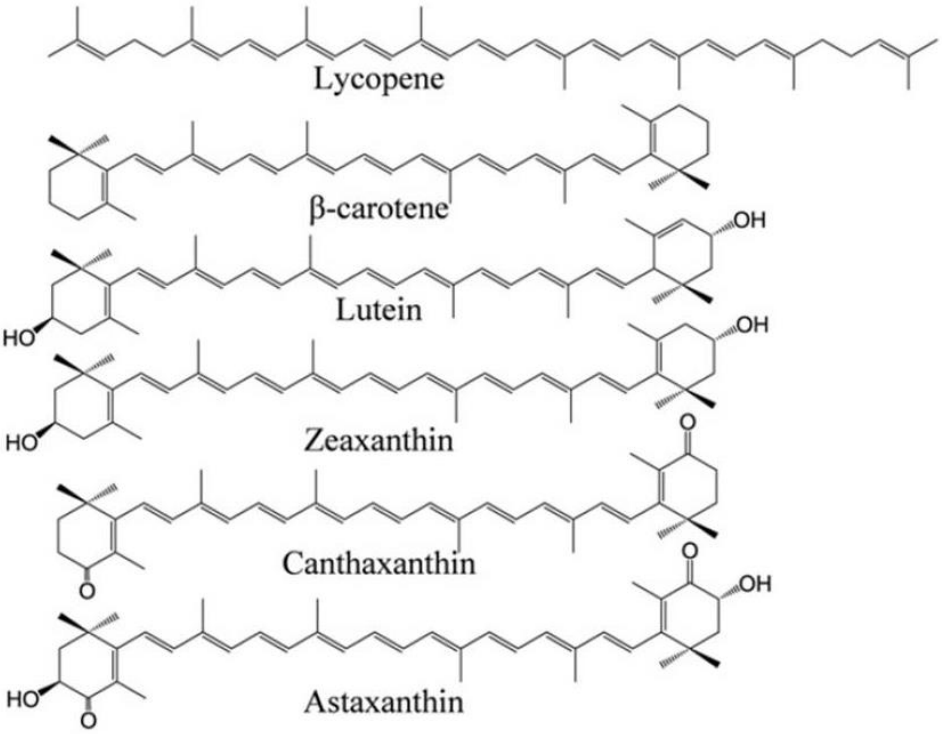

Fig. 18 Chemical structure of some common carotenoids found in microalgae. Source Gong and Bassi (2016).

Phycobilins or phycobiliproteins - are soluble accessory pigments that collect light during photosynthesis and are mainly found in cyanobacteria, Rhodophyta (red algae), Glaucophytes and some cryptomonads, while green algae including all Chlorophyta are not natural producers of these chromophores (Koller *et al.* 2014; D'Alessandro and Antoniosi Filho 2016). In cyanobacteria and red algae, the phycobiliproteins are organized in supramolecular complexes, called phycobilisomes which are assembled in regular arrays on the outer surface of the thylakoid membranes (MacColl *et al.* 1999; Zhao and Qin 2006; Sekar and Chandramohan 2008; Mishra *et al.* 2010; Lage-Yusty *et al.* 2013). Chemically, constituted by open-chain tetrapyrroles, phycocyanin is a blue pigment primarily found in cyanobacteria. Phycoerythrin is a pigment occurring in Cyanobacteria,

Rhodopyta, and Cryptophyta, and is responsible for the characteristic red coloration (Fig. 19) (Koller *et al.* 2014). Due to their absorption properties, phycobilins are widely used in industry, in immunology laboratories and in molecular biology where are often employed in as fluorescent markers for immunoassays and as fluorescent dyes for microscopy (Koller *et al.* 2014; D'Alessandro and Antoniosi Filho 2016). Besides these applications as chemical tags, phycobilins are also used as food colorants and in cosmetics due to their high coloration effects (Arad and Yaron 1992; Koller *et al.* 2014).

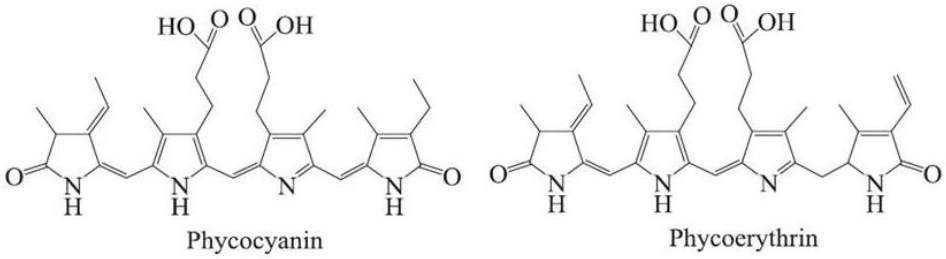

Fig. 19 Phycobilin structures. Source D'Alessandro and Antoniosi Filho (2016).

Chlorophylls - are the most prominent fat-soluble algal pigments, with a porphyrin ring in their structure. They are responsible for converting solar energy into chemical energy in photosynthesis, absorb light mainly in the blue zone and, to a minor extent, in the red zone of the electromagnetic spectrum causing the typical and well-known green coloration of the green algae (Chlorophyta) (Koller *et al.* 2014; D'Alessandro and Antoniosi Filho 2016). These pigments are approved as additive (E140) of a variety of foodstuffs and beverages (Koller *et al.* 2014). A derivative of chlorophyll, the chlorophyllin, demonstrated its high effectiveness as a chemopreventive agent against colon cancer cells if provided as dietary supplement (Chimploy *et al.* 2009; Du *et al.* 2014; Koller *et al.* 2014; D'Alessandro and Antoniosi Filho 2016), while due to the high deodorant capacity, both chlorophyll a and chlorophyllin may also be used as deodorants in items of personal hygiene (Koller *et al.* 2014; D'Alessandro and Antoniosi Filho 2016).

Polyphenols - The term includes more than 8000 compounds with great diversity in structure, that are recognized as important natural antioxidants (Safafar *et al.* 2015). Chemically, polyphenols can be divided into several classes, such as phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids), flavonoids (flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanins), isoflavonoids (isoflavones, coumestans), stilbenes, lignans, and phenolic polymers (proanthocyanidins—condensed tannins and hydrolysable tannins) (Machu *et al.* 2015). Polyphenols act as antioxidant through single electron transfer and through hydrogen atom transfer

and are considered as one of the most important classes of natural antioxidants (Machu *et al.* 2015; Safafar *et al.* 2015). Recent studies found several classes of flavonoids, such as isoflavones, flavanones, flavonols, and dihydrochalcones in microalgae, but only few publications regarded the identification and quantification of phenolic composition in microalgae species (Safafar *et al.* 2015). The content of total phenols of several microalgae has been reported to vary from 12.48±0.09 acid in *Fischerella ambigua* to 19.46±1.95 mg eq. of gallic in *Chlorella vulgaris* (Hajimahmoodi *et al.* 2010; Esquivel-Hernández *et al.* 2017). Among species recently investigated for their phenolic content and antioxidant capacity there are: *Tetraselmis suecica*, *Botryococcus braunii*, *Neochloris oleoabundans*, *Isochrysis* sp., *Chlorella vulgaris*, and *Phaeodactylum tricornutum* which showed the highest antioxidant capacities and, thus, could be potential new sources of natural antioxidants (Goiris *et al.* 2014; Safafar *et al.* 2015). Phenolic compounds have been studied for their therapeutic properties that include anticancer, antioxidative, antibacterial, anti-allergic, anti-diabetes, anti-aging, anti-inflammatory and anti-HIV activities (Machu *et al.* 2015). The main bioactivities are as potent antioxidants, fungicides, with antimicrobial and antiproliferative functions against certain types of cancer (Esquivel-Hernández *et al.* 2017). Recent reports revealed that phenolic compounds such as gallic and chlorogenic acids exhibit cholinesterase inhibitory activities and protect the brain against metal-induced lipid peroxidation (Olasehinde *et al.* 2017), which may be relevant to the management and/or treatment of Alzheimer's Disease. Polyphenolic compounds have become very common constituents of human diet and received an increasing interest from consumers and also from food manufacturers for many reasons, the health benefits, mentioned above, being the most significant (Machu *et al.* 2015).

Lipids - Microalgae can accumulate a high percentage of lipids, up to approximately 30–50% of their total weight (Chew *et al.* 2017), and the composition of this bioproduct can differ considerably in different species (de Souza *et al.* 2018). Under optimal cultivation conditions, several species especially such belonging to the genera *Botryococcus*, *Chlorella*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Scenedesmus*, *Dunaliella* and *Schizochytrium* are described to show exceptionally high amounts of lipids in their cell mass (Koller *et al.* 2014). Recently, algae have been being considered seriously as sources of biodiesel (Harwood and Guschina 2009; Dickinson *et al.* 2017; de Souza *et al.* 2018; Duran *et al.* 2018). Biodiesel is typically made up of fatty acid methyl ester (FAME) whose lipid precursors are stored in lipid bodies in the cytoplasm and function as secondary energy sources for microalgae (Dickinson *et al.* 2017). In any case, biodiesel is not the only product accessible from algal lipids. Algal sterols are of increasing significance for bivalve hatcheries, e.g.

for oyster cultivation (Jo *et al.* 2004; Koller *et al.* 2014). Recent interest in microalgae has really focused on them as producers of PUFAs. Polyunsaturated, fatty acids (PUFAs) with high market values, such as EPA, DHA, GLA, and AA can be commercialized for pharmaceutical and therapeutic applications, at higher prices than the ones at which can be sold as biofuels (Desboi *et al.* 2009; Koller *et al*. 2014). Polyunsaturated fatty acids (PUFA) are widely recognized as essential nutritional components that assist in the prevention of various cardiac disorders (Chew *et al.* 2017). Furthermore, ω3-PUFAs are important for good health in lowering the risk of diseases where chronic inflammation plays an important role. This includes cardiovascular disease, various cancers, arthritis and dementia (Harwood and Guschina 2009). A broader discussion about microalgal lipids will be held in chapter II.

Proteins - Due to its chemical composition and its high protein content, microalgae biomass positively affects humans and animals health, it is able to enhance the nutritional content of conventional food preparations and hence, can be applied as dietary or "health food" (Spolaore *et al.* 2006; Koller *et al.* 2014). The first microalgal strain commercially produced as source of human food has been *Chlorell*a sp. (Vigani *et al.* 2015; Khanra *et al.* 2018). The predominant genera cultivated and harvested for foodstuff are *Spirulina*, *Nostoc*, *Porphyra* and *Chlorella vulgaris* (Becker 2007; Colla *et al.* 2007; Koller *et al.* 2014). Several studies demonstrated that microalgae represent a reliable and safe source of vegetable proteins (Becker, 2007), and essential amino acids (Hosseini *et al.* 2013; Gutierrez-Salmean *et al.* 2015; Khanra *et al.* 2018). The protein content from different microalgae species and cyanobacteria may vary from about 6 to 70% of its dry biomass (Becker 2007; Khanra *et al.* 2018), and they are of fundamental importance in human and animal feed (de Souza *et al.* 2018). Spolaore *et al.* (2006) reported that *Arthrospira* is used in human nutrition because of its high protein content, its excellent nutritive value and various possible health-promoting effects such as the alleviation of hyperlipidemia, suppression of hypertension. The market price for algal biomass of protein-rich strains like *Spirulina* sp. or *Chlorella* sp. that can be applied for human nutrition has been evaluated around 40 to 50 US-$/kg with a global market volume of 1.25 billion US-$ (Koller *et al.* 2014). Due to their protein content and their nutritional value, microalgae have been recently employed as feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals (Spolaore *et al.* 2006). A large number of nutritional and toxicological evaluations demonstrated their suitability as supplement or substitute of conventional protein sources (soybean meal, fish meal, rice bran, etc.) (Becker 2007). In the case of animals feed

the price of the final product is estimated about 10 US-$/kg, corresponding to a high global market volume exceeding 4 billion US-$ (Koller *et al.* 2014).

Carbohydrates - are molecules composed of carbon, hydrogen, and oxygen, such as sugars, sugar and their polymers (Markou *et al.* 2012; de Souza *et al.* 2018) and are mainly represented of this by glucose, starch, cellulose, and polysaccharides. Microalgae can have approximately 50% of their dry weight as carbohydrates depending on the species (Chew *et al.* 2017; de Souza *et al.* 2018). Several microalgal species, such as *Porphyridium cruentum* (40–57 %), *Spirogyra* sp. (33–64 %), etc., are characterized by an innate high carbohydrate content (Markou *et al.* 2012). Glucose or starch are conventionally used for the production of biofuels, such as bioethanol and biohydrogen, in addition they can also modulate the immune system to inflammatory reactions, making them highly favorable to act as sources of active molecules for insertion in cosmetics, food ingredients and as natural therapeutic agents (Chew *et al.* 2017; de Souza *et al.* 2018). The most important carbohydrate classes are β-glucans since they are responsible for initiating host defense reactions in response to the molecules of pathogens. *Chlorella* sp. is recognized for its rich content of β-1,3-glucan, which is an active immunostimulator, a free radical scavenger and a reducer of blood lipids (Spolaore *et al.* 2006; de Souza *et al.* 2018).

7 CONCLUDING REMARKS

The wide range of products accessible from the primary and secondary metabolism of diverse microalgal species clearly demonstrates the importance of these versatile microbes as cellular factories. So far, algae have been considered mainly as feedstocks for sustainable fuels like biodiesel and biofuel, but recently, microalgae cultivation has gained attention for the production of larger amounts of high-value compound such as pigments, vitamins, PUFAs, anti-oxidant and so on. The growing demanding of market bioproducts from microalgae and their subsequent utilization in food nutrition may be a suitable option for the future development of microalgae cultivation at industrial scale. If exploited in an effective way, the efficient cultivation of microalgae for valuable bioproducts may contribute combating climate change by CO_2 sequestering. However, some barriers must be overcome to achieve technologies suitable for large-scale cultivation and accumulation of bioproducts. In this context, genetically modified microalgae may help to solve some constraints and improve the targeted bioproducts in industrial scale. However, the development of an efficient processes technology for large-scale production of algal biomass and

valuable chemicals needs the availability of expertise from different scientific fields. In conclusion, research efforts should focus on reducing product loss and minimizing energy costs while heading towards an environmental friendly large-scale downstream processing for the extraction of high-value compounds from microalgae.

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CHAPTER II

Introductive overview

1. GENERAL OVERVIEW

Microalgae are an extremely diverse group of primary producers present in almost all ecosystems on Earth, ranging from marine, freshwater, desert sands, and hot springs, to snow and ice (Guschina and Harwood 2006b; Rajkuma and Yaakob 2013). Among these habitats are several places which are, from the anthropocentric view, inhospitable and different from the "normal" places. The microbes occurring in those environments are referred to as "extremophiles." These extremophiles can provide important answers to the ecology and biochemistry and lead to biotechnological applications and industrial aspects (Seckbach 2007). In this context, extremophile microalgae have gained a growing interest because of their ability to grow under extreme conditions, allowing outdoor cultivation with negligible contamination risks and thus permitting to suitably exploit the economic advantages linked to the use of open raceways (Hirooka *et al*. 2014; Varshney *et al*. 2015; D'Alessandro and Antoniosi Filho 2016; Souza *et al.* 2017).

1.1 Extreme environments

An extreme environment is a habitat characterized by harsh environmental conditions beyond the optimal range for the development of humans, for example, pH 2 or 11, −20°C or 113°C, saturating salt concentrations, high radiation, and pressure, among others (Gómez 2011). “Extremes” include physical extremes, such as temperature, radiation or pressure, and geochemical extremes, such as desiccation, salinity, pH, oxygen species or redox potential (Rothschild and Mancinelli 2001). Extremophiles can be defined as organisms that thrive in habitats which for other terrestrial life-forms are intolerably hostile or even lethal (Rampelotto 2013). Kristjansson and Hreggvidsson (1995) defined an extremophile as one whose optimal growth conditions are found beyond their “normal” environments, “normal” meaning those that have a temperature between 4 and 40°C, a pH between 5 and 8.5, and salinity between that of freshwater and that of seawater (Walsh and Seckbach 1999; Seckbach and Oren 2007). Extremophiles can thrive in extreme hot niches, ice, and salt solutions, as well as acid and alkaline conditions; some may grow in toxic waste, organic solvents, heavy metals, or in several other habitats that were previously considered inhospitable for life (Isken and de Bont 1998; Nies 2000; Rothschild and Mancinelli 2001; Rampelotto 2013). A variety of organisms have shown that they not only tolerate these conditions but also often require those conditions for survival (Rampelotto 2013).
Extremophiles include members of all three domains of life i.e., bacteria, archaea, and eukarya (Rothschild and Mancinelli 2001; Rampelotto 2013).

Extremophiles may be divided into two broad categories: extremophilic organisms which require one or more extreme conditions in order to grow, and extremotolerant organisms which can tolerate extreme values of one or more physicochemical parameters but grow optimally under normal conditions (Rampelotto 2013).

Oxygenic phototrophic microorganisms can be both extremophilic and extremotolerant, and include prokaryotes (cyanobacteria) and eukaryotes (microalgae).

Oxygenic phototrophic microorganisms are abundantly found in environmental extremes of temperature, pH, salt concentration, and radiation (Seckbach and Oren 2007). Phototrophs of extreme environments can be distinguished as thermophiles (lovers of high temperature), psychrophiles (cold-loving organisms), halophiles (high salt-loving organisms), acidophiles (cells thriving at low pH), alkaliphiles (cells thriving at high pH), and radiation-resistant phototrophs (Seckbach and Oren 2007). We should not forget situations, e.g., the intertidal where conditions,

especially related to water availability, fluctuate on a daily basis (Seckbach and Oren 2007). Some extremophilic phototrophs are strictly dependent on their specialized environment and will not survive even a short exposure to “normal” conditions (Seckbach and Oren 2007). In addition, “polyextremophilic” phototrophs are simultaneously adapted to multiple forms of environmental stress (Rothschild and Mancinelli 2001; Seckbach and Oren 2007; Rampelotto 2013).

Microalgae produce biomass for food, feed, and fine chemicals, using solar energy. Microalgae are found all over the world. They are mainly distributed in the waters but are also found on the surface of all type of soils. Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms (Tomaselli 2004).

Extremophile algae can be easily isolated from highly acidic environment contaminated by acid mine drainage (AMDs). Such environments are known to support an unsuspected microbial biodiversity (bacteria, archaea, microalgae), although the validated species of acidophilic organisms are still very limited compared to those in neutral environments (Hirooka *et al.* 2014; Škaloud *et al.* 2014; Hirooka and Miyagishima 2016).

1.2 Freshwater algae associated with Acid Mine Drainage (AMD)

Particular attention in this thesis will be given to acidic mine drainage (AMD) environments. Acid mine drainage (AMD) is a phenomenon commonly associated with mining activities throughout the world (Novis and Harding 2007). Acidic solutions can be formed by natural decomposition of sulfide materials through oxidative processes in effluent wastewater (Bwapwa *et al*. 2017). Distinctly, however, extremely acidic habitats from anthropogenic sources are associated with a massive burden of spoil and heavy metals (Novis and Harding 2007). The main source of AMD is oxidation of sulfide mineral ores, which are initially exposed to the environment by intensive mining activities (Kefeni *et al.* 2017). In particular, among the metal sulfides, pyrite ore (FeS_2, commonly known as fool's gold) is one of the main mineral responsible for generation of AMD due to its ease of oxidation when exposed to oxygen, water, and microorganisms (Chen *et al.* 2014; Plante *et al.* 2014; Han *et al.* 2015; Pierre Louis *et al.* 2015; Kefeni *et al.* 2017). Regarding acid mine drainage (AMD), the high concentrations of heavy metals cause major environmental concerns characterized by contamination of various ecosystems due to its leaching capacity and the presence of very active bacteria, which makes the process self-perpetuating (Gross 2000; Kalin *et al.* 2006; Bwapwa *et al.* 2017). AMD can occur in surface runoff (particularly in opencast mines), in the groundwater associated with underground mines, supply and contaminate streams (Harding and Boothroyd 2004; Novis and Harding 2007), and lakes within the catchment (Wicks *et al*. 1991; Niinioja *et al.* 2003; Tittel *et al.* 2005; Novis and Harding 2007). Freshwater environments exposed to AMD typically have extremely low pH and high concentrations of dissolved metals (Novis and Harding 2007). Sulfuric acid is the prominent acid in mining areas; it is formed from decomposition of pyrite, which is most often associated with coal (Gross 2000). Strip (or opencast) mining, in particular, exposes the pyrite to oxidative conditions leading to chemical oxidation (Gross 2000). The ferric ion acts as the major oxidant of the mineral according to the following equation (Novis and Harding 2007):

$$FeS_2 + 6Fe(H_2O)_6 + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_2O)_6^{2+} + 6H^+$$

Oxidative dissolution of sulfide minerals generates acidity and releases sulfate, iron, and associated metals. However, the chemistry of drainage from a mine site is the result of the competing processes of acid generation and acid neutralization (Seal II and Piatak 2012). In fact, the buffering capacity of the receiving water, and the extent to which the water body can dilute the contamination, largely influence the impact of AMD (Gray 1997; Novis and Harding 2007). This is partially because the

solubility of metal ions varies greatly with pH (Harding and Boothroyd 2004; Novis and Harding 2007), and also because dilution may reduce metal concentrations while not markedly influencing pH (Novis and Harding 2007). At higher pH (>4.0), precipitation of metal hydroxides can smother biota with precipitates, whereas at lower pH the toxicity of dissolved metals, which can cross membranes (Van Ho *et al.* 2002; Novis and Harding 2007), combined with acidity, may have the greatest impact (Novis and Harding 2007). This complexity of impact has led to a view that AMD is "multifarious" affecting organisms in "numerous interactive ways" (Gray 1997; Novis and Harding 2007). The impact of strong acidity on biota is associated with the high H^+ concentrations, the limited supply of carbon dioxide for photosynthesis, because of the absence of a bicarbonate pool (Gross 2000), the nutrient deficiency (Johnson 1998; Novis and Harding 2007) because of precipitation of nutrients (Gross 2000) and with the toxicity of heavy metals due to the generation of reactive oxygen species (ROS) (Pinto *et al.* 2003; Novis and Harding 2007). Several studies on the heavy metal effect on the biosphere evidenced that metals are transferred to some vegetal species that grow on the dumps or in nearby areas (Barbafieri and Dadea 1999; Biddau *et al.* 2001). It was also recognied a degradation effect on detritivores communities of the main rivers of the area (Dadea *et al*. 1996; Biddau *et al.* 2001). Some algal taxa are particularly able to adapt to low pH and are a recurrent presence in acidic habitats such as species of the genus *Chlamydomonas*, *Euglena*, *Ochromonas, Pinnularia, Klebsormidium,* and *Coccomyxa* (Novis and Harding 2007; Amaral-Zettler 2013; Škaloud *et al.* 2014; Barcytė and Nedbalová 2017). The resistance of many algae to AMD might be due either to their ability to complex metals outside the cells (Podda *et al.* 2000; Garcia-Meza *et al.* 2005; Novis and Harding 2007), and to accumulate and sequestrate in thylakoids (Soldo *et al.* 2005; Novis and Harding 2007). Several studies have shown that algae can adsorb heavy metals, suggesting their possible use in remediation (Novis and Harding 2007; Bwapwa *et al.* 2017). However, attempts to turn this observation into remediation technologies have met with mixed success, and require a greater understanding of AMD effects on biota (Novis and Harding 2007). Although the generic term acid mine drainage (or acid rock drainage) is used frequently to describe mine water discharges, the pH of these waters may be above 6, particularly at the point of discharge (where dissolved oxygen concentrations are frequently very low) (Johnson and Hallberg 2005). When mine and mine waste drainage has neutral pH, with a high concentration of sulfate and dissolved metals, the term neutral (or alkaline) mine drainage is applied (Scharer *et al.* 2000; Iribar 2004; Frau *et al.* 2015, 2017). Net acidity in AMD needs to be offset against any alkalinity present; this is mainly in the form of bicarbonate (HCO_3^-) deriving from the dissolution of basic minerals (e.g., calcium carbonate), though, as noted below, biological processes may also generate alkalinity

in AMD streams (Johnson and Hallberg 2005). Neutral mine drainage (NMD) has received less attention than AMD, but NMD can have environmental adverse effects caused mainly by precipitation of dissolved Fe and Zn (Iribar 2004). However, neutral mine drainage (NMD) conditions may persist in an abundance of carbonate minerals (Majzlan *et al.* 2011; Kuang *et al.* 2013; Lindsay *et al.* 2015; Kisková *et al.* 2018). Near-neutral drainages may be highly contaminated and represent a major risk to the hydrographic system, heavily modifying water chemistry and composition of stream sediments (Frau *et al.* 2015, 2017).

1.3 Arburese mining district (Sardinia, Italy)

An example of neutral mine drainage (NMD) is river Irvi located in the Arburese mining district (Fig.1). The Arburese mining district (SW Sardinia, Italy) is mainly composed of the abandoned mine workings of Montevecchio and Ingurtosu. Montevecchio mine is the most important case of groundwater discharge from a flooded mine in Sardinia (Frau *et al.* 2015, 2017). Casargiu is one of the mines belonging to the Montevecchio mining system. After mine closure in the 1980s and subsequent shutdown of the dewatering system, groundwater rebound led to drainage outflow from the Casargiu gallery beginning in 1997 (Caboi *et al.* 1999; Biddau *et al.* 2001; Cidu and Fanfani 2002; Frau *et al.* 2015). The Casargiu drainage (20–70 L/s; pH 6.0±0.2; Zn-Mg-Ca-SO_4 composition) flows into the Rio Irvi whose hydrological basin has an area of about 15.4 km^2 and a length of about 11 km (Frau *et al.* 2015). West of Montevecchio (Rio Irvi-Piscinas and Rio Naracauli) pollution is essentially the result of interaction between waters and tailings piles. The pH values in this area are close to the neutrality (6-8), due to the buffering effect of carbonate gangue minerals (Biddau *et al.* 2001). At the Casargiu outflow and along the Rio Irvi ochreous precipitates can be observed (Fig. 2). Abundant precipitation of amorphous Fe(III)-(oxy)hydroxides occurred (Frau *et al.* 2015, 2017). Moreover, sulfate-bearing green rust was observed to flocculate in the reach of the Rio Irvi where pH was still circumneutral (Frau *et al*. 2015, 2017). The green rust is a mixed Fe(II)/Fe(III) layered double hydroxide forming under weakly acidic to alkaline conditions in sub-oxic environment. Being an unstable phase under atmospheric conditions, there is no persistent accumulation of green rust in the Rio Irvi streambed, even where stream water is clearly oversaturated with respect to green rust (Frau *et al.* 2017). Water sampling along this stream for about 6 km almost to its mouth in the Mediterranean Sea showed a pH decrease from 6.0 to 4.0 and a significant removal of Fe (46 %) and As (96 %), while sulfate, Zn, Mn, Co, Ni, and Cd showed small variations downstream (Frau *et al.* 2015). To the best of our knowledge, no studies have been carried out on the microbial diversity of the river Irvi. However, previous researches carried out in a nearby river flowing from the same dismissed mining district, the river Naracauli, evidenced the presence of a photosynthetic community adapted to toxic metals and involved in the mechanism of metal sequestration (Podda *et al.* 2000). A microbial community composed by a photosynthetic filamentous bacterium, classified as *Scytonema* sp. strain ING-1, associated with a microalga *Chlorella* sp. strain SA1 was found responsible for the biological processes of natural polishing of heavy metals in the water stream by co-precipitation with hydrozincite (Podda *et al.* 2000; Biddau *et al.* 2001).

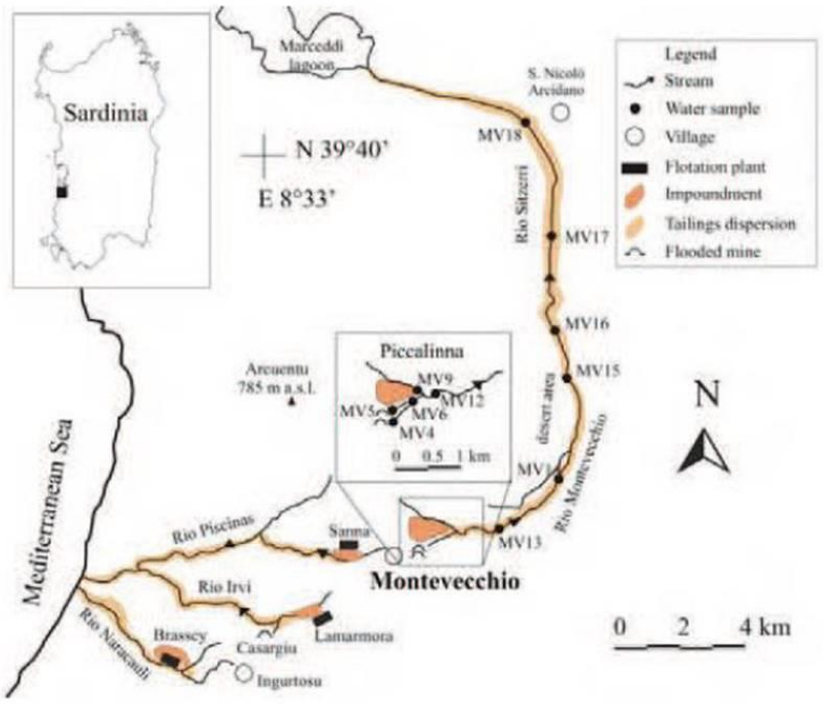


Fig. 1 Study area located in the Arburese mining district (SW Sardinia, Italy). Lead and zinc sulfide ore deposits were mined until 1968. Source Cidu and Frau (2010).

Fig. 2 The Rio Irvi river, Locality Montevecchio-Ingurtosu, Sardinia, Italy. Source Malavasi *et al.* (2016).

1.4 Economic potential of extremophiles

Microalgae are an extremely heterogeneous group of organisms which represents a potentially rich source of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries (Olaizola 2003). Microalgae grow much faster and show greater photosynthetic efficiency compared with land plants, however, a key challenge in mass culturing of micro-algae is to find strains that not only produce marketable products or biomass but also grow well under industrially relevant outdoor conditions (Varshney *et al.* 2015).

There are currently relatively few micro-algae grown commercially, i.e. profitably, at large-scale, and the few taxa making up the bulk of the algal biomass market are mostly extremophiles (*Spirulina* and *Dunaliella*) (Slocombe *et al.* 2015; Varshney *et al.* 2015).

A key factor in the commercial success of these two species is their ability to grow under specific extreme conditions that help in reducing the contamination by other algal species (Avron and Ben-Amotz 1992; Varshney *et al.* 2015).

For several applications, this can be beneficial, even when the growth rates of extremophilic algae are sometimes slower compared with "common" algae (Pulz and Gross 2004).

In view of this, extremophilic microalgae have the potential to play an important role in the eventual commercial exploitation of microalgae-based biofuels, bioproducts, and agriculture (Varshney e*t al.* 2015). As a result of thriving in such diverse and extreme environments, they produce an array of unique bioactive, complex, exotic acyl lipids and fatty acids that are not generally present in terrestrial plants (Kumari *et al.* 2013). In this framework, collections of extremophilic microorganisms could allow the selection of strains that produce high-value compounds with specific features as thermo-resistance or cryo-resistance; this may also prevent environmental microbial contaminants that would not survive the selected growth conditions (Ruiz *et al.* 2016; D'Elia *et al.* 2018).

Literature reported several examples of potential biotechnological applications of extremophile microalgae. For example, the halophile *Dunaliella salina* is used as a natural source of β-carotene (Santos *et a*l. 2001; Arakawa *et al.* 2012; Varshney *et al.* 2015), while *Spirulina* is employed as food and feed additive in human and animal nutrition (Varshney *et al.* 2015). Massive accumulation of β-carotene in *Dunaliella salina* is triggered by environmental stresses such as high irradiance, salinity, nutrient starvation or extreme temperatures (Ben-Amotz and Avron 1983; Borowitzka *et al.* 1990; Garbayo *et al.* 2008). The psychrophilic algae *Chlamydomonas nivalis* and *Raphidonema* sp. produce Astaxanthin and Tocopherol (vitamin E) and pigments respectively (Remias *et al.* 2005; Leya *et al.* 2009; Varshney *et al*. 2015). The thermophilic *Desmodesmus* sp. F51, *Desmodesmus* sp.

F2 and F18 are known for lutein and high lipid content respectively (Pan *et al.* 2011; Xie *et al.* 2013; Varshney *et al.* 2015). Algae and cyanobacteria capable of growing well under extreme light radiation (PAR) and UVB, might be excellent sources of antioxidants and protective compounds such as the β-carotene production by *Dunaliella tertiolecta* and astaxanthin by *Haematococcus* (Varshney *et al.* 2015). Extremophiles microalgae, like *Dunaliella bardawil*, are often involved in carotenoids photoproduction thus gaining biotechnological relevance (Fujiwara 2002; Podar and Reysenbach 2006; Garbayo *et al.* 2008). Highly acidic environments facilitate metal solubility, resulting in an environment in which metal-tolerant acidophiles reside (Novis and Harding 2007; Varshney *et al.* 2015). It is thought that among the methods of dealing with metal toxicity are binding to the cell wall, sequestration within the cell, or by complexation with organic compounds and storage in the vacuole (Rai and Gaur 2001; Varshney *et al*. 2015). Also, the formation of extracellular polymeric substances (EPS) may be potentially responsible for the detoxification of the metal as well as an aid for the formation of the biofilm (Garcia-Meza *et al.* 2005; Varshney *et al.* 2015). This ability to accumulate metals and other pollutants can be harnessed in creating remediation solutions for treatment of wastewater (Varshney *et al.* 2015; Bwapwa *et al.* 2017). The use of various algae strains to remove heavy metals from AMD has been investigated (Sheoran and Bhandari 2005; Suresh Kumar *et al.* 2015; Delrue *et al*. 2016). In particular, the possible synergistic role of acidophilic and non-acidophilic microalgae together with bacteria, all contained in biofilms, has been reported for bioremediation of AMD (Abinandan *et al.* 2018; Marques 2018) and at this regard Hammed *et al.* (2016) evidenced the necessity to direct the strains selection towards identification of algal that are extremophiles. *Galdieria sulphuraria,* from sulfuric acidic hot springs, was shown to selectively recover rare earth elements (Minoda *et al.* 2015; Hirooka and Miyagishima 2016). An extreme form of metal tolerance is found in those organisms that can grow in the presence of radionuclides and which are therefore extremely resistant to ionizing radiation (Varshney *et al.* 2015). For example, Rivasseau *et al.* (2013) isolated *Coccomyxa actinabiotis* that is able to withstand gamma-ray radiation and to accumulate, and remove from solution, a range of nuclides. Earlier work had also shown cyanobacteria to possess a degree of radiation resistance (Kraus 1969; Varshney *et al.* 2015). Acidophilic extremophiles have the potential to provide much knowledge of cellular tolerance mechanisms to advance and optimize their bioremediation potential, as well as providing many potential species for the construction of bioremediation systems (Varshney *et al.* 2015).

Furthermore, extremophile microalgae from mine drainages have shown to be able to produce high-value products under specific conditions, such for example the acidophiles *Coccomyxa onubensis*

and *Chlamydomonas acidophila* that increased their production of the antioxidants lutein and β-carotene in the presence of copper (Garbayo *et al.* 2008; Vaquero *et al.* 2012; Varshney *et al*. 2015) and the green alga *Pseudochlorella* sp. YKT1 (Trebouxiophyceae), recently isolated from an acidic mine drainage in Japan which is able to accumulates a large amount of storage lipids (~30% of dry weight) under nitrogen-depleted condition and low pH (Hirooka *et al.* 2014; Hirooka and Miyagishima 2016).

2 MICROALGAL LIPIDS

Microalgae have great potential as a source of pigments, antioxidants, lipophilic compounds and biofuels for industrial applications (Milledge 2012; Hirooka and Miyagishima 2016). For example, they contain phycobilins (Singh *et al.* 2005), carotenoids (Borowitzka 2010) and long-chain polyunsaturated fatty acids (Abedi and Sahari 2014; Hirooka and Miyagishima 2016).

Algal lipids are of immense commercial value as alternative sources of nutritionally important n-3 polyunsaturated fatty acids (PUFAs) and are, therefore, widely employed as ingredients in functional food formulations (Mendis and Kim 2011; Misurcovà *et al.* 2011; Kumari *et al.* 2013).

Lipids and polyglucans are the energy and carbon reserves in microalgal cells, but polyglucans represent less concentrated stores of metabolic energy than lipids (Vitova *et al.* 2015; Zhu *et al.* 2016). Both lipids and polyglucans not only ensure the survival of microalgal cells such as in night periods as well as in periods with variable light intensities but also supply energy for biological processes associated with the multiplication of microalgal cells, such as the replication of DNA, division of nuclear, cytokinesis, and formation and liberation of daughter cells (Zhu *et al.* 2016).

Microalgal lipids can be divided into two groups according to their structures (Fig. 3):

- nonpolar (NLs) such as acylglycerols, sterols, free fatty acids, wax, and steryl esters;
- polar lipids such as phosphoglycerides, glycosylglycerides, and sphingolipids.

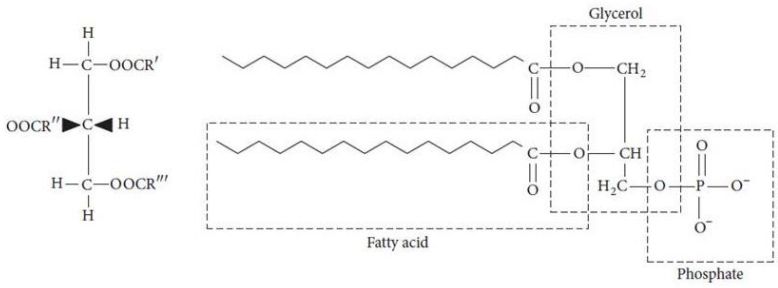


Fig. 3 Lipid molecules. Triacylglycerol (NL) on the left. Phospholipid (polar lipid) on the right. R', R'', and R''' in the triacylglycerol molecule represent fatty acid chains. Source Chen *et al.* (2018b).

Fig. 3 shows the structural formula of the polar lipid and NLs (Guschina and Harwood 2007; Halim *et al.* 2012; Chen *et al.* 2018b). These lipids play different but important roles in microalgal metabolism and growth period (Chen *et al.* 2018b).

Phospholipids are characterized by the presence of a phosphate group at sn-3 position which is further linked to a hydrophilic head group that classifies individual phospholipid molecules (Kumari *et al.* 2013). The basic structure of glycerolipids consists of a glycerol backbone metabolically

derived from glycerol 3-phosphate to which hydrophobic acyl groups are esterified at sn-1 and sn-2 positions (Kumari *et al.* 2013).

Lipids such as phosphoglycerides, glycosylglycerides, and sterols are imperative structural components of biological membranes, while lipids like inositol lipids, sphingolipids, and oxidative products of polyunsaturated fatty acids may act as key intermediates in the cell signaling pathways and play a role in sensing changes in the environment (Borowitzka and Moheimani 2013; Chen *et al.* 2018b). The quantities of these microalgal lipids vary with the type of species, growth conditions, and ambient environments (Chen *et al.* 2018b). It was reported that the lipid contents ranged at 20–50% of dry biomass including *Chlorella*, *Crypthecodinium*, *Cylindrotheca*, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum*, *Porphyridium*, *Schizochytrium*, and *Tetraselmis* (Mata *et al.* 2010; Chen *et al.* 2018b).

The main storage lipids and energy reservoir in microalgae are neutral lipids (NLs) or triacylglycerols (TAG) (Kumari *et al.* 2013; Slocombe *et al.* 2015; Zhu *et al.* 2016; Chen *et al.* 2018b). Algal lipids are mostly characterized by saturated and monounsaturated fatty acids but many oleaginous algae exhibit the potential to accumulate long-chain PUFAs (AA, EPA, and DHA). *Parietochloris incisa* accumulates AA, *Phaeodactylum tricornutum*, *Porphyridium cruentum*, *Nitzschia laevis*, and *Nannochloropsis* sp. accumulate EPA; *Pavlova lutheri* accumulates both AA and EPA; *Schizochytrium mangrovei*, and *Isochrysis galbana* accumulate DHA (Bigognoa *et al.* 2002; Meireles *et al.* 2003; Chen *et al.* 2007; Fan *et al.* 2007; Patil *et al.* 2007; Khozin-Goldberg and Boussiba 2011; Kumari *et al.* 2013). TAGs are mostly synthesized in light, stored in cytosolic lipid bodies and reutilized for polar lipid synthesis in the dark (Thompson 1996; Kumari *et al.* 2013). PUFA-rich TAGs act as reservoirs for FAs and donate acyl groups for polar lipid biosynthesis especially under adverse conditions, when de novo syntheses of PUFAs are impaired (Khozin-Goldberg *et al.* 2000; Kumari *et al.* 2013). Algae contain a wide variety of fatty acids that basically are carboxylic acids with long aliphatic chains that may be straight or branched, saturated or unsaturated (Kumari *et al.* 2013). Most of the naturally occurring FAs contain even carbon numbers (C4–C28); however, odd-chain FAs are also prevalent in algae (Kumari *et al.* 2013). The major saturated fatty acid in algae is invariably palmitate while oleate is much less abundant than in higher plants (Harwood and Guschina 2009). On the basis of the number of double bonds present, FAs are classified as monounsaturated FAs (MUFAs, with 1 double bond), and polyunsaturated FAs (PUFAs, with ≥2 double bonds). Further, PUFAs are classified as n-3 or n -6 FAs depending on the position of the first double bond from the methyl end. n-3 PUFAs are of nutritional importance as these cannot be synthesized by humans and thus obtained through diet (Kumari *et al.*

2013). Algae are extensively explored for fatty acids, especially PUFAs (representing 10–70% of total fatty acids; TFAs) due to their chemotaxonomic and nutritional importance, with their compositions varying even within the same phyla (Kumari *et al.* 2013). Many algae have very long chain (VLC) (>18C) polyunsaturated fatty acids (PUFAs) as major components (Harwood and Guschina 2009). Long-chain PUFAs are indispensable for proper growth and development of organisms with n-3 PUFAs (ALA, STA, and EPA) being beneficial for the prevention of cardiovascular and other chronic diseases such as diabetes, hypertension and autoimmune diseases, DHA for visual and neurological health, while AA and EPA are precursors of bioregulators prostaglandins, thromboxanes and other eicosanoids, which influence inflammation processes and immune reactions (Calder and Grimble 2002; Kumari *et al.* 2013). Triacylglycerols (TAGs) that can be esterified to FAMEs with the primary profiles of C16 and C18, proven to be the most suitable for biofuel production (Ge *et al.* 2017; Chen *et al.* 2018b).

2.1 Lipid biosynthesis

Several microalgal species are able to accumulate appreciable lipid quantities, and therefore are characterized as oleaginous (Bellou *et al.* 2014). Understanding lipid metabolism (cfr Fig. 4) and how it is controlled during algal growth is of great importance for maximizing lipid production (Bellou *et al.* 2014). Through photosynthesis, CO_2 is converted to glycerate-3-phosphate (G3P) (Bellou *et al.* 2014). Metabolism of both starch and lipid begins with an identical initial pool of molecules containing three carbons such as glyceraldehyde 3-phosphate (GAP) and 3-phosphoglycerate (3PG) (de Jaeger *et al.* 2014; Zhu *et al.* 2016). The conversion of G3P to pyruvate and thereafter to acetyl-CoA, via a reaction catalyzed by the pyruvate dehydrogenase complex (PDC), initiates the lipid biosynthetic pathway, which occurs in the plastid. The committing step in fatty acid biosynthesis is the carboxylation of acetyl-CoA to form malonyl-CoA, reaction catalyzed by the acetyl-CoA carboxylase (ACCs) located either in the plastid or in the cytosol (Khozin-Goldberg and Cohen 2011; Lei *et al.* 2012; Baba and Shiraiwa 2013; Bellou *et al.* 2014).

Briefly, in the plastid, the malonyl-CoA is introduced in the fatty acid synthesis cycle and converted via the sequential reactions to butyryl-ACP. The cycle is repeated until the formation of palmitoyl-ACP. The fatty acids are released from the chloroplast envelope and transferred in the cytosol, where they become available for lipid synthesis. Those transferred in the cytosol acyl-CoA chains are esterified with structural phospholipids of the endoplasmic reticulum (ER) to be converted into higher derivatives (PUFAs). The modified or not in the ER fatty acids are used as building blocks for the formation of TAGs via the Kennedy pathway.

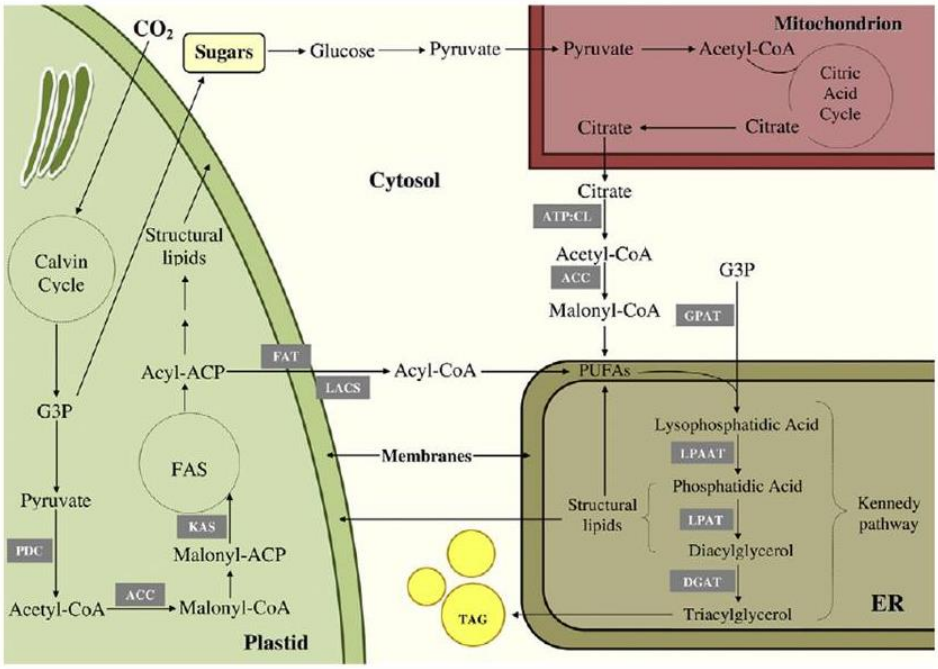


Fig. 4 A simplified scheme showing lipid synthesis in microalgae. Source Bellou *et al.* (2014).

2.2 PUFA biosynthesis

The synthesis of long-chain unsaturated fatty acids requires the presence of specific elongases and desaturases enzymes (Fig. 5), which act primarily on palmitic, stearic and oleic acids. Fatty acid elongation occurs in both plastids and ER (Ohlrogge and Browse 1995; Kunst and Samuels 2009; Bellou *et al.* 2014) and requires acyl-CoA and malonyl-CoA as substrates plus 1 ATP and 2 NADPH molecules per C2-unit elongation of the carbon chain. For the synthesis of the very long-chain PUFAs, such as arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, the "elongase of very long-chain fatty acid" (ELOVL) is required (Bellou *et al.* 2014). Desaturases are specialized in the location, number and stereochemistry of double bonds in fatty acids (Heinz 1993; Pereira *et al.* 2003; Bellou *et al.* 2014). The biosynthesis of EPA, for example, occurs through a series of reactions that can be divided into two distinct steps. First is the de novo synthesis of oleic acid (18:1 ω9) from acetate, followed by conversion to linoleic acid (18:2 ω-6) and α-linolenic acid (18:3 ω-3). The subsequent stepwise desaturation and elongation steps form an ω-3 PUFA. Inside the cell, EPA is normally esterified (by cyclooxygenase and lipooxygenase activities) to form complex lipid molecules and plays an important role in higher animals and humans as the precursor of a group of eicosanoids, hormone-like substances such as prostaglandins, thromboxanes and leukotrienes that are crucial in regulating developmental and regulatory physiology (Fig. 5) (Wen and Chen 2003; Cardozo *et al.* 2007). An aspect of PUFA biosynthesis which should not be passed over is that, in general, desaturation is increased by lower temperatures (Guschina and Harwood 2006b; Harwood and Guschina 2009). Indeed, an increase in membrane unsaturation at low temperatures is a common adaptation process to maintain fluidity (Gurr *et al.* 2002; Harwood and Guschina 2009).

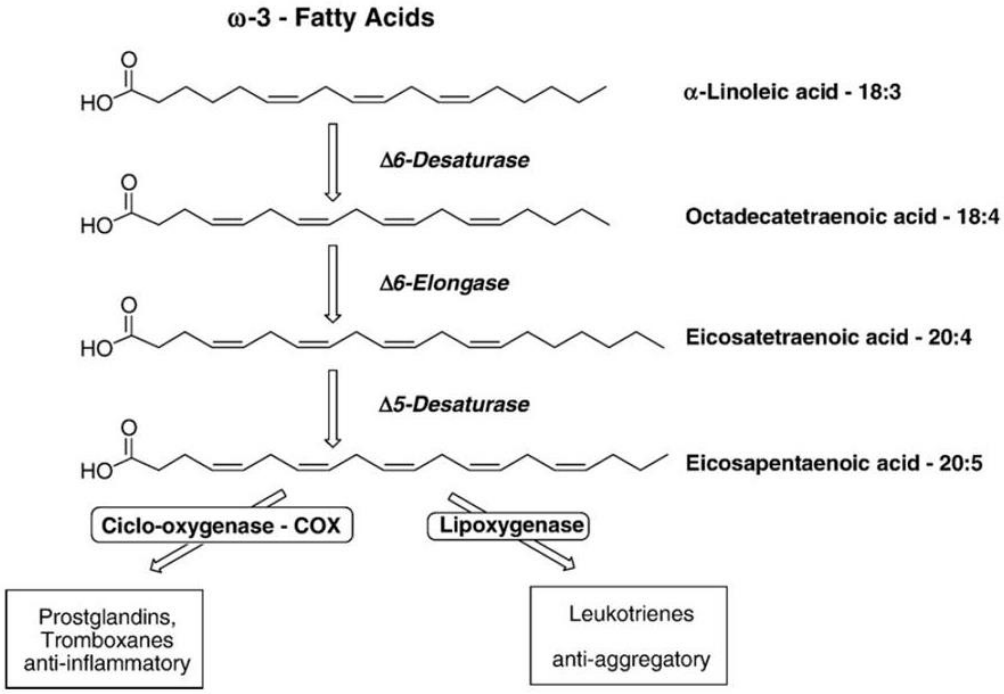


Fig. 5 A simplified biosynthesis scheme of eicosapentaenoic acid and eicosanoid (prostaglandins, thromboxanes, leukotrienes) Source Cardozo *et al.* (2007).

2.3 Cultivation under stress conditions

When microalgae are cultivated under sub- or supra-optimal conditions they react and change their metabolic pattern and strategies, in order to cope with the difficulties under the specific environmental conditions. This dynamic change on the metabolic strategy affects the biomass composition, fluctuating the relative content of the biomass compounds (Hu 2004; Markou and Nerantzis 2013). The lipid, fatty acid compositions often vary with the seasonal changes owing to the combined influence of environmental factors such as temperature, light, nutrient availability and the physiological state of the algae (Kumari *et al.* 2013). Various physiochemical parameters are known to influence the lipid content of microalgae such as light intensity, pH, salinity, temperature and nitrogen (Liang *et al.* 2011; Yeesang and Cheirsilp 2011; Behnaz *et al.* 2013; Rai *et al.* 2015).

Several methods are currently being investigated for boosting lipid biosynthesis in microalgae, and the most of these have in common processes that lead the microalgae to face stress conditions which trigger lipid synthesis (Fig. 6) (Concas *et al.* 2016). Specifically, techniques exploiting extreme pH and temperature conditions, high radiation, osmotic stress, high heavy metals concentration, and nutrient starvation, are currently under investigation (Sharma *et al.* 2012; Concas *et al.* 2016). However, regarding the criteria of choosing a technique, low energy consuming techniques such as nutrient starvation might be preferred in comparison to high energy consuming ones such as high artificial light (Lamers *et al.* 2010, 2012; Markou and Nerantzis 2013). Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and FA composition. Environmental stress condition when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is enough light and CO_2 available for photosynthesis (Thompson 1996; Sharma *et al.* 2012). Nutrient limitation, which generally causes a reduced cell division rate in algae, surprisingly activates the biosynthesis of storage lipids, primarily TAGs (Kumari *et al.* 2013). In Fact, although microalgae generally use starch as their primary carbon storage compound, some strains accumulate neutral lipids, mainly in the form of triacylglycerols (TAG) under environmental stress conditions such as nitrogen limitation (Radakovits *et al.* 2010; Tan and Lee 2016). Microalgae share common carbon precursors for starch and lipid biosynthesis, and thus blocking of starch synthesis has been suggested as a way to increase oil accumulation in algal cells (Kumari *et al.* 2013). When algal growth (as measured by cell divisions) slows down and there is no requirement for the synthesis of new membrane compounds, the cells instead divert and deposit fatty acids into TAG. Under these conditions, TAG production

might serve as a protective mechanism (Sharma *et al.* 2012). The accumulation of TAG likely occurs as a means of creating an energy deposit that can be readily utilized in response to a more favorable environment allowing for rapid growth (Yu *et al.* 2011; Tan and Lee 2016). Nutrient starvation is one of the most widely used and applied lipid induction techniques in microalgal TAG production and has been reported for many species (Sharma *et al.* 2012; Concas *et al.* 2016). Since nitrogen is the most growth-limiting factor for eukaryotic microalgae and would be one of the first nutrients to be depleted during algae cultivation, hence, nitrogen starvation is the most successful lipid inducing technique at present (Sharma *et al.* 2012; Concas *et al.* 2016). However, stress in microalga cultivation increases lipid accumulation but reduce growth rate, affecting lipid productivity (Converti *et al.* 2009; D'Alessandro and Antoniosi Filho 2016). A serious concern about the cultivation of microalgae under stress conditions is the decrease or the arrest of growth rates and consequently the decrease of the total production and productivity (Markou and Nerantzis 2013). In some cases, it is possible that the productivity of an accumulated compound cannot achieve the values obtained under regular conditions because of the decrease in the growth rates (Adams *et al.* 2013; Markou and Nerantzis 2013). While an increase in TAG production during nitrogen deprivation is ideal, reduced growth caused by nutrient deficiency hampers the use of this strategy for producing biofuel as the decrease in biomass productivity would reduce overall yield (Griffiths and Harrison 2009; Tan and Lee 2016). This inverse relationship between biomass productivity and lipid content makes the choice of the suitable nitrogen concentration not straightforward since a trade-off value should be assured in order to maximize lipid productivity (Concas *et al.* 2013, 2014).

Also, fluctuations of the pH in the medium results in lipid accumulation and may alter the lipid composition of microalgae. For example, alkaline pH stress was reported to induce TAG accumulation and a decrease in membrane lipids that was not dependent on nitrogen or carbon limitation levels (Guckert and Cooksey 1990; Sharma *et al*. 2012). Based on morphological observations, alkaline pH inhibited the growth of microalgae, thus diverting the energy to form TAG (Guckert and Cooksey 1990; Sharma *et al.* 2012). The actual mechanism of lipid accumulation due to pH-induced stress is not yet known (DeBhowmick *et al.* 2015). But a few reports like that of Guckert and Cooksey (1990) predicted that alkaline pH reduces the autospore release from cell thereby inducing lipid accumulation. This finding was further supported by the morphological observations by Gardner *et al.* (2011) and also by Shah *et al.* (2013) (Rai *et al*. 2015). The effects of pH on the lipid and FA composition were also studied in an extremophile

strain *Chlamydomonas* sp. isolated from a volcanic acidic lake (Tatsuzawa *et al*. 1996; Sharma *et al.* 2012), and FAs of polar lipids resulted more saturated than in the control. The increase in saturation of fatty acids in membrane lipids of such alga has been suggested to represent an adaptive reaction at low pH to decrease membrane lipid fluidity (Tatsuzawa *et al.* 1996; Sharma *et al.* 2012).

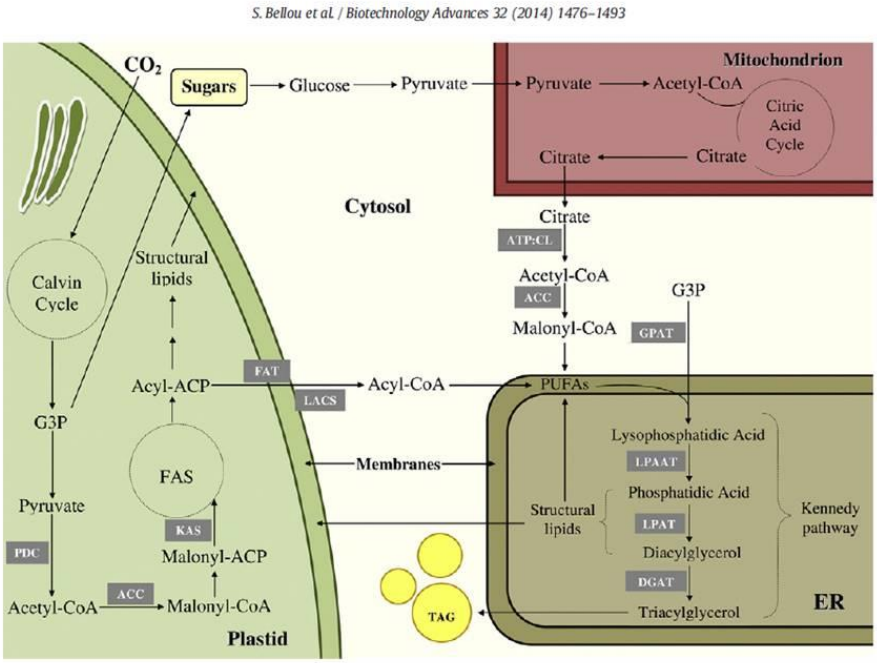


Fig. 6 Depiction of physicochemical parameters leading towards lipid accumulations in microalgal cell factory. Source DeBhowmick *et al.* (2015).

3 STRAIN SELECTION

The unialgal strain *Coccomyxa melkonianii* SCCA 048, studied in this work was selected from the Sardinian Culture Collection of Algae (SCCA) (Fig. 7) for its capability of growing under extreme cultivation conditions (high heavy metals) and producing high valuable bioproducts such lutein (Pasqualetti *et al.* 2015).

Fig. 7 *Coccomyxa melkonianii* SCCA 048 in the Sardinian Culture Collection of Algae (SCCA) at CINSA (University of Cagliari).

Algal culture collections are irreplaceable and unique source providing consistency and standardized quality material for many types of investigations such as comparative taxonomic, physiological, ecotoxicological and *ex-situ* ecological studies (Day *et a*l. 2004; Koreivienė *et al.* 2012). The Sardinian Culture Collection of Algae (SCCA) structured at Interdipartimental Center of Environmental Science and Engineering (CINSA), University of Cagliari, is characterized that all the strains are from Sardinian region. In fact, the primary mission of SCCA is to accomplish the isolation, identification, characterization, selection and in vitro cultivation of photosynthetic microorganisms from several habitat of interest in Sardinia (Malavasi and Cao 2015). The strains of the collection represent an extremely useful source of algae from Sardinia which can be exploited for laboratory experimentation and future biotechnological applications.

3.1 *Coccomyxa melkonianii* SCCA 048

The unicellular extremophile green alga, named *Coccomyxa melkonianii* SCCA 048, was used in this thesis. *C. melkonianii*, isolated from the highly heavy metals-contaminated river "Irvi" (Arburese mining district, Sardinia, Italy) (Pasqualetti *et al.* 2015; Malavasi *et al.* 2016), was elected as a promising microalga for biotechnological applications principally because of its capability of living at extreme conditions (i.e. high heavy metal concentration) which would represent an advantage for outdoor cultivations. Furthermore, in previous investigations, the strain has been yet identified for its rich carotenoid profile, especially lutein (up to 80% of total carotenoids) (Pasqualetti *et al.* 2015).

The strain SCCA 048 was taxonomically characterized by phylogenetic analysis on 18S SSU rDNA and ITS rRNA sequences, and the newly obtained gene region was deposited in the GenBank database under accession number KU696488.1 (Malavasi *et al.* 2016), and finally classified as follows:

Empire:	**Eukaryota**
Kingdom	Plantae
Subkingdom	Viridiplantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	Trebouxiophyceae ordo incertae sedis
Family	Coccomyxaceae
Genera	*Coccomyxa*
Species	*Coccomyxa melkonianii* V.Malavasi & P.Skaloud

3.2 Trebouxiophyceae

The green algae are photosynthetic eukaryotes characterized by the presence of chloroplasts with two envelope membranes, stacked thylakoids and chlorophyll **a** and **b**. The green algae are one of the most diverse groups of eukaryotes with a worldwide distribution. The green algae represent one of the most successful groups of photosynthetic eukaryotes, but compared to their land plant relatives, surprisingly little is known about their evolutionary history. This is in great part due to the difficulty of recognizing species diversity behind morphologically similar organisms (Lemieux *et al.* 2014). Early hypotheses on green algal phylogeny were based on morphology and ultrastructural data derived from the flagellar apparatus and cell division (Pröschold and Leliaert 2007). These

ultrastructural features, which apply to most green algae, supported the existence of the Streptophyta and Chlorophyta. The Chlorophyta includes the most investigated species of green algae. The Streptophyta consist of charophytes, a paraphyletic assemblage of freshwater algae, and land plants (Leliaert *et al.* 2012). On the other hand, traditionally, four distinct classes can be recognized within the Chlorophyta, i.e. the freshwater or terrestrial Trebouxiophyceae and Chlorophyceae, the coastal Ulvophyceae, and the unicellular, predominantly marine planktonic Prasinophyceae (cf. Fig. 8).

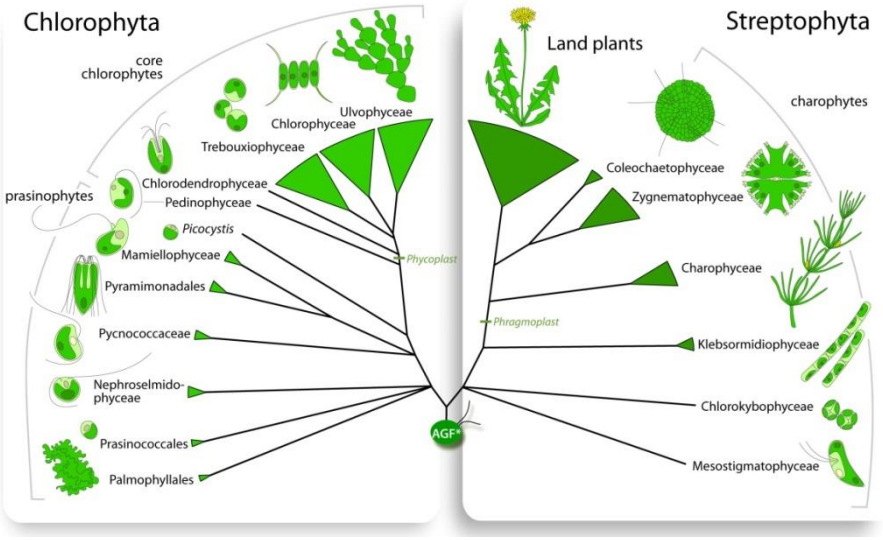


Fig. 8 Overview phylogeny of the green lineage (top) and spread of green genes in other eukaryotes (bottom). Source (Leliaert *et al.* 2012).

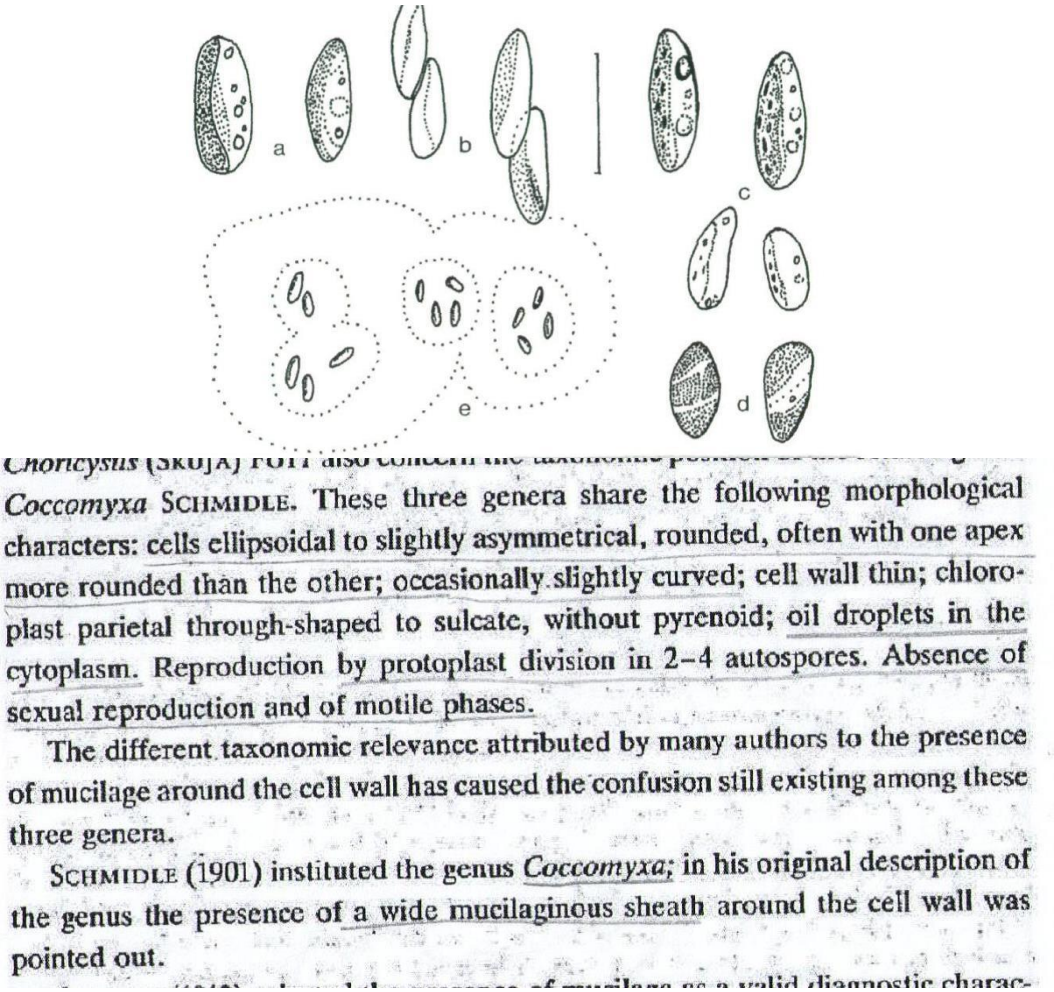
It has been postulated that the Prasinophyceae have given rise to the Ulvophyceae, Trebouxiophyceae, and Chlorophyceae (UTC). Later, phylogenetic analyses based on the nuclear-encoded small subunit rRNA gene (18S rDNA) largely corroborated these hypotheses. Moreover the class Trebouxiophyceae "*sensu stricto*" is not a monophyletic group (Lemieux *et al.* 2014). This species-rich class displays remarkable variation in both morphology (comprising unicells, colonies, filaments, and blades) and ecology (occurring in diverse terrestrial and aquatic environments). Moreover, there are some genera that include unicellular non-flagellated parasites/pathogens that still retain vestigial plastids (Figueroa-Martinez *et al.* 2014).

The class Trebouxiophyceae includes organisms that participate in lichen symbioses (lichen phycobionts), and a growing number of free-living planktonic or terrestrial species (Lewis and McCourt 2004). Members of Trebouxiophyceae reproduce asexually by autospores or zoospores.

Sexually reproductive stages have not been directly observed in any of the trebouxiophyte algae (Lewis and McCourt 2004). The Trebouxiophyceae also involves species that have lost photosynthetic capacity and have evolved towards free-living or parasitic heterotrophic lifestyles (e.g. *Prototheca* and *Helicosporodium*). Aside from their intrinsic biological interest, trebouxiophycean algae have drawn the attention of the scientific community because of their potential utility in a variety of biotechnological applications such as the production of biofuels or other molecules of high economic value (Hannon *et al.* 2010; Mata *et al.* 2010).

3.3 The genus *Coccomyxa*

The genus *Coccomyxa* comprises unicellular species that occur either as free-living cells (Verma *et al.* 2009; Blanc *et al.* 2012; Hrdinka *et al.* 2013; Mthakathi *et al.* 2015; Malavasi *et al.* 2016; Barcytė and Nedbalová 2017) in soil (Ismail *et al.* 2015) or aquatic ecosystems (Hrdinka *et al.* 2013; Barcytė and Nedbalová 2017) or in symbiotic or parasitic association with fungi, higher plants and animals (Peveling and Galun 1976; Guschina *et al.* 2003; Guschina and Harwood 2006a; Trémouillaux-Guiller and Huss 2007; Syasina *et al.* 2012; Wieners *et al.* 2012). *Coccomyxa* genus has a worldwide distribution, can also form biofilms, can be dominant in certain ecosystems and displays a remarkable versatility in habitat and lifestyle (John *et al.* 2002; Darienko *et al.* 2015; Malavasi *et al.* 2016; Barcytė and Nedbalová 2017). Species of *Coccomyxa* were common contaminants in laboratory chemical solutions (Darienko *et al.* 2015; Pasqualetti *et al.* 2015) and have been isolated in extreme environments such as polar regions (Blanc *et al.* 2012), extreme acidic- or heavy metal polluted habitats (Garbayo *et al.* 2012; Barcytė and Nedbalová 2017) and even in the spent fuel cooling pool of a nuclear reactor (Rivasseau *et al.* 2013, 2016). This genus became a model organism because its whole genome sequence has been published (Blanc *et al.* 2012) and several biotechnological patents have been registered for it (Darienko *et al.* 2015). The genus *Coccomyxa* was described by Schmidle (1901) (Fig. 9), and the holotype species is *Coccomyxa dispar* Schmidle now become *Coccomyxa confluens* (Kützing) Fott.



Choricystis (SKUJA) FOTT also concern the taxonomic position of the [illegible]
Coccomyxa SCHMIDLE. These three genera share the following morphological characters: cells ellipsoidal to slightly asymmetrical, rounded, often with one apex more rounded than the other; occasionally slightly curved; cell wall thin; chloroplast parietal through-shaped to sulcate, without pyrenoid; oil droplets in the cytoplasm. Reproduction by protoplast division in 2–4 autospores. Absence of sexual reproduction and of motile phases.

The different taxonomic relevance attributed by many authors to the presence of mucilage around the cell wall has caused the confusion still existing among these three genera.

SCHMIDLE (1901) instituted the genus *Coccomyxa;* in his original description of the genus the presence of a wide mucilaginous sheath around the cell wall was pointed out.

[illegible] of mucilage as a valid diagnostic charac-

Fig. 9 Description of the *Coccomyxa* genus. Source Albertano *et al.* (1990); Gartner and Ernet (1993).

Representatives of this genus are characterized by a small size (6–14 x 3–6 μm), an irregular elliptical to globular cell shape, a parietal chloroplast without a pyrenoid and the absence of any flagellated stages (Jaag 1933; Darienko *et al*. 2015; Malavasi *et al.* 2016). The taxonomy of this genus has long been difficult. Due to the scarcity of morphological characters useful for taxonomic purposes and to the environmentally morphological variability, this genus (and its separation from the similar genera *Pseudococcomyxa* and *Choricystis*) using only morphological features has been traditionally problematic. Recent DNA-based and ecological analyses split this genus into 27 species much more narrowly defined (Fig. 10) (Malavasi *et al.* 2016).

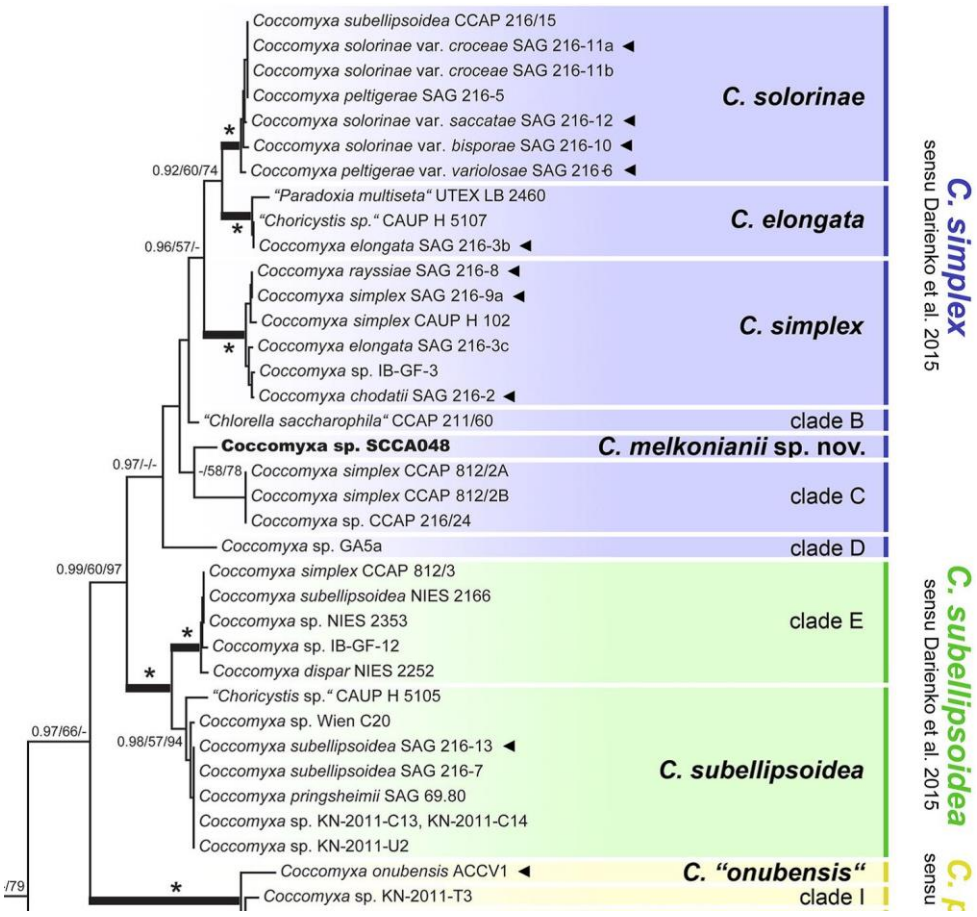


Fig 10 A part of phylogeny of the genus *Coccomyxa* (Bayesian inference of the concatenated SSU and ITS rDNA dataset). Source Malavasi *et al.* (2016)

Information upon physicochemical characterization of this microalgal genus is available in literature. Honegger and Brugger (1981) reported the study of the cell walls of *Coccomyxa* phycobionts of various lichens investigated with cytological and chemical methods. Three different wall layers were observed: an outer wall layer, uniformly thick, appears electron-dense and exhibits short, probably cellulosic fibrils embedded in an amorphous matrix. Beyond these is an outermost trilaminar wall layer of uniform thickness that contains sporopollenin in its middle part. Proteinlike particles are embedded in an amorphous, carbohydrate-containing matrix on its electron-dense inner and outer surfaces. IR spectrophotometry yielded data comparable with those of other sporopollenin-containing algal walls (Honegger and Brugger 1981). The free-living *Coccomyxa dispar*, type-species of the genus, differs from the lichen phycobionts for the presence of a gelatinous sheath (Honegger and Brugger 1981).

3.4 *Coccomyxa melkonianii* SCCA 048 morphology

The morphology of *Coccomyxa melkonianii* SCCA 048, described by Malavasi *et al.* (2016), is briefly reported in what follows (Fig. 11). The vegetative cells are narrowly ellipsoidal and slightly asymmetrical, 3–5.4 wide and 6–8.5 µm long, regularly curved, with rounded apices and without mucilaginous sheath. The chloroplast is parietal, cup-shaped and covers much of the inner cell wall surface. The pyrenoid is absent. The nucleus is positioned in the central part of the cell and the protoplast is filled with lipid droplets.

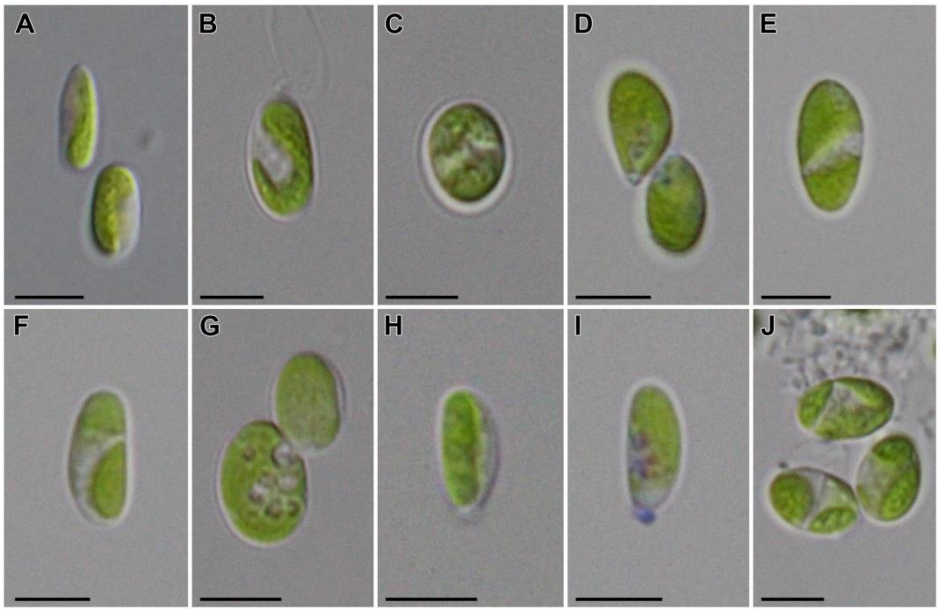


Fig. 11 Morphology of *Coccomyxa melkonianii* SCCA048. A-D. Morphological plasticity of vegetative cells, E-F. Cells containing two plastids, G. Accumulation of lipid droplets in the cytoplasm, H-I. Formation of small mucilaginous caps, J. Mature cells (note the autospore formation at the bottom left cell). Scale bars = 5 µm. Source Malavasi *et al.* (2016).

3.5 Growth medium and biomass measurements

The growth medium used for the experiment carried out in our research was the Bold Basal Medium (BBM) (Bischoff and Bold 1963), which is a broad-spectrum medium for freshwater algae. The BBM composition is reported in the following Fig. 12.

(Bold 1949, Bischoft & Bold 1963)

#	Component	Final concentration	Stock solution ($g \cdot L^{-1}$ dH_2O)	Addition per 1 Litre culture medium
1	$NaNO_3$	2.94 mM	25.00	10 ml
2	$CaCl_2 \cdot 2H_2O$	0.17 mM	2.50	10 ml
3	$MgSO_4 \cdot 7H_2O$	0.3 mM	7.50	10 ml
4	K_2HPO_4	0.43 mM	7.50	10 ml
5	KH_2PO_4	1.29 mM	17.50	10 ml
6	NaCl	0.43 mM	2.50	10 ml
7	Alkaline EDTA solution EDTA (Titriplex III) KOH	 17.10 mM 55.30 mM	 50 g 31 g	1 ml
8	Acidified Iron solution $FeSO_4$ x 7 H_2O H_2SO_4	 0.179 mM	 4.98	1ml
9	Boron solution H_3BO_3	 18.50 mM	 11.42	1ml
10	Trace Metals solution $ZnSO_4$ $7H_2O$ $MnCl_2$ $4H_2O$ MoO_3 $CuSO_4$ $5H_2O$ $Co(NO_3)_2$ $6H_2O$	 8.82 1.44 0.71 1.57 0.49	 0.307 µM 7.28 µM 4.93 µM 6.29 µM 1.68 µM	1ml

Fig. 12 Chemical composition of the BBM growth medium

The freshwater medium was prepared from premixed stock solutions. Reagent grade chemicals and bidistilled water were used to make stock solutions of enrichment. Aliquots from these stocks were then measured and added to the given volume of water.

The growth of microalgae was monitored through *in vivo* absorbance spectrophotometric measurements (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham), of the Chlorophyll a optical density (OD) of the culture, at 663 nm wavelength (Geis *et al.* 2000; Podda *et*

al. 2000; Hosikian *et al.* 2010) with 1 cm light path. The biomass concentration Cb (g_{dw} L^{-1}) was calculated from such OD measurements using the Cb vs. OD calibration curve reported below (Fig.13).

The following calibration curve (Fig. 13) was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes that were previously centrifuged at 4000 rpm for 15 min and then dried in an oven at 105°C for 24 h.

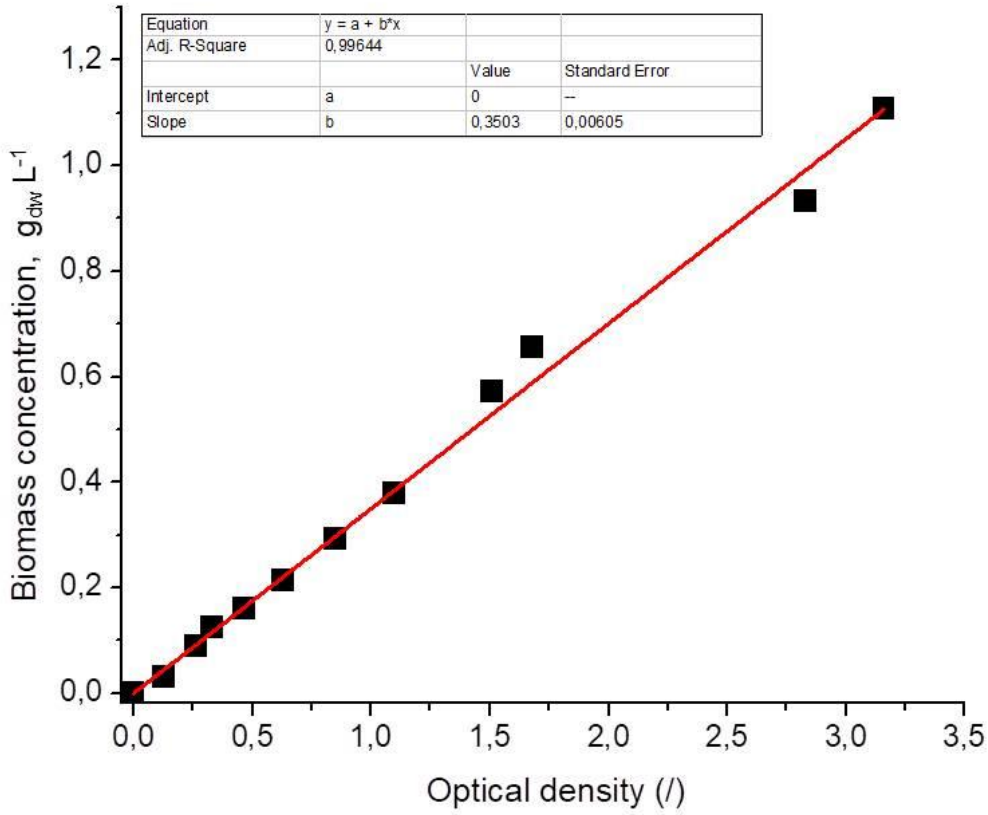


Fig.13 Calibration line showing the correlation between the optical density (OD) at 663 nm measurements and the corresponding biomass dry weight content (experimental data).

3.6 Spectrophotometric analysis of lipid content during growth

As far, multiple methods have been reported for the quantification of microalgal lipids, mainly including the conventional gravimetric method using extraction solvents, Nile red lipid visualization method, SPV, and TLC (Chen *et al.* 2009, 2018a; Cheng *et al.* 2011; Sitepu *et al.* 2012; Mishra et *al.* 2014). In the present study, the rapid colorimetric method proposed in the literature (Mishra *et al.* 2014) and based on the use of Sulfo-Phospho-Vanillin (SPV) has been adopted to quantify the lipid content of microalgae during their growth. It is based on the capability of sulfo-phospho-vanillin (SPV) to react with lipids to generate a reaction product which is characterized by a typical pink color whose intensity can be quantified using spectrophotometric methods, i.e. by measuring absorbance at 530 nm (cf. Fig. 14). This colorimetric SPV method is a rapid alternative for lipid measurement because of its fast response and relative ease in sample handling (Inouye and Lotufo 2006; Chen *et al.* 2018b).

Sulfo-phospho-vanillin (SPV) reaction was first introduced by Chabrol and Charonnat in 1937 and was used as a standard routine for estimation of total lipids in human cerebrospinal fluid (Vatassery *et al*. 1981). An improved version of the SPV reaction was introduced; including a colorimetric method for the quantification of total lipid within a sample was developed by (Drevon and Schmit 1964). As of today, the sulfo-phospho-vanillin reaction is a widely utilized tool in medical field and has been successfully employed for a rapid quantification of intracellular lipid contents within *Chlorella* sp., *Monoraphidium* sp., *Ettlia* sp., and *Nannochloropsis* sp. (Mishra *et al.* 2014).
The procedure is schematically reported in Fig. 14. Phosphovanillin reagent was prepared by initially dissolving 0.6 g vanillin (Sigma-Aldrich, St. Louis, MO, USA) in 10 ml absolute ethanol; 90 ml deionized water and stirred continuously.

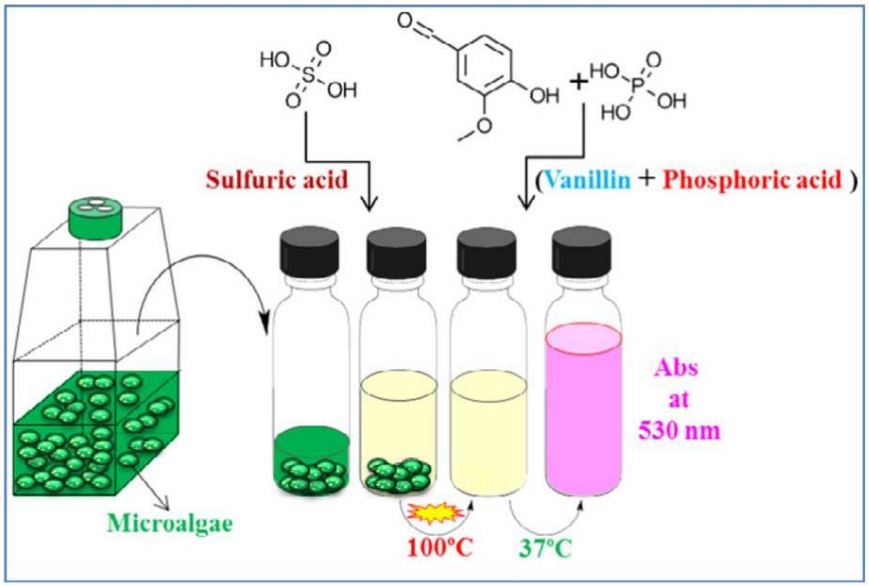


Fig. 14 Schematic representation of colorimetric method for lipids quantification which employs sulfo-phospho-vanillin Mishra *et al.* (2014).

Subsequently, 400 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run. For SPV reaction of the algal culture for lipid quantification, a known amount of dried biomass was re-suspended in 100 µl deionized water. A further step, wherein the algal cells were lysed by sonication using an ultrasonic bath for 30 min before quantification, was carried out with respect to the previous literature (Concas *et al.* 2016). Subsequently, 2 mL of concentrated (98%) sulfuric acid was added to the sample. The resulting solution was heated for 10 minutes at 100 °C and was cooled for 5 minutes in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 minutes at 37 °C incubator shaker at 200 rpm.

Afterward, the absorbance at 530 nm was measured and then translated in terms of lipid content of the sample by using a calibration curve, i:e: lipids (mg) = $0.209 \cdot OD_{530} + 0{,}017$; which was previously obtained by performing the SPV reaction with known amounts of lipid created by dissolving canola oil in chloroform according to the procedure proposed in the literature (Mishra *et al.* 2014; Concas *et al.* 2016) and reported below.

The calibration curve was obtained as follows. About 20 mg of commercial canola oil was dissolved in 10 ml chloroform (final concentration, 2 mg/ml) and the resulting solution was stored at −20 °C before use.

A known amount of lipids (oil) was added in the empty tube which was then kept at 60 °C for 10 minutes in order to evaporate the solvent. Subsequently, 100 µl of water was added to the lipid standard and the resulting solution was then subjected to the spectrophotometric analysis above described. Several samples with different amounts of lipids were assessed thus allowing to obtain the calibration curve shown in Fig. 15.

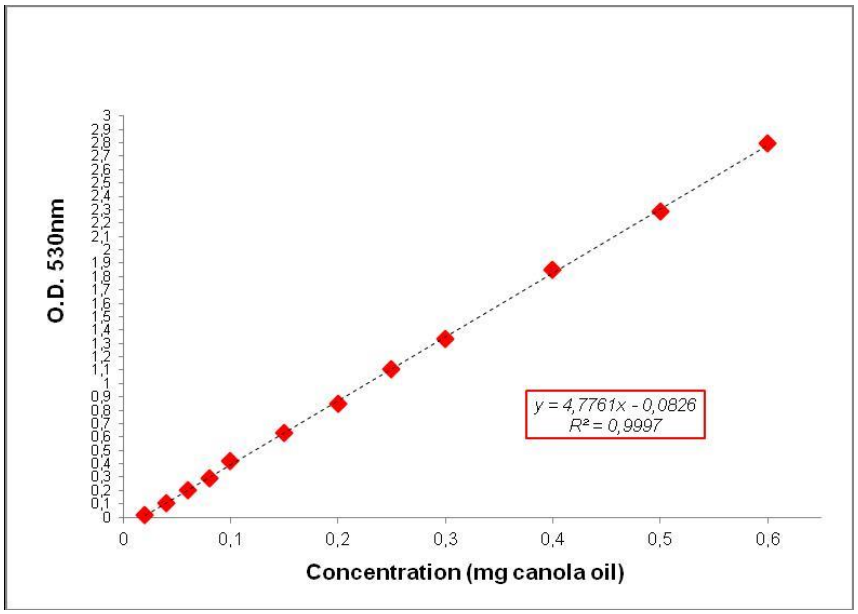


Fig. 15 Calibration curve of lipid standard solutions (canola oil) adopted in this thesis work.

As it can be observed a quite good correlation is achieved between optical density at 530 nm and lipid content of the sample. Therefore, the use of this calibration line allows to confidently assessing the lipid content of a sample by using spectrophotometric techniques.

The SPV method was then used to evaluate the lipid content of *C. melkonianii* during its batch growth.

3.7 Lipid extraction

Several types of lipid extraction processes exist, but there is not yet one ideal method, and the effectiveness of each process varies based on the lipid content of the microalgae cell (Dickinson *et al.* 2017). Conventionally, microalgae is harvested, dried and pre-treated for cell disruption and fed to the extraction unit where oil is extracted with some suitable solvent (Chaudry et al., 2015). . Typically, solvent extraction is carried out by contacting microalgal biomass with an organic eluting solvent which diffuses through the cell wall/membrane into the cytoplasm and interacts, through van der Waals type bindings, with the neutral lipids by forming organic solvent-lipids complexes. The latter ones, driven by a concentration gradient, counter-diffuses across the cell wall towards the bulk solvent from which they can be collected to be further processed (Halim *et al.* 2011, 2012). Solvent extraction of algal lipids can be performed starting from both wet and dry microalgal biomass and, depending upon which option is chosen, specific pre-treatments should be carried out. In fact, lipid extraction from untreated wet biomass is characterized by low yields due to the immiscibility of water with the organic solvents. Therefore, when solvent extraction is applied to wet biomass, the microalgal cells tend to remain in the water phase due to their surface charges and thus they cannot contact the organic solvent phase which is able to extract lipids (Kim *et al.* 2013). Fortunately, this phenomenon can be prevented by breaking the cell wall of microalgae to provoke the release of intracellular lipids into the extracting mixture, thus facilitating the access of solvent to lipids. Therefore, once released from the algal cell, lipids are able to pass to the solvent phase from which they can be collected after evaporation of the solvent. We use a simple and low energy consuming technique for cell disruption, based on the use of low toxicity and cheap reactants such as H_2O_2. The wet biomass was resuspended in 1 ml of a 1/40 (v/v) solution of H_2O_2 for 4 minutes under agitation. Hence, neutral lipid extraction was performed directly on the wet disrupted biomass according to a method proposed by Steriti *et al.* (2014) (Fig. 17). The method consists firstly of diluting 1/10 the mixture of wet-disrupted biomass and disruption solution with ethanol (96% v/v) while assuring the contact for 18 hours under continuous stirring. As mentioned above, this step allowed also stopping the disruption reaction. The resulting hydro-alcoholic solution was then subjected to centrifugation in order to separate solid residuals (i.e. pieces of broken cells, organelles, etc.) from the supernatant liquid where lipids were transferred.

As far as the specific reactive mechanisms involved during the cell wall disruption process, only some assumptions have been formulated by Steriti *et al.* (2014). Among them, the most realistic one is that H_2O_2 might generate the Fenton's reaction shown in Fig. with the Fe^{2+} ions present in the liquid solution. In fact, $FeSO_4$ was used to prepare the growth medium which constitutes the liquid

phase of the wet biomass subjected to disruption. According to Wu *et al.* (2010), the reaction between H_2O_2 and Fe^{2+} ions can produce hydroxyl radicals which in turn may attack and degrade the organic compounds constituting the cell wall according to the simplified mechanism shown in Fig. 16. Probably, such a reaction occurs preferentially in specific zones of the cell wall constituted by organic compounds that are easily oxidized by OH radicals. In fact, microscopic analysis confirmed that rupture took place in certain areas of the cell wall and led to the release of the intracellular material, including lipids, in the liquid bulk of the disrupting solution. Once transferred in the liquid bulk, even lipids might be attacked by hydroxyl radicals, as schematically shown in Fig. 16, thus generating degradation products such as for example lipid peroxides (González *e*t al. 2012). The extent to which such undesired reaction proceeds depends upon the residual concentrations of H_2O_2 and Fe^{2+} as well as upon the time elapsed before ethanol is added in order to stop it (Steriti *et al.* 2014).

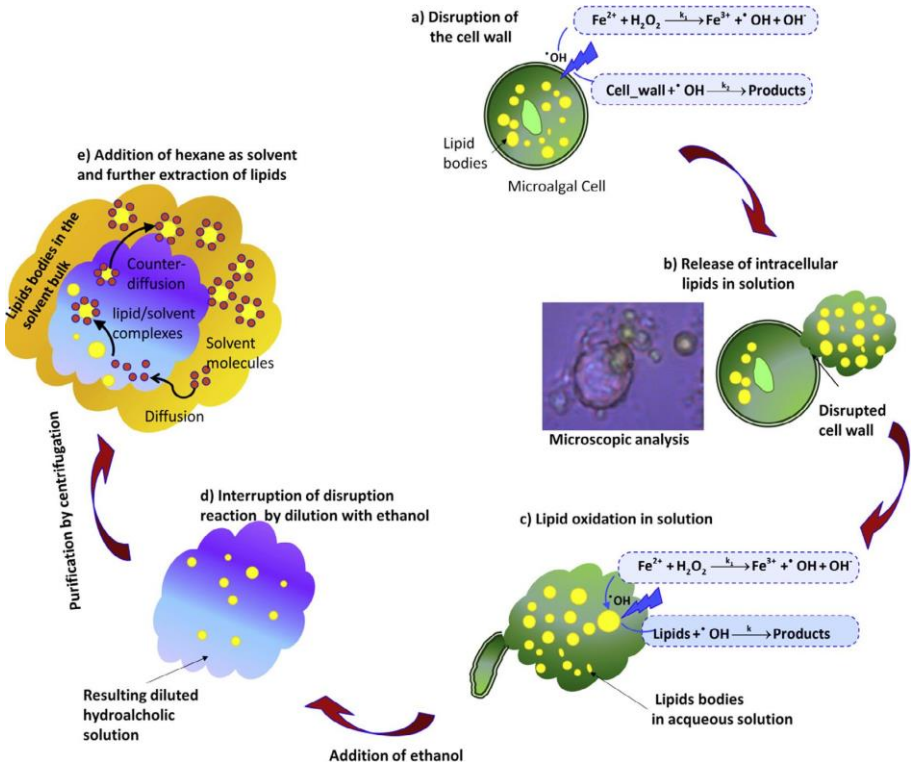


Fig. 16 Scheme of the mechanism assumed to influence cell disruption and lipid extraction yields. Source Steriti *et al.* (2014).

The lipid-rich supernatant was then suitably stored while the residual solid was further contacted with ethanol for 1 hour under stirring in order to extract residual lipids remained in the solid phase. Then, the polar and non-polar phases were separated (Fig. 17), the extracted oil was evaporated to dryness in a rotary evaporator flask and ready for GC-FID analysis of the fatty acid methyl esters composition.

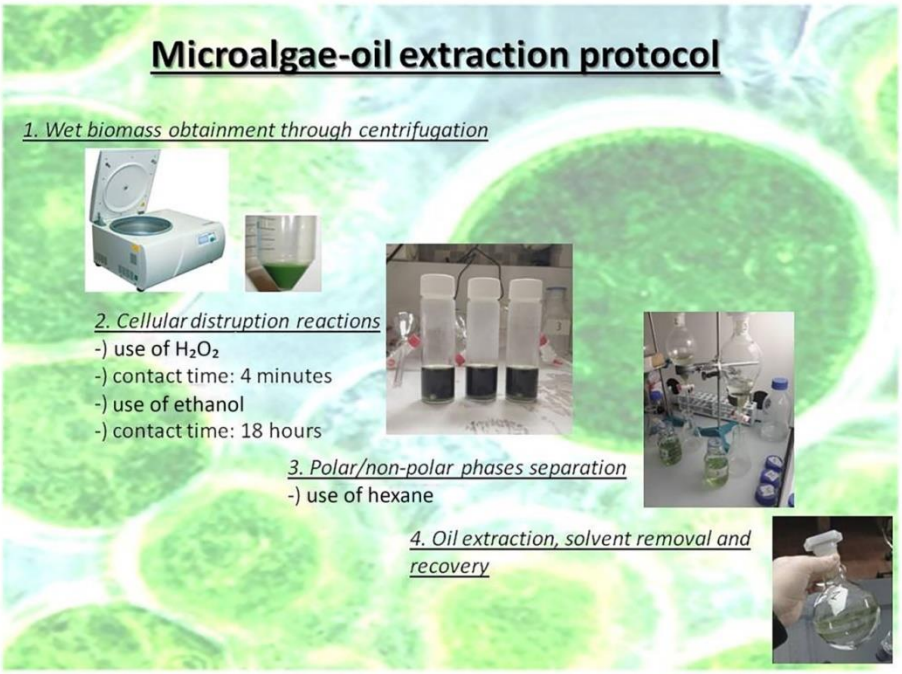


Fig. 17 Schematic representation of the lipid extraction method adopted in this thesis work.

4 Objectives and approach

The objective of this thesis is the characterization of the growth of the extremophile microalgae strain *Coccomyxa melkonianii* SCCA 048 from the Sardinian Culture Collection of Algae (SCCA) for the production of biomass and high-value compounds such as lipids and the identification of the better cultivation condition for the possible large-scale production.

The approaches adopted during the research are summarized in Fig.18.

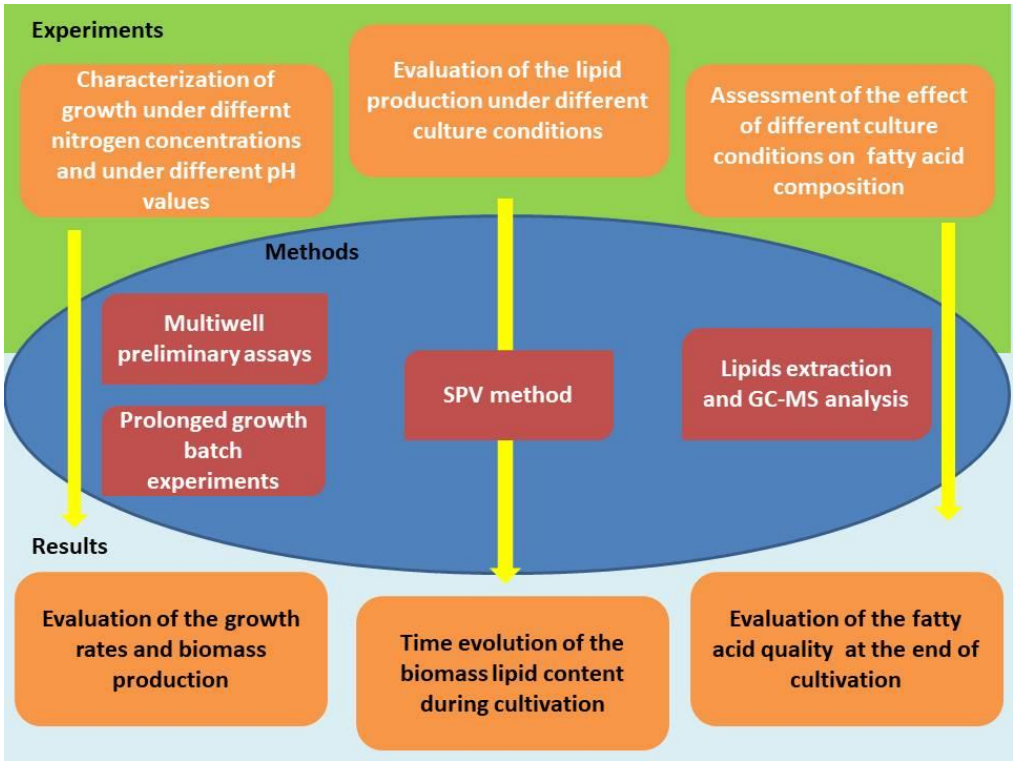


Fig. 18 Overview of the approaches taken during this thesis.

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Chapter III

Growth and lipid production of the extremophile microalga *Coccomyxa melkonianii* SCCA 048 under nitrogen starvation conditions

1. Introduction

Microalgae are currently considered a potential feedstock for the sustainable production of a wide range of consumer goods such as biofuels, nutraceuticals, pharmaceuticals, bioplastics, functional food, lubricants for industrial applications and food for aquaculture systems (Concas *et al.* 2016a; Hirooka *et al.* 2016). Moreover, when compared to crop, microalgae are characterized by higher photosynthetic efficiency and biomass productivity as well as the capability to grow in non-arable/arid lands by exploiting wastewaters and CO_2 as source of nutrients. Accordingly, microalgae cultivation may lead to positive side effects related to the reduction of water and atmospheric pollution (Larkum *et al.* 20112). Despite these aspects, the current technology is still affected by high production costs. As a result, the industrial scale exploitation of this technology has been so far limited (Concas *et al.* 2016b). Therefore, the current main challenge in the field of microalgae-technology is to identify low-cost technology to perform the cultivation and extraction steps. As far as the mass cultivation step is concerned, closed photobioreactors maximize the production of desired compounds but are still characterized by high operating costs. Open raceways are very simple to build and operate and currently represent the most economical solution in terms of both investment and management costs (Concas *et al.* 2014). Moreover, open raceways might use wastewater as a nutrient source to lower cultivation costs. The major problem in open pond systems is contamination by undesirable microorganisms, including algal predators, which could lower productivity. To face this challenge, several researchers have proposed a selective culturing strategy which uses extreme operating conditions that can be tolerated by the target strain, but are lethal for invasive or competitive species (Do Nascimento *et al.* 2012; Larkum *et al.* 2012; Ruiz-Dominguez *et al*. 2015; Hirooka and Miyagishima. 2016; Wang *et al.* 2017). Extremophile algae are considered to be very promising in this regard. Because these algae can tolerate extreme conditions, they can be cultivated in open ponds with lower contamination risks thus permitting to exploit the economic advantages of open raceways. Extremophile algae can be easily isolated from highly acidic environment contaminated by acid mine drainage (AMDs). Such environments are known to support an unsuspected microbial biodiversity (bacteria, archaea, microalgae) although the validated species of acidophilic organisms are still very limited as compared with those ones in neutral environments (Garbayo *et al.* 2012; Hirooka *et al.* 2014; Skaloud *et al.* 2014; Hirooka and Miyagishima. 2016;). In a floristic investigation carried out in a mining site of Sardinia (Italy), an extremophile microalga *Coccomyxa melkonianii* SCCA 048 was found, isolated and subsequently characterized from the morphological, phylogenetic and taxonomic point of view (Malavasi *et al.* 2016). In this site, several decades of intense mining left huge amounts of waste material and

tailings that have caused serious ground and surface water pollution problems (Fanfani *et al.* 2000; Cidu and Rau 2010; Frau *et al.* 2017). In particular, acid mine drainage (AMD) phenomena took place in the concerned environment and consequently high heavy metal concentrations were transferred from mining wastes to river waters. Previous studies were conducted in the Rio Naracauli creek, a contiguous river affected by the same phenomena (Podda *et al.* 2000; Biddau *et al.* 2001; Cidu and Rau 2010; Frau *et al.* 2015, 2017). In the Rio Naracauli creek, a photosynthetic microbial population was found to colonize sediments in spring and deposit a white mat on the creek bed itself. The two photosynthetic microorganisms discovered were *Scytonema* sp., a cyanobacteria and *Chlorella* sp., a green alga. These algae adapted to high levels of toxic metals and suggested the role of their photosynthetic metabolism in the mechanism of metal sequestration and pH modulation (Podda *et al.* 2000). The eukaryotic strain *C. melkonianii* can be seen as extremophile due to its capability to tolerate high concentrations of toxic heavy metals (Malavasi *et al.* 2016). It has been demonstrated that this strain is capable of expressing large amounts of lutein (Pasqualetti *et al.* 2015) which make it potentially useful in a bio-refinery framework. For these reasons, *C. melkonianii* represents a potential candidate for large-scale cultivation in open raceways. However, to the best of our knowledge, no information exists in literature about its growth kinetics and its capability to synthesize valuable lipids. As a result, no useful information is yet available in the literature about the performance of this strain in industrial frameworks. For these reasons, the pH-dependent growth kinetics of *C. melkonianii* have been quantitatively investigated in this work with the aim of obtaining crucial information about the profitability of its cultivation in large-scale devices under extreme conditions. Because lipid synthesis is affected by nitrogen availability, the nitrate-dependent growth kinetics were also investigated. Finally, lipids synthesized during growth have been quantitatively characterized and profiled in terms of fatty acids composition to verify whether valuable chemicals could be produced through this alga. It should be noted that only few studies have been devoted to the assessment of lipid accumulation and biomass production in extremophile *Coccomyxa* genus under different nitrogen concentrations (Msanne *et al.* 2012; Abe *et al.* 2014; Tevatia *et al.* 2014; Allen *et al.* 2015 Wang *et al*. 2017) and, only one of them involves an acid-tolerant strain *Coccomyxa onubensis* (Ruiz-Dominguez *et al.* 2015). A cultivation strategy of *C. melkonianii* based on the manipulation of nitrogen concentrations has been applied in this work, in order to boost lipid synthesis (Soru *et al.* 2018). As mentioned above, nitrogen starvation leads to the imbalance of carbon and nitrogen content within the cell which, as a response, activates specific metabolic processes aimed to store the excess carbon into high energy molecules, such as lipids (Concas *et al.* 2016a). While these complex

biochemical phenomena are still not completely understood, it is now well recognized and experimentally proved that nitrogen starvation can trigger lipid accumulation in microalgae cells. Hence, a deep investigation about the effect of nitrogen depletion on lipid productivity is required, especially for recently characterized species like *C. melkonianii*. Accordingly, a series of experiments have been performed where this strain was grown under different nitrogen concentrations and the synthesized lipids were correspondingly monitored in time. During such experiments, particular attention has been devoted to the assessment of the effect of nitrogen concentration on the final quality of FAMEs (Fatty Acid Methyl Esters) obtained by transesterification of lipids. The obtained experimental results have confirmed the possibility to exploit *C. melkonianii* for industrial applications (Soru et al. 2018).

2. Materials and methods

2.1. Microorganism sampling and maintenance conditions

The freshwater algal strain used in this work is *Coccomyxa melkonianii* SCCA 048, which was originally isolated from highly contaminated mine waters of river Irvi located in the abandoned mine area of Montevecchio (SW Sardinia, Italy) shown in Fig. 1. According to earlier environmental characterizations, the river shows pH values ranging from 3 to 7 along its course, as well as high concentrations of toxic heavy metals. Iron is primarily responsible for the reddish-brown color of the water (Malavasi *et al.* 2016). The alga was collected by scraping reddish ferrous material from a rock at the river edge, where the water pH was 6.85. The collected sample was stored in plastic flasks and then isolated as reported in the literature (Malavasi *et al.* 2016). Finally, stock cultures were propagated in Erlenmeyer flasks or in tubes with a Bold's Basal Medium (BBM) (Bischoff and Bold 1963), WARIS-H culture medium or modified WARIS-H (McFadden and Melkonian, 1986) culture medium without soil extract. The strain is maintained at the SCCA Sardinian Culture Collection of Algae (Malavasi and Cao 2015) under axenic conditions.

2.2 Screening experiments in multiwell plates

To test the ability of *Coccomyxa melkonianii* to grow under different nitrogen concentrations and pH values , and to select which conditions could led to the better growth rates, preliminary screening tests were performed. The microalgal bioassay was based on standard methodologies (ISO 8692/12; OECD 201/11). In particular, 72 h growth tests screenings were carried out by inoculating about 10^4 cell mL^{-1} of this strain into 24-welled multiwell plates (Primo® EUROCLONE ET3024, Italy) by using test solution volumes of 2 mL of BBM medium in each well. A pre-culture of 4-7 days was prepared as inoculum for the test and used when cells were exponentially growing. Such pre-culture was incubated and maintained under axenic conditions (incubator VELP® SCIENTIFICA, FOC 225E) at 25°C, with a photon flux density of 80-100 μE m^{-2} s^{-1} for a light/dark photoperiod of 12 h (Lightmeter Delta OHM HD2302.0). The pre-culture was continuously treated in an orbital shaker at 100 rpm (Stuart SSM1, Biosigma). Cells morphology was observed by optical light microscopy (Leica Microsystems DM750, Switzerland). Micrographs were acquired by a digital color camera (EC3, Leica Microsystems, Switzerland) equipped with LAS EZ 3.2.1 software (Leica Microsystems, Switzerland). Nitrogen was provided in the form of $NaNO_3$ (Sigma-Aldrich®, Germany) dissolved in bidistilled water and diluted in the BBM culture medium to reach the desired nitrate concentration. Each solution was sterilized in autoclave (Vapormatic mod. 770/A) at 121°C for 15 min before microalgae inoculation. The tests were carried out using nine series of nitrate concentrations ranging from a minimum of 2.50×10^{-4} to a maximum of 1.75 mg L^{-1} (cf. Table S1 in Supplementary materials for details). To evaluate the effect of pH, eight different pH values ranging from 3 to 12 were imposed in the BBM, using HCl or NaOH (cf Table S2 in Supplementary materials for the details). The pHs were not furtherly adjusted during the experiments. In order to preserve axenic conditions throughout the course of the tests, all the operation was conducted under a microbiological safety cabinet (MSC-ADVANTAGE 1,2 Thermo Scientific). The multiwell were incubated under the same conditions of pre-culture and continuously shaken at 30 rpm. Growth was evaluated by spectrophotometric measurements as reported in the following sections. Measurements were performed after 24, 48 and 72 h of cultivation. All experiments were repeated in triplicate for the sake of reproducibility.

2.3. Experiments in batch reactors

The multiwell screening experiments allowed to identify the nitrogen concentrations that inhibited the growth. To produce suitable amounts of algae from which appreciable quantities of valuable lipids could be extracted, further experiments were performed by manipulating nitrogen concentration. These experiments were performed on unialgal cultures, into 2 L Pyrex bottles with a culture media volume of 1.8 L. The experiments lasted for a sufficient time-lapse for the observation of all the main growth phases (lag, exponential, and steady phase). During cultivation, the culture was stirred at 500 rpm using magnetic PFTE stir bars and a magnetic stirrer. The bottles and the magnetic stir bars, as well as culture media, were sterilized in autoclave at 121°C before microalgae inoculation. The bottles were stoppered with plugs wrapped in cotton gauze to prevent external contamination while atmospheric CO_2 within the culture was introduced by an air pump (EHEIM 100 GmbH & Co KG, Germany). Algae were cultured at room temperature and under the same conditions described for the multiwell experiments, i.e. photon flux density of 80–100 $\mu Em^{-2} s^{-1}$ for a light/dark photoperiod of 12 h (Lightmeter Delta OHM HD2302.0) and with an incident light intensity (I_0) equal to 1.00×102 $\mu Em^{-2} s^{-1}$. All experiments were carried out in duplicate; the evaluated experimental error was within 3.0-13.8%. BBM standard was the growth medium used for the cultivation of *C. melkonianii* in the base-case batch experiments. The composition of BBM is reported elsewhere (Bischoff and Bold 1963) and is characterized by an initial concentration of nitrate equal to $0.25\ g_{NaNO_3^-}\ L^{-1}$. However, the nitrate concentration was varied to assess the effect of nitrogen starvation on lipid productivity of *C. melkonianii.* Specifically, the reduction by one-fifth (1/5N-BBM) and the increase by five times (5NBBM), respectively of the initial nitrogen concentration were investigated.

2.4 Biomass, pH and nitrate measurements

The growth of microalgae was daily monitored through *in vivo* absorbance spectrophotometric measurements (Genesys 20 spectrophotometer, Thermo Scientific, Walthman, USA) of the chlorophyll a optical density (OD) of the culture at 663 nm wavelength (Hosikian *et al.* 2010) with 1 cm light path. The biomass concentration *Cx* ($g_{dw}\ L^{-1}$) was calculated from OD measurements using the calibration line C_b vs OD shown and described in Supplementary Materials (Figure S1), which was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes that were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h (Steriti *et al.* 2014). The pH was measured daily by pH-meter (Basic 20, Crison). During the experiments in batch reactors, nitrate concentration was measured about twice per week using

HACH® Lange Probes LCK339 Cuvette-test (D-40549 Dusseldorf, Germany). The probes provide precise and reliable measurement values according to standard: ISO 7890-1-2-1986, DIN 38405 D9-2 and are based on the principle that nitrate ions in solutions containing sulphuric and phosphoric acids react with 2.6-dimethylphenol to form 4-nitro-2.6-dimethylphenol. Inorganic ions and COD below 200 mg/L do not cause interference to the test. Known volumes (i. e. 1 mL) of culture samples were periodically withdrawn from the reactors and then filtered through a Corning® 0.20-μm pore-size sterile syringe filter (Corning, NY 14831) to eliminate the microorganisms. Subsequently the samples were inserted in the cuvette test containing pre-dosed reactants according to the standardized procedure provided by the packaged equipped box. After fifteen minutes the absorbance was measured by spectrophotometer HACH® Lange DR 1900 (D-40549 Dusseldorf, Germany). When required, the filtered culture samples were diluted using demineralized water.

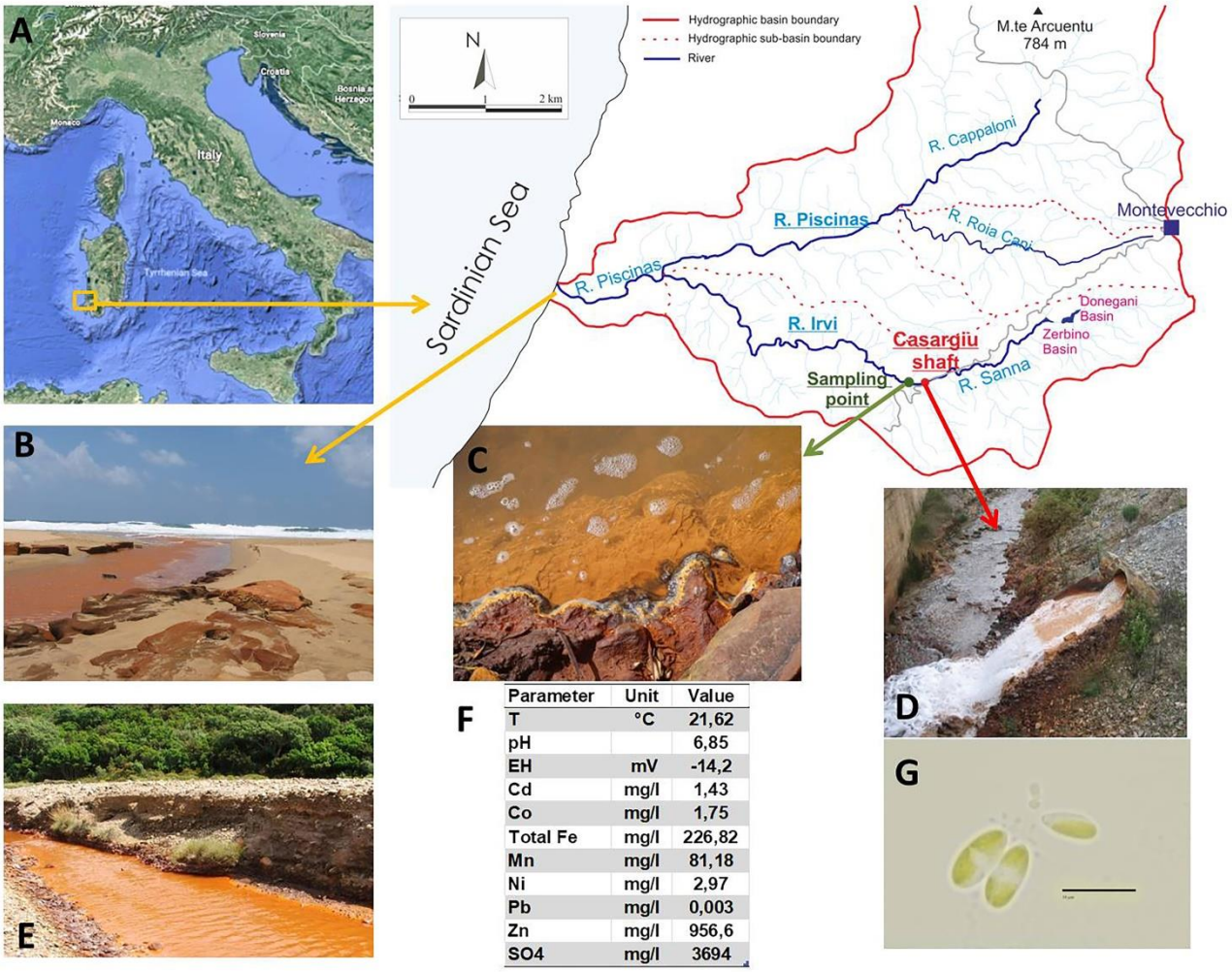
2.5 Lipid colorimetric quantification, cell disruption, lipid extraction, and fatty acid methyl esters analysis

During algae growth, 50 mL of culture were withdrawn about once per week and processed using a colorimetric procedure based on the use of Sulfo-Phospho-Vanillin (SPV) to quantify the lipid content of microalgae (Concas *et al.* 2016a). At the end of each experiment, microalgae were harvested and then centrifuged at 4000 rpm for 15 min (Thermo HERAEUS MEGAFUGE 1.0R) to obtain a concentrated pellet of wet biomass. The exact weight of dry biomass contained in the wet pellets was evaluated using a calibration line as described elsewhere (Steriti *et al.* 2014). Next, wet pellets containing known amounts of dry biomass were subjected to a cell disruption procedure, during which the wet biomass was contacted with selected volumes (1 mL per gram of wet biomass) of a specific solution of H_2O_2 (0.29 M) within a glass tube that was kept sealed and continuously shaken at 500 rpm at room temperature for four minutes. The disruption reaction was then quenched and neutral lipid extraction was performed according to the method reported elsewhere (Concas *et al.* 2015; Concas *et al.* 2016a). The chromatographic analysis of fatty acids of extracted microalgal oil was performed with a Gas Chromatographer Trace (Thermo Finnigan, Rodano, Milan, Italy) equipped with an FID detector, an AS 800 autosampler and a split-splitless injector. The capillary column was a CP-WAX 57CB from Varian (60 m long, 0.25 mm id, and 0.25 mm film thickness; Varian Inc., Palo Alto, CA) operating from 50 to 220°C (13 min) at 3°C/min. The injector and the detector were set at 200 and 280°C, respectively. A 1 μL volume of each sample was injected in the split mode (1:20). Helium was used as carrier gas, and nitrogen for

makeup at 120 and 80 Kpa, respectively. Standard compounds in extracted oil were identified by comparison of their relative retention times and with those of the blend FAME MIX C4-C24 CRM47885 reference substances.

3. Results and discussion

Coccomyxa melkonianii SCCA 048 is a freshwater microalga that has been isolated from the waters of the river Irvi, which is highly contaminated by water mine drainage phenomena and located in the abandoned mine area of Montevecchio (SW Sardinia, Italy), as shown in Fig. 1. The Casargiu drainage flows into the Rio Irvi and merges with the Rio Piscinas after 6 km. The Rio Piscinas then flows into the Mediterranean Sea after about 2 km. The outflow from the Casargiu gallery represents the main water contribution to the Rio Irvi throughout the year (Frau *et al.* 2017). River contamination is mainly caused by the continuous spill of groundwater containing a high concentration of ferrous sulfides into its course. As a result, very high heavy metal concentration was observed in the concerned river waters that eventually enter the sea (cf. Fig. 1B). Water pH and heavy metals concentrations in the sampling point are briefly summarized in the table embedded in Fig. 1F. While pH at the sampling point was equal to about 6.8, the iron oxidation phenomena taking place along the river course, and the subsequent precipitation of iron hydroxides, was able to lower the pH to values below, which, mobilized heavy metals from the tailings abandoned in the river banks (Concas *et al*. 2006; Pasqualetti *et al.* 2015; Malavasi *et al.* 2016).



Parameter	Unit	Value
T	°C	21,62
pH		6,85
EH	mV	-14,2
Cd	mg/l	1,43
Co	mg/l	1,75
Total Fe	mg/l	226,82
Mn	mg/l	81,18
Ni	mg/l	2,97
Pb	mg/l	0,003
Zn	mg/l	956,6
SO4	mg/l	3694

Fig. 1. Habitat, geographic context of the contaminated site where the green alga *Coccomyxa melkonianii* SCCA 048 was sampled and brief summary of water characteristics at sampling point. (A) geographic context of the Sardinia island in the Mediterranean Sea; (B) estuary of river Rio Piscinas spilling contaminated waters when entering the sea; (C) sampling site; (D) groundwater from the Casargiu shaft spilling into the river Rio Irvi; (E) Rio Irvi waters contaminated by water mine drainage; (F) pH, temperature, and concentrations of certain ions in the river water; (G) micrography of *C. melkonianii* SCCA 048 (scale bar: 10 µm).

Because the concerned strain was detected in a contaminated portion of the river course, *C. melkonianii* can be reasonably classified as an extremophile microalga capable of tolerating high heavy metal concentration which might represent a competitive advantage over other organisms that cannot survive under similar conditions.

3.1 Screening experiments in multiwell plates

In this study, the effect of nitrate concentration on the growth kinetics of *C. melkonianii* was investigated. To this aim, two specific sets of experiments were carried out by cultivating the strain

in multiwell plates under different initial concentrations of dissolved nitrates. The multiwell experiment set 1 investigated the nitrate concentration dilution series from 2.50×10^{-1} to 2.50×10^{-4} mg L^{-1}. The multiwell experiment set 2 analysed the nitrate concentration dilution series from 1.75×10^{-0} to 7.50×10^{-1} mg L^{-1} (Table 1). The cultures were then monitored daily up to a total cultivation time of 72 h, i.e. a time that was observed to be sufficient for the occurrence of exponential growth. Results of these experiments are reported in Table 1.

$C_{NO_3^-}$	Time (h)			
mg L^{-1}	0	24	48	72
[a]2.50 x 10^{-4}	4.83 ± 0.29	3.99 ± 0.29	8.295 ± 1.63	13.86 ± 3.56
[a]2.50 x 10^{-3}	4.83 ± 0.29	3.36 ± 1.78	8.19 ± 3.86	15.54 ± 7.72
[a]2.50 x 10^{-2}	4.83 ± 0.29	3.99 ± 2.07	7.35 ± 2.67	12.81 ± 4.45
[a]2.50 x 10^{-1}	4.83 ± 0.29	2.94 ± 1.78	8.40 ± 1.78	14.49 ± 4.45
[b]7.50 x 10^{-1}	3.78 ± 0.00	3.57 ± 0.29	11.97 ± 1.48	20.16 ± 1.18
[b]1.00 x 10^{0}	3.78 ± 0.00	4.62 ± 0.00	10.08 ± 0.59	21.21 ± 2.07
[b]1.25 x 10^{0}	3.78 ± 0.00	3.36 ± 0.00	11.76 ± 0.00	24.78 ± 0.00
[b]1.75 x 10^{0}	3.78 ± 0.00	4.41 ± 2.07	10.71 ± 1.48	19.53 ± 2.67

[a] Multiwell data set 1; [b] Multiwell data set 2.

Table 1 Time evolution of microalgal dry biomass concentration (mg L^{-1}) in the multiwell screening experiment performed with different initial concentrations of nitrate. The results are expressed as mean values ± SD.

From the latter result, it can be demonstrated that, after a lag time of about 24 h, cultures start to grow exponentially up to the end of cultivation. The observed growth delay might be caused by the physiological adjustments of the inoculum to the nitrate concentrations and other culture conditions. It should also be mentioned that these screening experiments in multiwell plates used very low concentrations of algae for a very short period of time, so that the occurrence of CO_2 limitation phenomena is negligible. While considering as starting time t_0 and naming C_0 the corresponding initial nitrate concentration, during the first 24 h of cultivation, a very good linear correlation of $\ln(C/C_0)\ Vs\ (t-t_0)$ was observed as shown in the Table S1 of Supplementary materials. Accordingly, the slope of the linear regression of experimental data, expressed just in terms of $\ln(C/C_0)\ Vs\ (t-t_0)$, provided a quite reliable estimation of the growth rates μ of microalgae under the different nitrates concentrations investigated (cf. Table S1 of Supplementary materials for the detailed description of the calculations). In Fig. 2a, the growth rates obtained as described above are plotted as a function of nitrate concentration. It can be observed that, at very low nitrate

concentration, the growth rate remains very low; thus, the corresponding concentration represents a controlling factor. On the contrary, for nitrate concentrations higher than 2.5×10^{-1} $(mg\ L^{-1})$ the growth rate achieves a kind of plateau of about to 4.00×10^{-2} (h^{-1}). To evaluate the capability of *C. melkonianii* to grow under different pH levels, additional multiwell screenings sets of experiments were performed. It should be noted that in this case, nitrate concentration was fixed at a value unable to trigger starvation phenomena during the 72 h of cultivation. Accordingly, the observed variations in the growth rates could be ascribed only to the different value of pH imposed in the plates. The results of these experiments are reported in Table 2. The obtained data were then elaborated according to same procedure already described for the experiments performed when changing nitrates concentration (cf. Table S2 of Supplementary Materials) and then reported in terms of growth rate as a function of pH in Fig. 2b. From this figure, it can be observed that growth rate is characterized by a bell-shaped trend with a maximum detected at a value close to neutrality, namely $pH = 6.8$. Such an outcome is corroborated by the recent literature (Soru *et al.* 2019). Moreover, the maximum growth rate achieved at neutral pH is very close to the one correspondingly observed in the experiments with changing nitrates concentration, i.e. 4.00×10^{-2} (h^{-1}), thus confirming the reliability of the obtained results. When moving to extreme values of pH, i.e greater than 8 and lower than 5, growth rates of *C. melkonianii* decrease slightly but unlike the majority of microalgal strains, the growth is not suppressed. On the contrary, growth rate stabilizes around asymptotic values of about 2.00×10^{-2} (h^{-1}) both for very high and very low pH, i.e. 12.0 and 3.0, respectively. In most of the tests the experimental error was below 15-20%. Only in two cases a major error percentage was observed. Further experiments and measures will be performed to validate those specific data. The obtained results demonstrate that, although extreme values of pH tend to stress *C. melkonianii*, the cells are capable to adapt and grow even to extreme pH. It is worth noting that the ability to adapt under acidic and alkaline conditions confirms that this strain should be considered as an extremophile microalga not only from the point of view of tolerating the presence of heavy metals, but also for its capability to grow under extreme pH values. Recent studies have shown that the strain is capable of exhibiting good biomass and lipid productivities when it is cultivated in the pH range from 4.0 to 8.0 (Soru *et al.* 2019). It should be mentioned that this algal strain is not an acidophile. In the current literature, the so-called acidophilic algae are able to grow at pH values as low as 0.05 and unable to survive at neutral pH, while the so-called acid-tolerant ones are also able to grow at neutral or even higher pH (Gross 2000; Fuentes *et al.* 2016).

	Time (h)			
pH	0	24	48	72
[a]3	8.54 ± 0.24	6.72 ± 1.45	12.74 ± 2.20	18.06 ± 10.32
[b]4	7.35 ± 2.38	7.77 ± 2.28	13.75 ± 3.96	19.42 ± 6.14
[a]5	8.54 ± 0.24	8.40 ± 1.68	13.72 ± 1.04	24.08 ± 7.56
[c]6.8	5.56 ± 1.98	6.35 ± 2.40	13.28 ± 1.67	29.19 ± 5.13
[d]8	7.14 ± 1.92	10.83 ± 2.74	21.16 ± 4.77	35.86 ± 2.99
[e]9	7.66 ± 1.76	11.34 ± 0.68	18.79 ± 2.79	36.33 ± 6.77
[f]10	5.04 ± 0.00	12.18 ± 0.00	17.22 ± 0.00	38.64 ± 0.00
[f]11	5.04 ± 0.00	8.82 ± 1.18	11.76 ± 1.68	28.14 ± 1.78
[f]12	5.04 ± 0.00	7.77 ± 0.89	10.92 ± 2.52	23.94 ± 0.00

[a] Multiwell data set 1; [b] Multiwell data set 2; [c] Multiwell data set 3; [d] Multiwell data set 4; [e] Multiwell data set 5; [f] Multiwell data set 6.

Table 2. Evolution of microalgal dry biomass concentration (mg L^{-1}) in multiwell screening experiments performed with different initial pH conditions. The results are expressed as mean values ± SD.

This peculiarity, coupled with the capability to tolerate high heavy metal concentrations, might be viably exploited for performing cultivation of *C. melkonianii* in raceway as already represented in the introduction.

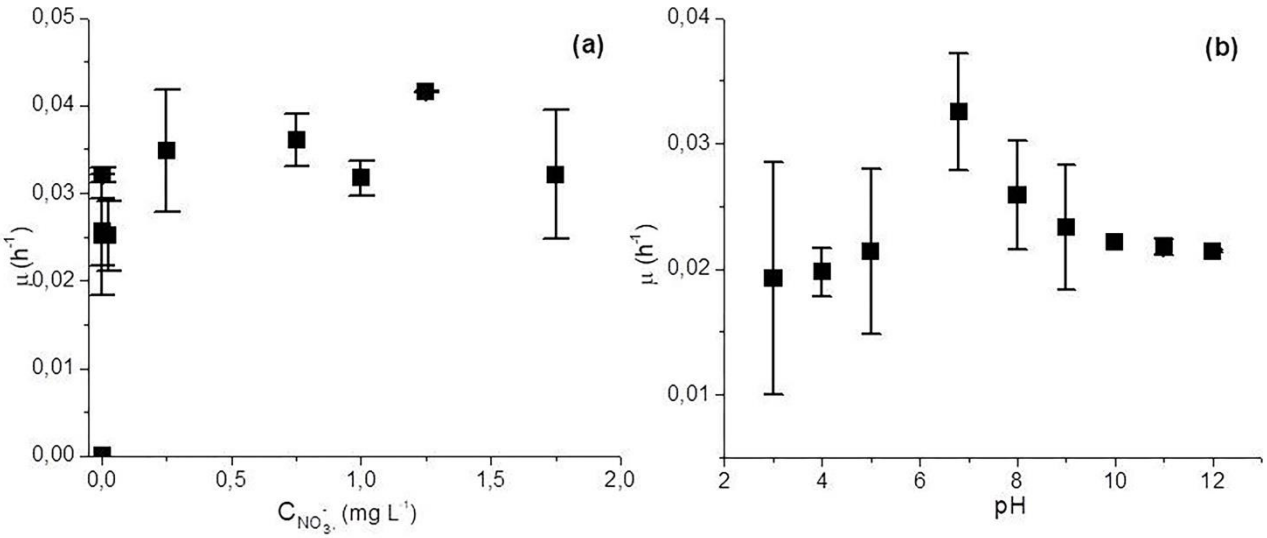


Fig. 2. Evaluation of growth rates as a function of nitrate concentrations (a) and pH (b) in multiwell screening experiments.

Therefore, the pH screening experiments in multiwell permitted to evaluate a crucial kinetic parameter such as growth rate and to confirm the extremophile character of *C. melkonianii*. It is well known that neutrophilic algae typically show an optimum pH, i.e. they are not able to grow under very acid or very alkaline pH conditions. However, as recently observed by Soru *et al.* (2019), from Fig. 2b it can be confirmed that *C. melkonianii* shows a quite good rate even at extreme pH values.

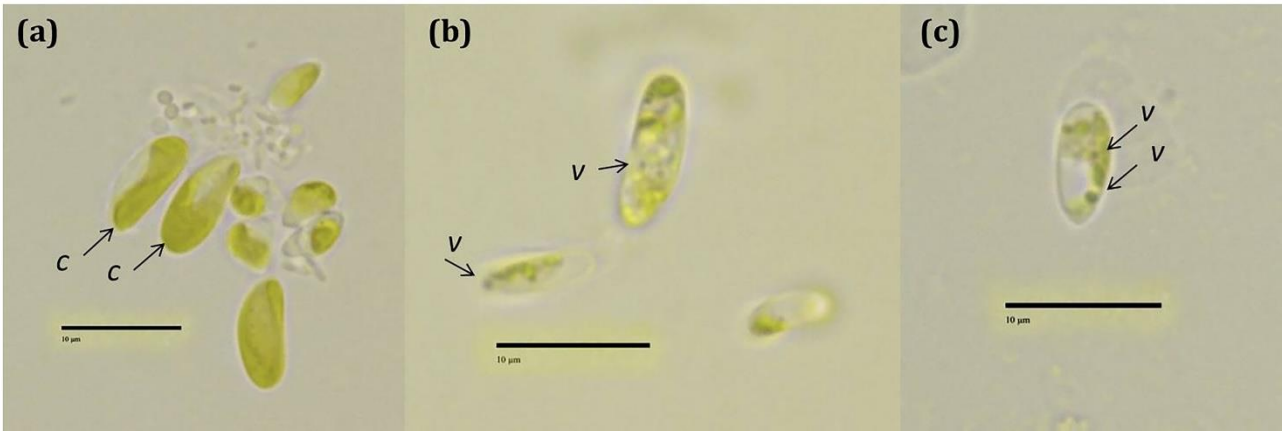


Fig. 3. Micrographies of *Coccomyxa melkonianii* SCCA 048 cultivated for 72 h with different concentrations of nitrate, i.e. 0.25 g L^{-1} (a), 1.75 mg L^{-1} (b), and 0.75 mg L^{-1} (c), respectively. Abbreviations: *c*, chloroplast; *v*, vacuole. Scale-bar: 10 µm.

As shown in Fig. 3a, the morphology of vegetative cells grown in the control medium i.e. BBM standard, containing 0.25 g L^{-1} of nitrate, exhibits usual elongated cylindrical or ellipsoidal drop-like shapes with single parietal chloroplast after 72 h of experiment. No vacuoles could be observed in this case. However, when cultivating *C. melkonianii* with lower nitrate concentration, i.e. 1.75×10^{-3} g L^{-1}, certain cells started to show some vacuoles that may be seen from Fig. 3b as round structures. Morphological changes, very pale chloroplasts, as well as several vacuoles, could be detected inside the cytoplasm, as a result of nitrogen limitation. When further reducing the nitrates concentration to 0.75×10^{-3} g L^{-1}, after 72 h of cultivation the strain showed a bleached and poorly visible chloroplast because most of the cells were strongly vacuolized (cf. Fig. 3c). Low nitrate concentration boosted lipid synthesis as shown in Fig. 3c, where numerous vacuoles can be observed within the cell. This observation is consistent with that reported by other authors for the *Coccomyxa* genus grown under stress-inducing conditions (Abe *et al.* 2014; Fuentes *et al.* 2016; Ohkubo *et al.* 2017).

3.2 Experiments in batch reactors

The use of multiwell microplate is not appropriate for evaluating long-term growth performances because alga growth in bottles and microplates is comparable only for a limited period of time. In order to better investigate the effects of nitrogen manipulation strategies on the prolonged growth and the lipid production of this strain further specific experiments were carried out by cultivating *C. melkonianii* in 2 L batch stirred bottles where the initial concentration of dissolved nitrates was suitably changed. The growth and lipid accumulation kinetics were first investigated using a nitrate concentration equal to 0.25 g L^{-1}. The time evolution of nitrate concentration and pH were also monitored during this experiment. The obtained results are shown in Fig. 4.

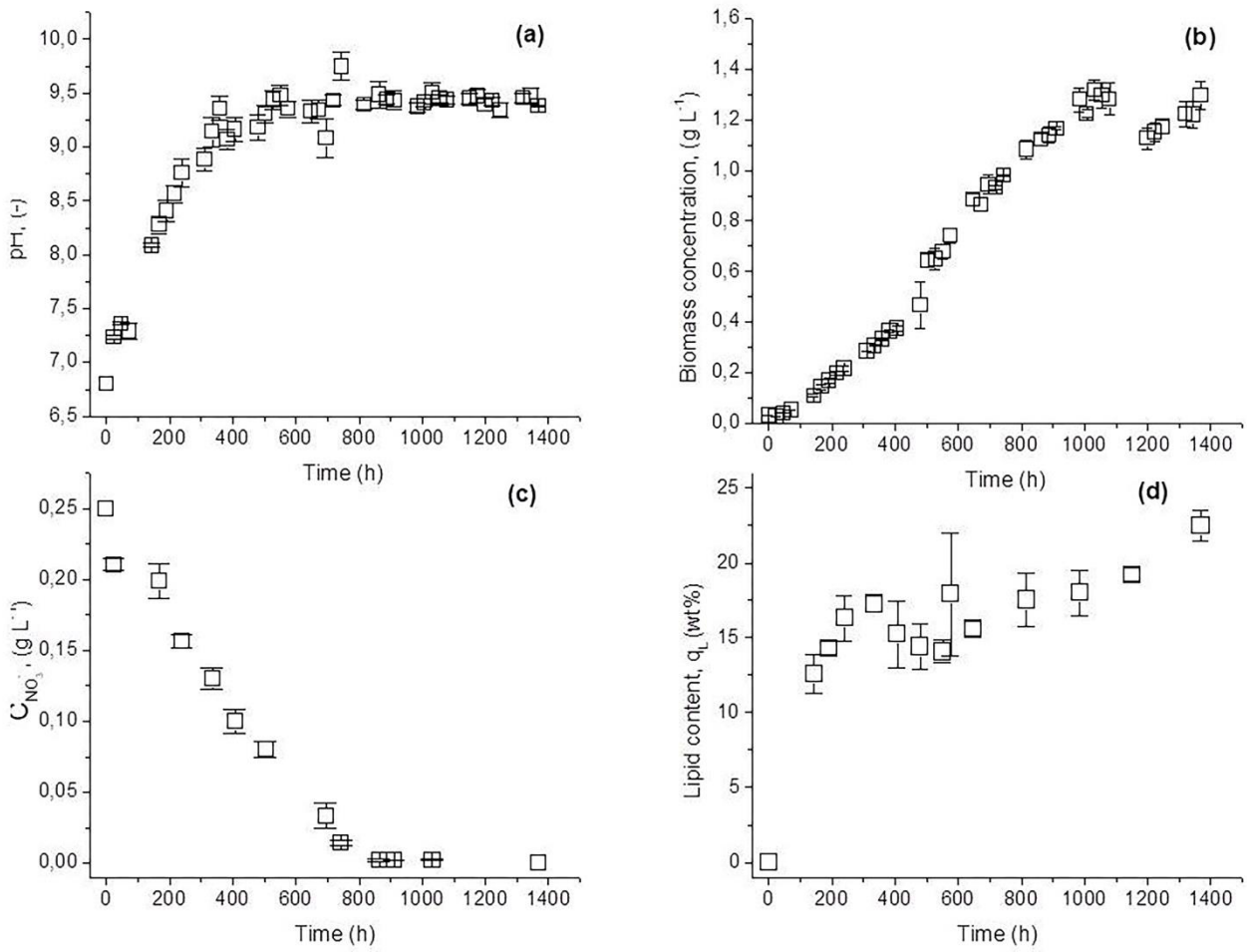


Fig. 4. Evolution of (a) pH and (b) total dry biomass concentration (g L^{-1}); (c) dissolved nitrate concentration, and (d) lipid content as a function of time during the experiment with BBM medium and initial nitrate concentration of 0.25 g L^{-1}.

From Fig. 4a it can be observed that, as expected, pH increases during the experiment as a result of dissolved CO_2 consumption by algae. In particular, by starting from the initial value of 6.8 it monotonically grows to reach a constant value of about 9.5 after about 25 days of cultivation. It should be noted in passing that the capability of microalgae to increase water alkalinity might be suitably exploited for remediating highly acidic waters contaminated by heavy metals; the increase of pH can promote the precipitation of heavy metals as hydroxides, thus allowing their removal through suitable operations by means of solid-liquid separation. Similar observations were described also by Podda *et al.* (2000). When considering the evolution of biomass concentration shown in Fig. 4b, it can be observed that, after a short lag phase, the culture starts growing almost exponentially until about 33 d (~ 800 h) when a decelerating growth takes place. After 45 d (~ 1080 h) of cultivation, the culture achieves a sort of steady state corresponding to a biomass concentration of about 1.4 g L^{-1}. This behavior is a result of the corresponding lack of nitrogen in

solution shown in Fig. 4c. In particular, it can be observed from the latter one that, by starting from the initial imposed value of 0.25 $g\,L^{-1}$, nitrate concentration in solution decreases as a result of algae uptake and they are completely consumed just after about 1080 h. The corresponding time evolution of lipid content during the concerned experiment is shown in Fig. 4d. The lipid content significantly increases during the first cultivation days, achieving a value close to 17 %wt after only 12 d ($\sim$ 300 h) of cultivation. Subsequently, after a slight decrease, it reaches an almost constant value of about 15 %wt until total consumption of nitrogen occurs, i.e. 1080 d. Next, photosynthesis and growth become decoupled and thus lipids start to increase at a higher rate by reaching the value of about 22,5 %wt at the end of cultivation. These outcomes are consistent with those ones observed in other extremophile *Coccomyxa* (Abe *et al.* 2014). Furthermore, the lipid evolution trend shown in Fig. 4d is qualitatively consistent with the one already obtained by Concas *et al.* (2016) in similar experiments with *Chlorella sorokiniana*. Accordingly, the biochemical phenomena responsible for this lipid behaviour could be similar to the phenomena discussed in that work (Concas *et al.* 2016) and reported in what follows. The culture is initially nitrate-replete and nitrogen is absorbed from solution for the synthesis of functional biomass (i.e. proteins). The high initial photosynthetic rate causes an excess of the internal carbon with respect to the maximum C: N stoichiometric ratio needed to synthesize proteins. The resulting carbon excess within the cell is used for the lipids production (Mairet *et al.* 2011). This effect is confirmed by the rapid increase of lipid content during the first cultivation days (Fig. 4d). As cell number increases, the optical density of the culture augments and, consequently, the carbon fixation driven by photosynthesis reduces. At some point, the internal nitrogen and carbon are almost balanced, thus the latter one is preferably used along with nitrogen to produce functional compounds, i.e. proteins, rather than lipids synthesis. This phenomenon is likely at the base of the slight reduction and stabilization of lipids content observed in Fig. 4d in the period of time ranging from 400 to 1080 hours.As growth proceeds further under batch conditions, nitrate in solution is consumed and thus the corresponding intracellular qN concentration becomes very low (Fig. 4c). The minimum stoichiometric ratio N:C needed to synthesize proteins is not longer available, and thus, all the carbon internalized by photosynthesis is used to produce lipids. In fact, given the high energy density of lipids, this mechanism permits cells to store the energy excess deriving from the high values of the light incoming flux. This explains how the lipid content starts to increase again, albeit at a lower rate, when nitrate is completely consumed, i.e. from 1080 h untill the end of cultivation (i.e.1368 h), as shown in Fig. 4c.

The kinetic parameters useful for quantitatively describing the growth of this strain have been obtained for the first time in this work. To evaluate the effect of nitrate on microalgae growth rate and lipid accumulation, further experiments were performed by increasing the initial concentration of dissolved nitrate 5 times (5N) with respect to the corresponding value in the BBM standard medium. In the 5N experiment, the initial nitrate concentration was set to 1.25 g L^{-1}. The corresponding experimental results are those marked with the tag 5N in Fig. 5 in term of pH, biomass, nitrate and lipid dynamics.

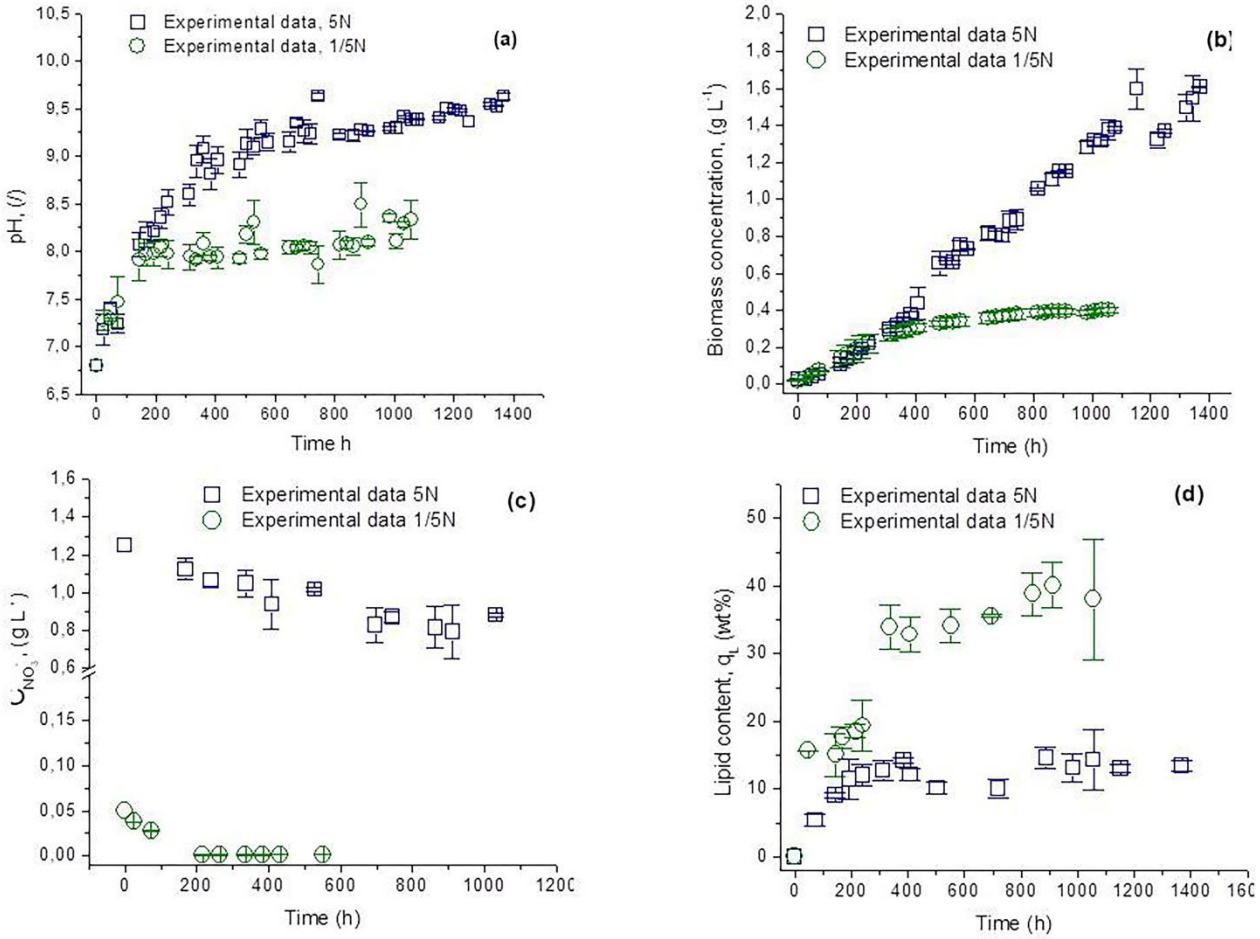


Fig. 5. Evolution of (a) pH, (b) dry biomass concentration, (c) dissolved nitrate concentration, and (d) lipid content as a function of time during the experiments with initial nitrate concentration of 1.25 g L^{-1} (5N), and 0.05 g L^{-1} (1/5N), respectively.

From Fig. 5a it can be observed that under such operating conditions, pH increases with time following a trend similar to the one already detected for BBM medium. In this case, it reaches an almost constant value, albeit slightly higher, of 9.6, as a result of continuous algae growth during the whole experiment, as shown in Fig. 5b. The value of biomass concentration at the end of the experiment (1368 h), i.e. 1.6 $g\,L^{-1}$, is slightly higher than the one observed in the base case experiment, despite similarities between data observed until 1080 hours of cultivation. Such a behaviour is due to the fact that microalgae can prevent the decrease of their nitrogen cell quota by taking advantage of nitrates available in solution which allows the sustainment of microalgal growth for a prolonged period of time. Similar behavior is shown in Fig. 5c, where it can be observed that, while nitrate concentration decreases during growth, it is never completely consumed. Accordingly, proteins can be always synthesized by cells and lipids do not accumulate. The evolution of lipid accumulation observed in the framework of the same experiment is shown in Fig. 5d. Similar to the BBM medium experiment, the lipid content increases significantly to about 13 wt% after 13 days of cultivation (312 h). This is probably caused by the phenomenon described above according to which, during the first cultivation days, the low optical density of the culture allows an effective penetration of light which in turn boosts the photosynthetic fixation rate of carbon. As shown in Fig. 5d, after 13 days the lipid content of microalgae reaches an almost constant value of about 13.9% wt which is lower than the one correspondingly observed in the experiment with standard BBM. This is likely caused by constant nitrogen availability in solution. when nitrogen is constantly available, the increased optical density of culture leads the algae growth to become photosynthesis-limited. Because the influx of carbon is lower than the corresponding influx of nitrogen, the fixed carbon is used to synthesize functional biomass (i.e. proteins) rather than fatty acids. In the Figures from 5a-d, the experimental results obtained when using an initial nitrate concentration reduced by five times (1/5N) with respect to the one of the BBM standard medium, are also shown. As seen in Fig. 5a, the medium pH achieves an almost constant value of 8 after about 240 h (10 d). The resulting pH is lower than the one attained for the case of the 5N experiment. This is because, as it can be observed from Fig. 5b, microalgae exponential growth stops after about 10 days when the biomass concentration has reached a value of about 0.4 $g\,L^{-1}$. Accordingly, CO_2 consumption is reduced and consequently pH increase is inhibited. As shown in Fig. 5c, this early achievement of culture stability is caused by complete nitrogen consumption and starvation phenomena. On the contrary, the corresponding evolution of lipid shows a growing trend for the entire investigated period of time i.e. 1056 h (cf. Fig. 5d). During the first 15 days of cultivation, lipid accumulation was similar to acccumulation observed under base-case conditions.

After total nitrogen consumption, lipid concentration greatly augments, reaching quite high values, i.e. ~40 %wt, at the end of cultivation. Lipid accumulation occurs after nitrogen consumption because all carbon is converted to lipids, rather than to structural molecules such as proteins, which require nitrogen. Therefore, when the growth of biomass stops, lipids accumulate significantly within the cell.

3.3 Lipid productivity and fatty acid methyl esters analysis

From the results so far discussed, it can be concluded that, the reduction of nitrogen concentration generally causes the intracellular content of lipids to increase, while the biomass concentration decreases, To evaluate the most favorable conditions for producing lipids, the total concentration of lipids in the reactor at the end of cultivation and the corresponding production rate should be evaluated. The final lipid concentration C_L (mg L^{-1}) provides an immediate estimate of the quantity of lipids that can be actually recovered from the reactor at the end of the investigated batch cycle. Productivity establishes the rate at which lipids are produced per unit of reactor volume and thus represents a more useful parameter for evaluating the potential scalability of the photobioreactors. The final lipid concentrations shown in Fig. 6 have been evaluated from the experimental data as $C_L(gL^{-1}) = C_x q_L$.

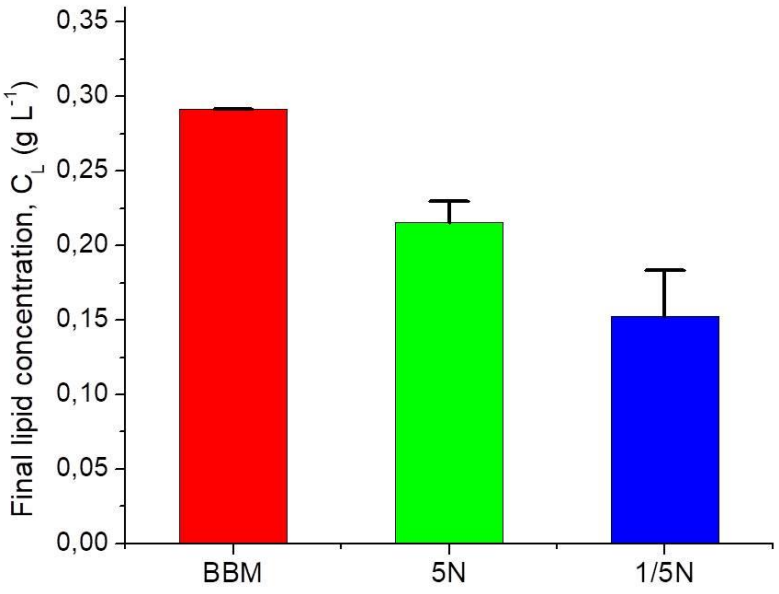


Figure 6. Total lipid concentration achieved at the end of cultivation.

From Fig. 6 it can be observed that, while the maximum intracellular content (i.e. 40 %wt) was obtained under nitrogen starvation conditions (cf. 1/5N in Fig. 5d), when considering the total lipid

concentration C_L, the best result was obtained using the standard BBM, i.e. with an initial nitrate concentration equal to 0.25 g L^{-1}. Furthermore, when using 5N growth medium, the intracellular content of lipids q_L was significantly lower than the one observed under nitrogen starvation (i.e., 14 % vs 40% respectively), but the lipid concentration recovered from the reactor was significantly higher. Microalgae in BBM standard and 5N cultures continued to grow and thus, albeit characterized by lower intracellular lipid content, the higher number of cells in the reactors caused the total lipid to increase. While the 5N was the optimal growth medium, it should be noted that the increase of lipid concentration is negligible with respect to BBM, and the cost associated to the availability of nitrate to be added would be 5 times higher. Thus, it can be reasonably stated that the best compromise is represented by the BBM medium. Nonetheless, the exploitation of waste waters rich in nitrates might represent a strategy to be applied to open pond culture systems for commercial sustainability in the future. In order to verify whether the different cultivation conditions had affected the quality of microalgal lipids, the content of FAMEs obtained after lipid extraction and transesterification was analyzed. The extraction procedure was performed at the end of each cultivation experiment. This investigation was aimed also to verify the potential exploitability of the extracted lipids in specific markets. The comparison among FAMEs profiles is shown in Fig. 7 in terms of weight percentage of each fatty acid (WF_i) with respect to the total amount of lipids which underwent transesterification.

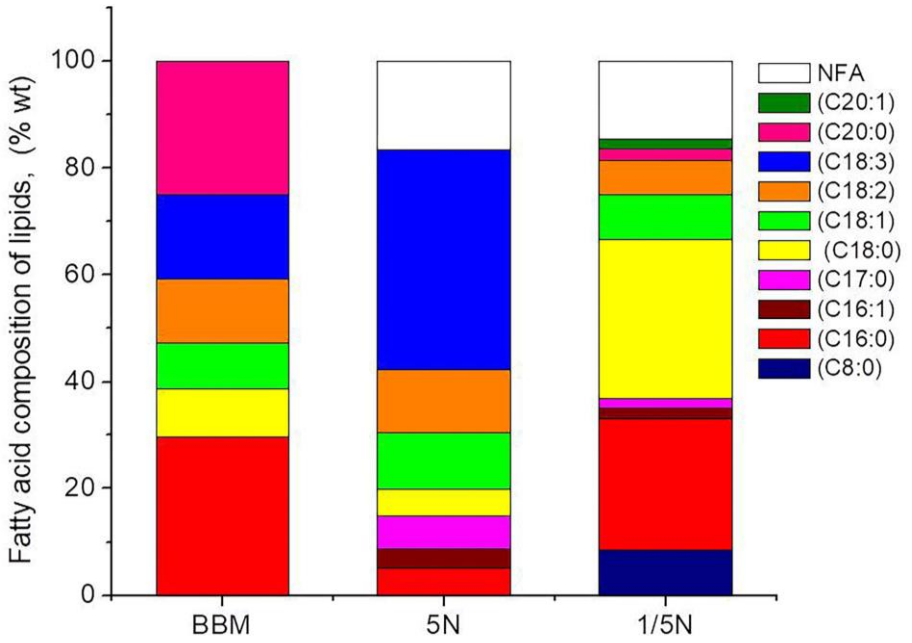


Fig. 7. $FAME_S$ composition of lipids in terms of weight. NFA refers to species not identified as fatty acids.

It can be noted that when using 5N and 1/5N media, a certain amount of extracted lipids, i.e. 16 %wt and 14 %wt respectively, could not be classified as fatty acids. They are most probably glycolipids from cell wall or lipids peroxides obtained as the result of peroxidation phenomena. On the contrary, all FAMEs extracted from algae grown in BBM standard could be identified as fatty acids indicating that such operating conditions promote the highest levels of exploitable fatty acids. The predominant fatty acids in the BBM strandard meium were palmitic (C16:0) and arachidic (C20:0). The former is typically used to produce soaps, cosmetics, and industrial mold release agents, while the latter one might be suitably exploited for the production of lubricants. In addition, high amounts of oleic (C18:1), linoleic (C18:2), and stearic (C18:0) acids were found which can be suitably exploited for producing biofuels, food supplements, and cosmetics (emollients). Finally, significant amounts of linolenic acid (C18:3) were also found in the lipids obtained from BBM. The predominant fatty acids detected in the lipids produced in the 1/5N medium are the palmitic and the stearic fatty acids, with about 25 %wt and 30%wt, respectively. Stearic acid can be suitably exploited to produce cosmetics, detergents, lubricants, and biofuels. The amount of arachidic acid detected in the case of 1/5N was significantly lower than that from microalgae cultivated under standard BBM medium. A relatively high amount of caprylic acid (C8:0) was detected in the lipids from the 1/5N culture. This fatty acid can be commercially used in the production of esters, antimicrobial pesticides in commercial food handling establishments, as well as disinfectants in health care facilities. FAMEs obtained from microalgae cultivated under nitrogen excess (5N-BBM) displayed a shift towards poly-unsaturated fatty acids, namely linolenic acid (~41 %wt), which represents a dietary precursor for the long-chain omega-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and is hence exploitable in the farmaceutical and nutraceutical market. In particular, poly-unsaturated fatty acids such as linolenic acid are anti-inflammatory and are pharmacologically important for diet and/or therapy as an alternative to fish oil in food (Draaisma *et al.* 2013; De Morais *et al.* 2015; Vaz *et al.* 2016). Moreover, according to Navarro *et al*. (2017), palmitic, oleic, linoleic, and linolenic acids, either free or combined in glycerides, appear to be involved in antimicrobial activity in another acid-tolerant strain *C. onubensis.* However, it should be noted that the cultures were in different growth phases at the end of experiments: BBM standard culture was at the beginning of the stationary phase, 5N culture was at the end of the exponential phase, and 1/5N culture was in advanced stationary phase; this might also have influenced the qualitative composition of lipids reported in Fig. 7.

In conclusion, of the three growth conditions tested, algae grown using BBM standard medium produced the highest amounts of almost all fatty acids except for stearic acid. This result suggests that BBM contains the optimal nitrogen concentration for valuable lipid production using *C. melkonianii*. On the contrary, 1/5 N growth medium should be used when the target of production is just stearic acid, while 5 N medium is preferable when oleic and linolenic acids are the target products.

4. Conclusions

Cultivation of extremophilic microorganisms has gained interest due to their ability to accumulate and produce high-value compounds. *Coccomyxa melkonianii* SCCA 048 is an extremophile green microalga able to live under extremely oxidative conditions**.** In this work, the kinetics of *Coccomyxa melkonianii* SCCA048 were investigated for the first time. By assessing the effect of pH on its growth rate in multiwell devices, it was demonstrated that *C. melkonianii* is an extremophile microalga capable of tolerating heavy metals and extreme pH conditions (Malavasi *et al.* 2016; Soru *et al.* 2018, 2019). This aspect makes the cultivation of this strain more economically sustainable in raceways-type photobioreactors by adopting selective culturing strategies that avoid contamination by undesired organisms. The effect of initial nitrogen concentration on the growth and lipid synthesis in two-liter photobioreactors operated in batch mode was investigated for the first time for this strain. By considering the experimental results, the best nitrate concentration for maximizing lipid productivity in batch photobioreactors was identified to be BBM standard medium. Finally, the quality of obtained FAMEs was investigated and the possibility to exploit them in different sectors was briefly discussed. In particular, it was found that while the obtained lipids could be further treated for producing biofuels, their FAMEs composition is very promising in the food and/or lubricant industries. Specifically, the caprylic acid (C8:0) production in nitrogen starvation conditions might be of significant interest for its antibacterial properties and could be used for various applications in medicine, agriculture, and food preservation, especially where the use of conventional antibiotics is undesirable or prohibited (Desbois *et al.* 2010).

Supplementary materials

S.1 Calibration line for converting OD measurements into biomass concentration

The biomass concentration Cx (gdw L^{-1}) was calculated from OD measurements using the calibration line C_b vs OD shown in Figure S1, which was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes that were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h.

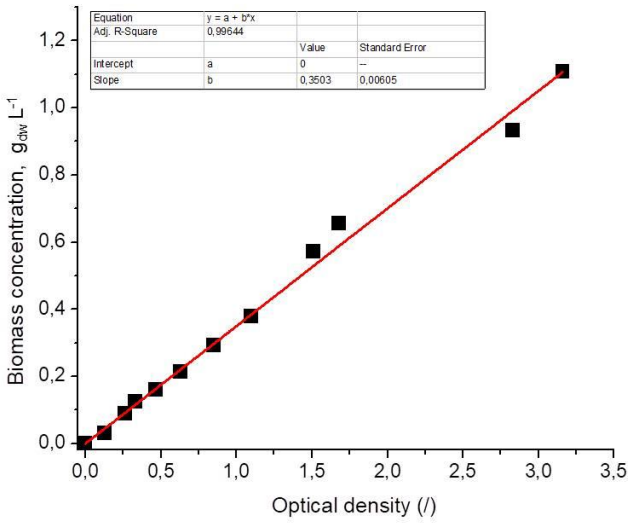


Figure S1. Calibration line for correlating optical density measurements to the biomass concentration (g L^{-1} wt)

S.2 Methods adopted to evaluate the pH and nitrate dependent kinetics from multiwell experiments.

Coccomyxa melkonianii SCCA 048 was batch cultured in multiwell plates using different concentrations of nitrogen (cf. Table 1 in the text) in order to assess the effect of this nutrient on its growth rate. The latter one was evaluated by considering that, for the short cultivation times and highly diluted solutions adopted in multiwell experiments, light and pH do not significantly change during cultivation and thus growth rate is affected only by the different values of nitrate concentrations imposed in the different plates. Moreover, during exponential growth of microalgae the following mass balance holds true:

$$\frac{dC_x}{dt} = \mu \cdot C_x \qquad \text{(S1)}$$

where μ is the total growth rate that, according to what above, is a function of only nitrate concentration. By integrating equation (S1) from the time t0 at which exponential growth starts and the generic cultivation times t, the following expression can be obtained:

$$\ln\left(\frac{C_x}{C_{x,0}}\right) = \mu \cdot (t - t_0) \quad \text{(S2)}$$

Accordingly, by performing a linear regression of experimental data expressed in terms of ln(C/C_0) *Vs* (t-t_0) the growth rate μ can be evaluated as the slope of the fitting line while the goodness of this evaluation is provided by the correlation coefficient. In order to carry out such an analysis, the starting time t_0 has been visually evaluated to be equal to 24 h for all the investigated nitrogen concentrations. In fact during the first 24 hours of cultivation a kind of lag phase was observed. In Table S1 the different values of ln(C/C_0) are reported as a function of (t-t_0) as well as of the considered nitrogen concentration. In the same Table even the obtained values of the growth rates and the corresponding correlation coefficients are reported.

A similar procedure has been adopted to assess the effect of pH on the growth rate of *C. melkonianii*. However in this case, different values of pH were imposed in the plates of multiwells as described in the materials and methods while the initial nitrate concentration was fixed to a value that assured nitrogen-replete growth for all the 72 h of cultivation. Accordingly, the variation of growth rates observed in this experiment can be ascribed only to the variation of pH. In Table S2 the values of ln(C/C_0) obtained under the different pH conditions are reported as a function of (t-t_0) along with the corresponding values of the growth rates evaluated through the linear fitting procedure already described. As it can be observed from this Table quite good correlation coefficient was obtained for all the investigated pH values thus demonstrating the reliability of the growth rates values estimated through the fitting procedure.

Table S1. Evaluation of the growth rates during the multiwell screening experiments with different initial nitrate concentrations.

	$t - t_0$				
	0 h	24 h	48 h		
C_{NO3} (mg L^{-1})	$\ln (C_x/C_{x,0})$ (/)			r (/)	μ (h^{-1})
1.75	0.000	1.190	1.792	0.983	0.037
1.75	0.000	0.693	1.293	0.999	0.027
1.25	0.000	1.253	1.998	0.989	0.042
1.00	0.000	0.738	1.452	1.000	0.030
1.00	0.000	0.821	1.591	1.000	0.033
7.50×10^{-1}	0.000	1.237	1.631	0.958	0.034
7.50×10^{-1}	0.000	1.179	1.833	0.987	0.038
2.50×10^{-1}	0.000	1.447	1.910	0.958	0.040
2.50×10^{-1}	0.000	0.833	1.435	0.996	0.030
2.50×10^{-2}	0.000	0.773	1.344	0.996	0.028
2.50×10^{-2}	0.000	0.526	1.073	1.000	0.022
2.50×10^{-3}	0.000	0.956	1.569	0.992	0.033
2.50×10^{-3}	0.000	0.860	1.514	0.997	0.032
2.50×10^{-4}	0.000	0.636	1.099	0.996	0.023
2.50×10^{-4}	0.000	0.811	1.361	0.994	0.028

Table S2. Evaluation of the growth rates during the multiwell screening experiments with different initial pH values.

	$t - t_0$				
	0 h	24 h	48 h		
pH (/)	$\ln (C_x/C_{x.0})$ (/)			r (/)	μ (h^{-1})
3.0	0.000	0.496	1.331	0.989	0.026
3.0	0.000	0.501	1.115	0.998	0.023
3.0	0.000	0.916	0.069	0.068	0.009
4.0	0.000	0.578	0.925	0.990	0.020
4.0	0.000	0.636	1.001	0.988	0.022
4.0	0.000	0.573	0.879	0.985	0.019
4.0	0.000	0.435	0.821	0.999	0.017
5.0	0.000	0.470	0.668	0.973	0.015
5.0	0.000	0.811	1.288	0.989	0.028
5.0	0.000	0.223	1.139	0.943	0.021
6.8	0.000	0.494	1.309	0.990	0.026
6.8	0.000	0.969	1.680	0.996	0.036
6.8	0.000	0.916	1.674	0.999	0.036
6.8	0.000	0.728	1.580	0.999	0.032
8.0	0.000	0.779	1.062	0.966	0.024
8.0	0.000	0.892	1.324	0.981	0.030
8.0	0.000	0.470	0.944	1.000	0.020
8.0	0.000	0.663	1.498	0.998	0.030
8.0	0.000	0.536	1.276	0.996	0.026
9.0	0.000	0.465	0.938	1.000	0.020
9.0	0.000	0.751	1.454	1.000	0.030
9.0	0.000	0.504	1.133	0.998	0.023
9.0	0.000	0.260	1.086	0.957	0.020
10.0	0.000	0.346	1.154	0.974	0.022
11.0	0.000	0.234	1.214	0.942	0.022
11.0	0.000	0.330	1.113	0.974	0.021
12.0	0.000	0.163	1.210	0.921	0.022
12.0	0.000	0.470	1.047	0.998	0.021

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Chapter IV

Behavior of the extremophile green alga *Coccomyxa melkonianii* SCCA 048 in terms of lipids production and morphology at different pH values

1 Introduction

Microalgae are a potential source of bio-active compounds such as proteins, lipids, pigments, and antioxidants (Cuaresma *et al.* 2006; Mata *et al.* 2010; Sahu *et al.* 2013; Khanra *et al.* 2018). The high quantity and quality of some of these molecules have stimulated the production of high-value products, including human health food, animal feed, biofuels, fine chemicals, and pharmaceuticals (Spolaore *et al.* 2006; Bux 2013; Richmond and Hu 2013; Concas *et al.* 2016; Dixon and Wilken 2018). Open ponds are the simplest and less expensive systems for mass cultivation of microalgae (Mata *et al.* 2010; Concas *et al.* 2014; Hirooka *et al.* 2014; Schneider *et al.* 2018). However, the massive production of microalgal biomass is partially limited by competition with invasive species, which diminish the corresponding productivity. In this context, extremophile microalgae have gained interest because of their ability to grow under extreme conditions, thus allowing outdoor cultivation with negligible contamination (Hirooka *et al.* 2014; Varshney *et al.* 2015; D'Alessandro and Antoniosi Filho 2016; Souza *et al*. 2017). It is worth noting that extremophile microorganisms are sources of enzymes and other cellular products which offer a wide range of applications in a variety of industrial and biotechnological operations, including medical ones (Pulz and Gross 2004; Anitori 2012; Navarro *et al.* 2016, 2017; Kisková *et al.* 2018). Furthermore, microalgae have the advantage of growing in minimal culture media with an inorganic carbon source which prevents cultures from bacterial contamination (Olaizola 2003; Cuaresma *et al.* 2006). Previous studies recognized that the cultivation of extremophile microalgae might have the major advantage of minimizing the contamination risk by undesirable organisms or predators in outdoor cultures (Pulz and Gross 2004; Eibl *et al.* 2014; Hirooka *et al.* 2014). Extremophiles may be divided into two broad categories: extremophilic organisms which require one or more extreme conditions in order to grow, and extremotolerant organisms which can live at extreme values of one or more physicochemical parameters, while optimally growing under normal conditions (Rampelotto 2013).

Recent studies have attempted to increase biomass productivity or change the profile and content of fatty acids, carotenoids, and antioxidants of microalgae collected in extreme environments or in conventional ones, albeit adopting extreme growth conditions, such as thermal, acidic, alkaline, salty, or eutrophic waters (Abe *et al.* 2007; Teoh *et al.* 2012; An *et al.* 2013; Ghozzi *et al.* 2013; Skorupa *et al.* 2014; Ruiz-Domínguez *et al.* 2015; D'Alessandro and Antoniosi Filho 2016; Bermejo *et al.* 2018).

The pH is one of the most important environmental parameters governing the growth rates and regulating species competition.

To date, several investigations have been focused on establishing pH tolerance and pH effects on growth, cell morphology as well as biomass, lipids and carotenoids productivity. Regarding this topics, members of the genera *Chlorella, Chlamydomonas, Pseudochlorella,* and *Coccomyxa* (Hargreaves and Whitton 1976; Azov 1982; Gerloff-elias *et al.* 2005; Moser and Weisse 2011; Hirooka *et al.* 2014; Vaquero *et al.* 2014; Fuentes *et al.* 2016) are the most frequently investigated species belonging to Chlorophyta.

A green alga of the genus *Coccomyxa* (Trebouxiophyceae) was isolated from the Rio Irvi (SW Sardinia, Italy), a river which flows in the abandoned area of Montevecchio mining district (Concas *et al.* 2006; Pasqualetti *et al.* 2015; Malavasi *et al.* 2016), the most important case of groundwater discharge from a flooded mine in Sardinia (Frau *et al.* 2015, 2017). Mining activities and ore processing result in permanent source of toxic substances, especially heavy metals which contaminate various ecosystems due to their leaching capacity. When mine waste drainage has neutral pH, with a high concentration of sulfate and dissolved metals, the term neutral (or alkaline) mine drainage is applied (Scharer *et al.* 2000; Iribar 2004). In particular, this extremophile microalga *Coccomyxa melkonianii* SCCA 048, which belongs to the Sardinian Culture Collection of Algae (SCCA) (Malavasi and Cao 2015), is able to live in highly selective environment characterized by extreme conditions since it has been isolated in fresh waters contaminated by high amounts of heavy metals (Malavasi *et al.* 2016). This strain has been already elected as a promising microalga particularly because of its rich carotenoids profile, especially lutein (up to 80% of total carotenoids) (Pasqualetti *et al.* 2015).

In this work (Soru *et al.* 2019), for the first time in literature, we have been analyzing the effect of pH on the growth and lipids productivity of *C. melkonianii*. A preliminary screening has been performed on a wide pH range to assess the tolerance limits of the strain. Then, three specific pH values have been selected for the experiments on prolonged growth. Additionally, the morphological effects of pH and its influence on fatty acids composition have been investigated. Furthermore, this study aims to support that this extremophile microalgae is able to grow and produce lipids at similar productivities of non-extremophile microalgae.

2 Materials and methods

2.1 Microorganism and culture conditions

The freshwater alga used in this work is *Coccomyxa melkonianii* SCCA 048 which has been originally isolated from the river Rio Irvi located in the abandoned mine area of Montevecchio (SW Sardinia, Italy) (Pasqualetti *et al.* 2015; Malavasi *et al.* 2016). The alga was found in a station where water pH was 6.85 (Malavasi *et al.* 2016). This strain is maintained at the SCCA (Malavasi and Cao 2015). Growth experiments were carried out in 2 L Pyrex bottles under unialgal conditions. The culture media volumes were 1.8 L, which were agitated by a magnetic stirrer at 500 rpm using magnetic PFTE stir bars (6 mm diameter and 30 mm length). Bottles and magnetic stir bars as well as culture media were sterilized in autoclave at 121°C (Vapormatic mod. 770/A) for 15 min prior to microalgae inoculation. Bottles were stoppered by means of cotton plugs wrapped in cotton gauze during cultivation in order to prevent external contamination while at the same time assuring atmospheric CO_2 within the culture by an air pump (EHEIM 100 GmbH & Co KG, Germany). To preserve unialgal conditions, all operation were conducted under a microbiological safety cabinet (MSC-ADVANTAGE 1,2 Thermo Scientific). The green alga was cultured at room temperature and under the photon flux density of 80-100 μmol $m^{-2}s^{-1}$ for a light/dark photoperiod of 12 h (Lightmeter Delta OHM HD2302.0). All experiments were carried out in duplicate. The evaluated experimental error was within 3.8-13.5%. The initial cell concentration in each experiment ranges from 0.018 to 0.060 g L^{-1}respectively. The BBM medium (Bischoff and Bold 1963) was employed in the present study.

2.2 Experiments in multiwell plates

To evaluate the *Coccomyxa melkonianii* SCCA 048 ability of growing under different pH values conditions, preliminary screening tests were performed. Specifically, the microalgal bioassay was based on standard methodologies (ISO 8692/12; OECD 201/11). In particular, 72 h growth tests screenings were carried out by inoculating about 10^4 cell mL^{-1} of this strain into 24-welled multiwell plates (Primo® EUROCLONE ET3024, Italy) by considering test solution volumes of 2 mL of BBM medium in each well. A pre-culture of 4 to 7 days was prepared as inoculum for the test and used when cells started their exponential growth. Such pre-culture was incubated and maintained under axenic conditions (incubator VELP® SCIENTIFICA, FOC 225E) at 25°C as well as photon flux density of 80-100 μmol m^{-2} s^{-1} for a light/dark photoperiod of 12 h (Lightmeter Delta OHM HD2302.0) and continuously treated in an orbital shaker at 100 rpm (Stuart SSM1, Biosigma). Five different pH values, i.e. 3.0, 4.0, 6.8, 8.0 and 12.0, were imposed in the BBM by

the addition of HCl or NaOH. To preserve axenic conditions, all the operation were conducted under a microbiological safety cabinet (MSC-ADVANTAGE 1,2 Thermo Scientific). The multiwell were incubated under the same conditions of pre-culture and continuously shaken at 30 rpm. Growth was evaluated by spectrophotometric measurements, performed after 24, 48 and 72 h of cultivation, as reported in what follows. All experiments were repeated in triplicate for the sake of reproducibility.

2.3 Light microscopy

Cells morphology was observed by optical light microscopy (Leica microsystems DM750, Switzerland). At the end of each experiment, micrographs were acquired by a digital color camera (EC3, Leica Microsystems, Switzerland) equipped with LAS EZ 3.2.1 software (Leica microsystems, Switzerland).

2.4 Biomass and pH measurements

The microalgae growth was monitored through *in vivo* spectrophotometric measurements (Genesys 20 spectrophotometer, Thermo Scientific, Walthman, USA) of the chlorophyll *a* optical density (OD) of the culture at 663 nm wavelength (Hosikian *et al.* 2010) with 1 cm light path. The biomass concentration, C_x (g_{dw} L^{-1}), was calculated from OD measurements using a suitable C_x vs OD calibration curve which was obtained by gravimetrical evaluation of the biomass concentration of known culture medium volumes (Steriti *et al.* 2014). The pH of each culture was measured daily by pH-meter (Basic 20, Crison) and maintained constant by the addition of suitable amounts of either H_2SO_4 or NaOH, respectively.

2.5 Lipid colorimetric quantification, lipid extraction and fatty acid methyl esters analysis

During batch experiments, suitable amount of culture was withdrawn and then subjected to a colorimetric procedure based on the use of Sulfo-Phospho-Vanillin (SPV) to quantify the lipid content of microalgae (Concas *et al.* 2016). Afterward, the absorbance at 530 nm was measured and then translated in terms of lipid content of the sample (Mishra *et al.* 2014).

Neutral lipid extraction was performed directly on the wet disrupted biomass according to a method that was previously described elsewhere (Steriti *et al*. 2014; Concas *et al*. 2015) along with some minor modification.

The chromatographic analysis of fatty acids of extracted microalgal oil was performed with a Gas Chromatographer Trace (Thermo Finnigan, Rodano, Milan, Italy) equipped with an FID detector,

an AS 800 autosampler and a split-splitless injector. The capillary column was a CP-WAX 57CB from Varian (60 m long, 0.25 mm id, and 0.25 mm film thickness; Varian Inc., Palo Alto, CA) operating from 50 to 220°C (13 min) at 3°C/min. The injector and the detector were set at 200 and 280°C, respectively. A 1 μL volume of each sample was injected in the split mode (1:20). Helium was used as carrier gas, and nitrogen for make up at 120 and 80 kPa, respectively. Standard compounds in extracted oil were identified by comparison of their relative retention times and with those of the blend FAME MIX C4-C24 CRM47885 reference substances.

2.6 Evaluation of algae growth rate and productivity

The growth rate was evaluated by considering that during early exponential growth of microalga the following mass balance holds true:

$$\frac{dC_x}{dt} = \mu \cdot C_x \tag{1}$$

where μ (day^{-1}) is the total growth rate that is known to depend upon light, nutrient concentration, pH, and temperature (Concas *et al.* 2014) while C_x (g_{dw} L^{-1}) represents the dry biomass concentration. Since pH is the only operating condition modified during the experiments performed in this work, the observed differences in terms of growth rates could be ascribed only to its variation, thus providing an indication of the corresponding effect. By integrating Eq. (1) from the time t_0 at which exponential growth starts and the generic cultivation times t, the following expression can be obtained:

$$\ln\left(\frac{C_x}{C_{x,0}}\right) = \mu \cdot (t - t_0) \tag{2}$$

Accordingly, by performing a linear regression analysis of experimental data expressed in terms of $\ln(C/C_0)$ vs $(t - t_0)$ the growth rate μ can be evaluated as the slope of the corresponding straight line while the goodness of such regression is provided by the correlation coefficient. To carry out such an analysis, the time t_0 has been visually chosen to be equal to 24 h for all the investigated pH conditions.

On the other hand, to evaluate biomass (π_B) and lipid (π_L) productivities (g L^{-1} day^{-1}) at a specific cultivation time t, the following relationships were considered:

$$\pi_B(t) = \frac{C_x(t) - C_x(t_0)}{t - t_0} \tag{3}$$

$$\pi_L(t) = \frac{C_x(t)q_L(t) - C_x(t_0)q_L(t_0)}{t - t_0} \quad (4)$$

where q_L (%wt) represents the intracellular lipid content at a given cultivation time t which has been evaluated using the SPV method described above.

A statistical analysis using one-way ANOVA has been performed for the evaluation of the growth rate and the FAMEs composition. The corresponding value of the P parameter has been reported in the text.

3 Results

3.1 Screening in multiwell plates

In this study, we evaluated the effect of pH on the growth of the extremophile microalga *Coccomyxa melkonianii* SCCA 048. The results of the preliminary 72 h screening tests conducted in multiwell plates are shown in Fig. 1, where it can be observed that the alga was able to grow under the tested operating conditions. The maximum and the minimum value of biomass concentration was achieved at pH equal to 8.0 and 3.0, respectively, however, the overall response of *C. melkonianii* to different pH values appeared rather similar (Fig. 1a). In most of the tests the experimental error was below 15-20%. Only in two cases a major error percentage was observed. Further experiments and measures will be performed to validate those specific data. On the other hand, significant variations in morphology correlated with the different pH growth conditions were observed. Specifically, Fig. 1b shows the presence of spare cells at pH 3.0 and 12.0 solutions after 72 hours of treatments. As it can be seen, at the extremes pH tested (i.e. uppermost acidic pH 3.0 and uppermost alkaline 12.0), the algal growth was greatly inhibited and only some cells could be detected. In particular, it can be observed that at pH 3.0, the cells appeared bleached and poorly visible. At both pH conditions, cells expanded and intracellular organelles were released. The outcomes of this preliminary experiments suggested that these two pH values were to be excluded from the subsequent experiments described in what follows.

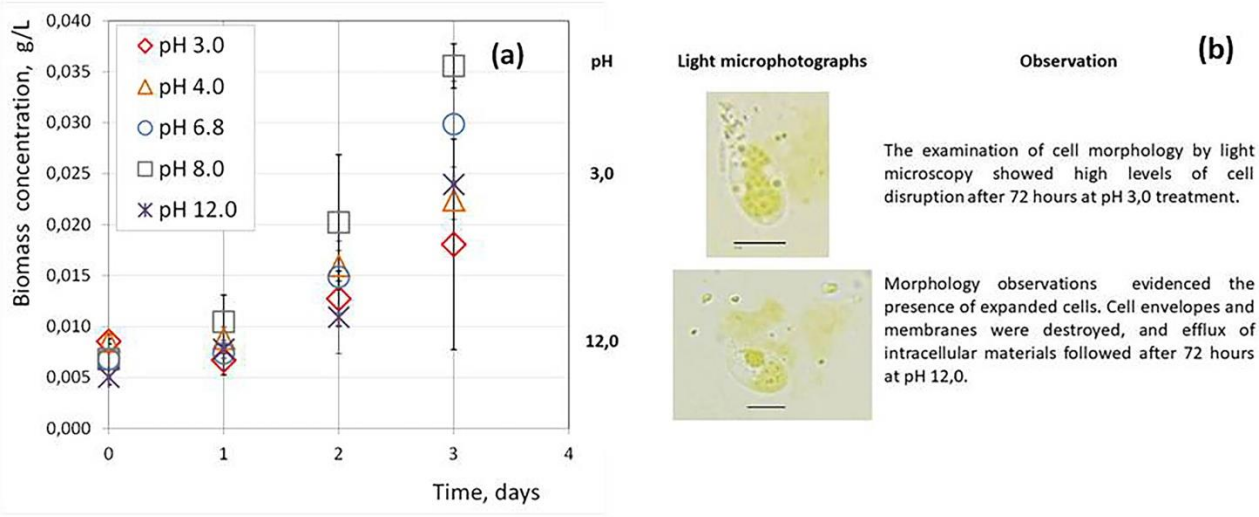

Fig. 1 Time evolution of biomass concentration of *Coccomyxa melkonianii* SCCA 048 as a function of five pH values in 72 h multiwell screening tests (a) and microphotographs of the damaged cells and the released chloroplasts after 72 h at pH 3.0 and 12.0 respectevely (b) (Scale-bar: 10 µm)

3.2 Growth rates and adaptation of *C. melkonianii* to acidic, neutral, and alkaline pH

The time evolution of biomass concentration observed during the batch experiments conducted at pH 4.0, 6.8, and 8.0, respectively is shown in Fig. 2. In all the experiments, the time courses of the cell growth exhibited the typical sigmoid pattern for batch cultures. We observed mainly three phases consisting of adaptation, exponential, and stationary phase. The adaptation phase occurred during the first 24 h of cultivation. In this time, a growth delay was observed due to the physiological adjustments of the inoculum to the pH and other culture conditions. From the second day, the cultures entered on the exponential phase where cells grow and replicate exponentially with time. During this early phase, a steep increase of growth rate and biomass content was observed in all experiments until about the 17th day. From the latter one, cultures slightly decelerate growth entering the late exponential phase. Then, all the tested solutions achieved the stationary phase approximately after the 40th day when the biomass concentration remained about constant as a result of the reduced availability of nutrients and light that lead the death rate to equal the growth one. While one can argue that no significant difference in growth behavior could be observed from Fig. 2, it should be noted (cf. grey box within Fig. 2) that such experiments started from different initial biomass concentration (C_x^0) value. In particular, pH 6.8 culture started with the lowest initial value but achieved similar biomass concentration of the other cultures, i.e. pH 4.0 and 8.0, after about 17 days of cultivation. This means that during the exponential phase, this culture was characterized by a slightly higher growth rate. Actually, as shown in Fig. 3a- c and confirmed by the one-way ANOVA test performed on the resulting growth rates, the latter ones appear to be affected by the pH of the culture medium ($P = 0.01$), at least when considering the investigated range. In Fig. 3d it can be observed that the value of μ was highest and optimum the one at pH 6.8 followed by that one for pH 4.0 culture, while the lowest one was achieved in pH 8.0 culture. In fact, the small difference in growth rate between pH 4.0 and 6.8 suggests that *C. melkonianii* prefers to perform photosynthesis over that pH range. On the other hand, alkaline pH (i.e. pH 8.0), does not significantly inhibit the growth of this alga which achieved a quite good rate (Fig. 3c, d), as compared to the one obtained under the other operating conditions.

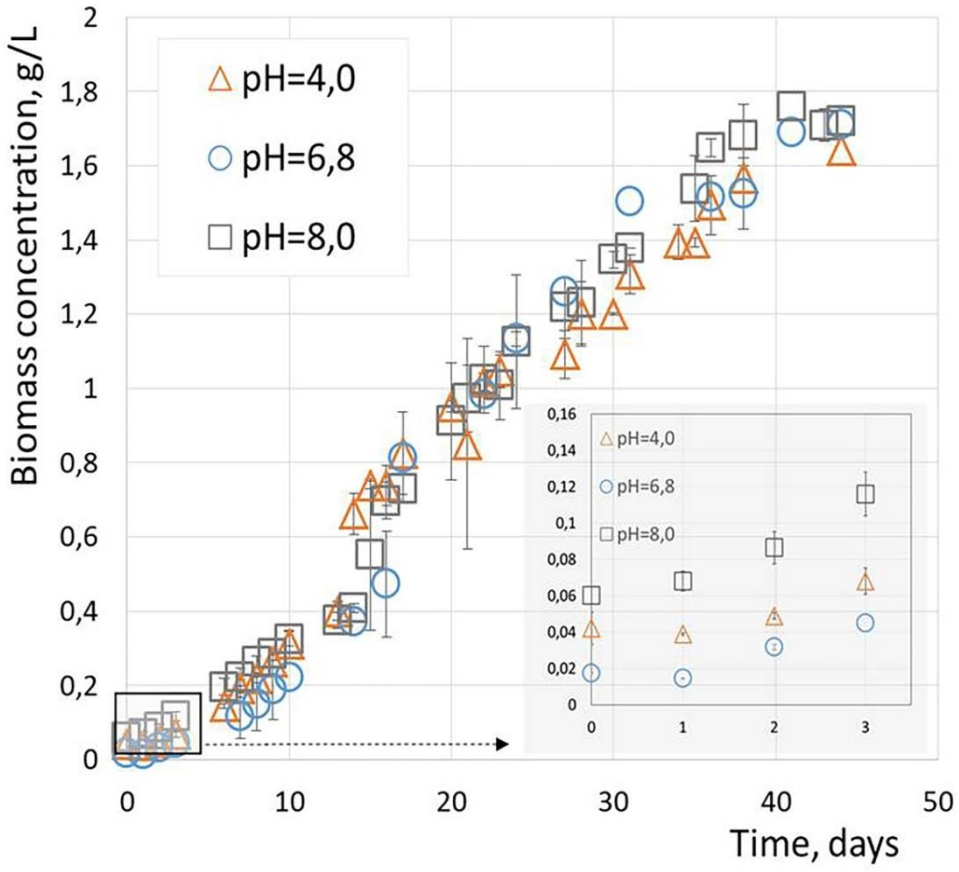

Fig. 2 Time evolution of biomass concentration as a function of pH of *Coccomyxa melkonianii* SCCA 048. In the grey box, initial values are highlighted

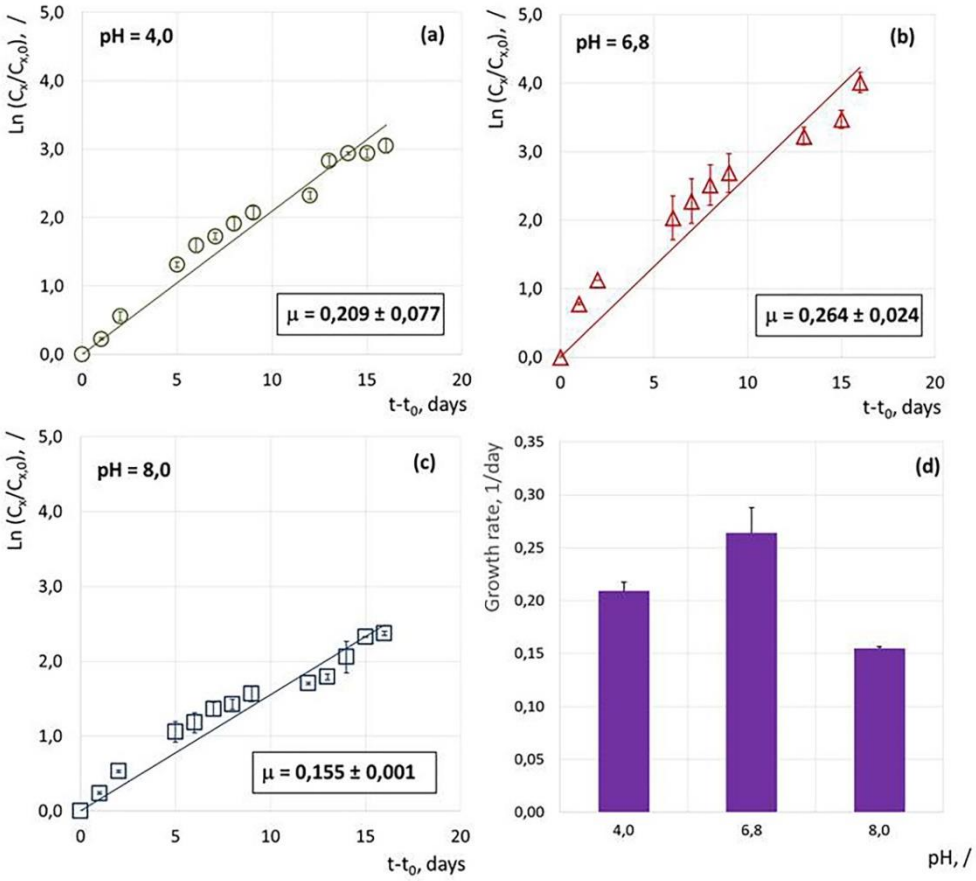

Fig. 3 Evaluation of microalgal growth rates under pH values of 4,0 (a), 6,8 (b), 8,0 (c) and comparison among the obtained growth rates (d)

3.3 Morphological plasticity at different pH

Morphological investigation of *C. melkonianii* cultivated in batch under the above three different pH conditions was performed after about six weeks of cultivation using light microscopy. The morphological effects of pH in the three cases are summarized in Fig. 4. Some differences in cells morphology were observed under the pH condition investigated, thus revealing the high phenotypic plasticity of this strain. At pH 6.8, vegetative cells exhibited the typical elongated cylindrical or ellipsoidal shapes in accordance with the observations of Malavasi *et al.* (2016). A few vacuoles were observed in the cytoplasm of each cell grown under standard conditions (pH 6.8). Morphological differences took place at pH 4.0 where cells appeared smaller and more spherical in shape than at higher pH values. Large vacuoles could be detected inside the cytoplasm as a result of the high lipid storage during the cultivation. Morphological differences were apparent also at the higher pH values investigated, although these differences were not always detectable in all cells. In fact, at pH 8.0 cells appeared curved and rounded in the apices and considerably longer than the

average length found at the other pHs despite the reduction in growth rate shown in Fig. 3c, d. The protoplast appeared filled with vacuoles, although less abundant with respect to lower pHs. This observation is consistent with the results obtained from lipid quantification analysis which showed a slightly lower lipid content compared to pH 6.8 (Fig. 5).

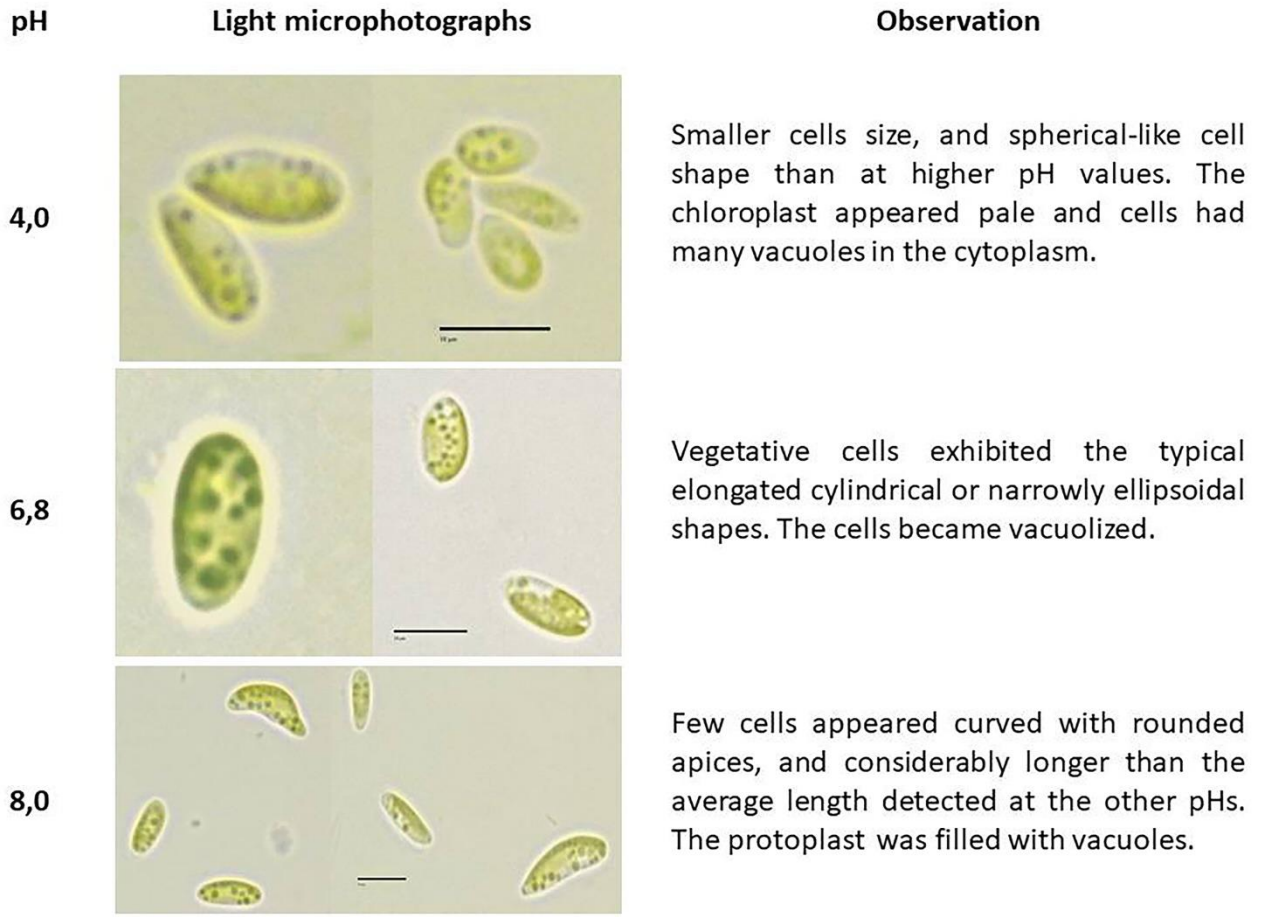

Fig. 4 Phenotypic plasticity of cells morphology of *Coccomyxa melkonianii* SCCA 048 under different pH. Light microphotographs (Scale-bar: 10 µm)

3.4 Effect of pH on lipid content (SPV)

The influence of pH on the total lipid accumulation of *C. melkonianii* during the batch experiments were also analyzed. Fig. 5a shows how the lipid concentration (% wt) changed with incubation time (days) for different pH values. As it can be observed, the evolution of lipid contents as a function of time at each pH treatment after 44 days of cultivation are quite similar. In particular, it can be observed from Fig. 5a that only a slight increase of lipid content could be observed up to the 20th cultivation day. Then, the lipids suddenly increase in the last cultivation days. In fact, after about 20 days, the lipid content raises almost linearly which is probably the result of the consumption of available nitrogen in solution that leads metabolism to shift towards the synthesis of lipids rather than the effect of pH cultivation values. This is confirmed by the observation that after about 20 days of cultivation, a plateau of biomass productivity is reached as it may be seen from Fig. 6a. On the other hand, as shown in Fig. 7a, time evolution of lipid productivity does not seem to be significantly influenced by pH variations.

Maximum lipid content achieved at the end of cultivation time, under the different pH conditions, are shown in Fig. 5b. The lipids accumulation does not appear to be significantly affected by culture pH and only slight differences could be observed. In particular, maximum lipid content under pH 6.8 was close to 23 % dw. On the other side, when using pH equal to 4.0 and 8.0, the corresponding lipid content was about 22 and 21 % dw, respectively (Fig. 5b).

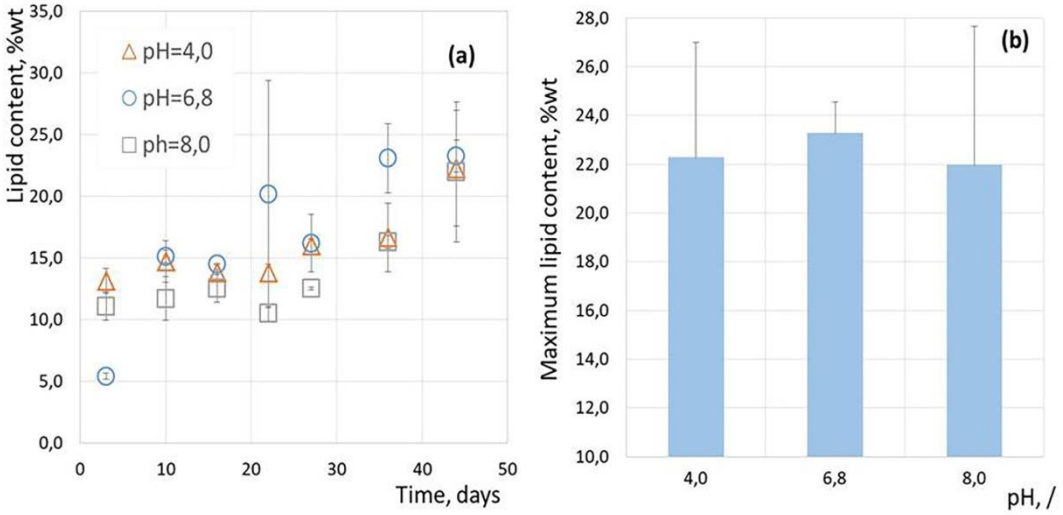

Fig. 5 Time evolution of intracellular lipid content (a) and comparison of its maximum values (b) achieved under the different pH values investigated

3.5 Effect of pH on biomass and lipids productivities

In Fig. 6a, the time evolution of biomass productivity, evaluated according to Eq. 3, is shown. As it can be observed, the system behavior is similar for all the investigated pH and is characterized by a relevant increase during the first days of cultivation which leads to the achievement of a sort of maximum after which the biomass productivity slightly decreases. The maximum biomass productivities attained under the different pH conditions are compared in Fig. 6b. It can be noted that only slight differences exist among the investigated conditions. However, the higher biomass productivity, i.e. 0.048 g L^{-1} d^{-1}, was achieved when using pH equal to 6.8.

In Fig. 7, lipid productivities, evaluated according to Eq,4, are shown. In particular, from Fig. 7a, it can be observed that, whatever the pH considered, the time evolution is characterized by an almost monotonic increase of lipid productivity which achieves its maximum value at the end of cultivation period. These maximum values are compared as a function of pH in Fig. 7b, where it is seen that, even in terms of lipid productivity, the maximum value of about 10 mg L^{-1} d^{-1} is achieved when using pH 6.8.

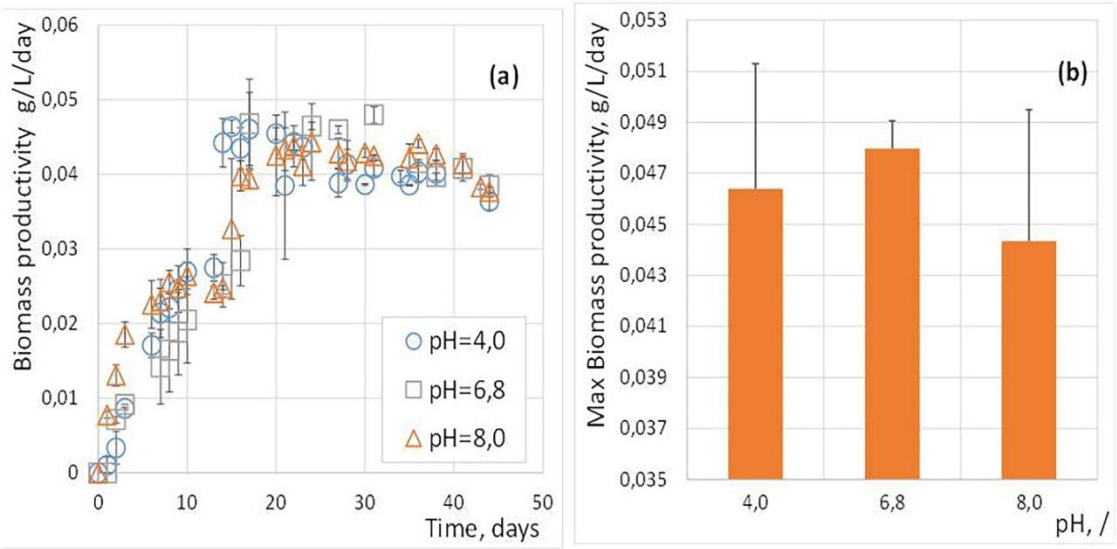

Fig. 6 Time evolution of biomass productivity (a) and comparison of its maximum values (b) as a function of pH medium

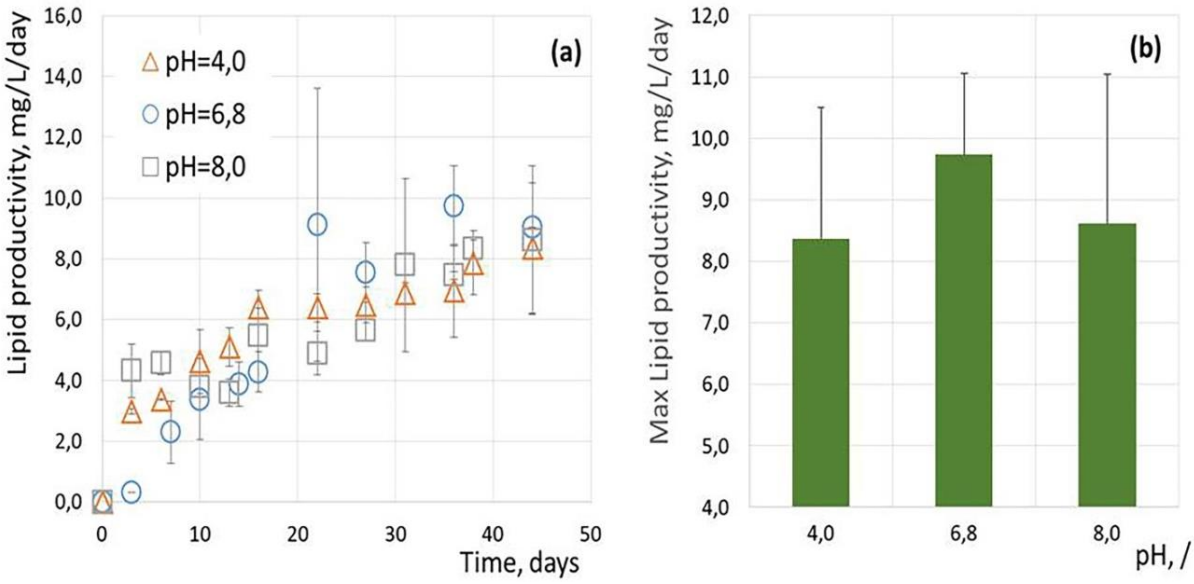

Fig. 7 Time evolution of lipid productivity (a) and comparison of its maximum values (b) as a function of pH medium

3.6 Effect of pH on FAMEs composition

In this study, we also attempted to verify whether the different pH conditions imposed during the batch experiments had affected the quality of microalgal lipids. Hence, the content of Fatty Acid Methyl Esters (FAMEs) obtained after lipid extraction and transesterification was analyzed and results are shown in Fig. 8. It can be seen that the prominent fatty acids found in all the three pH treatments were palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids. ANOVA results showed that statistical differences were detectable in the stearic ($P = 0.047$), oleic ($P = 0.001$), and linoleic ($P = 0.002$) fatty acids contents. Particularly, as it can be seen in the Fig. 8, oleic acid content was higher at pH 6.8 and pH 8.0 when compared to pH 4.0. Similarly, linoleic fatty acid showed an increase in pH 6.8 culture with respect to the amounts found at pH 4.0 and pH 8.0, although at the latter value the content was slightly higher than that one obtained at pH 4.0. It should be noted that at pH 4.0, a quite fair amount of cis-10-heptadecenoic acid (C17:1), i.e. 45.42%, was detected, which was not observed at any of the other pH values investigated. It should be also worth mentioning that the linolenic acid (C18:3) has been detected only at pH 4.0 and 8.0, respectively.

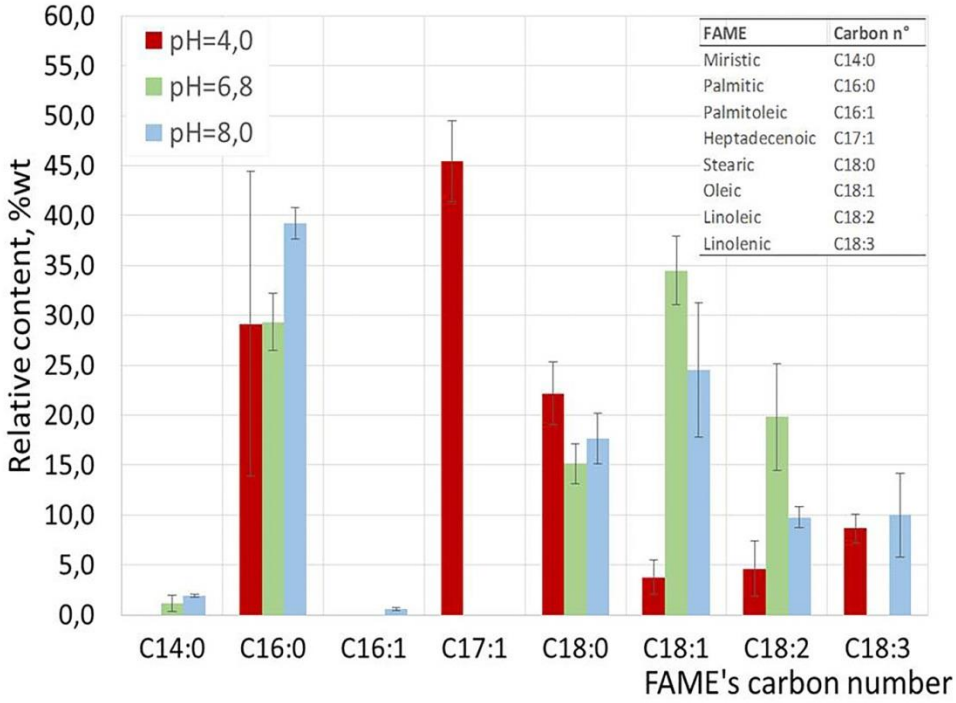

Fig. 8 FAMEs composition of lipids in terms of weight percentage extracted from *Coccomyxa melkonianii* SCCA 048 cultured at different pHs

4 Discussion

Members belonging to the genus *Coccomyxa* are found to be able to colonize a wide range of environmental habitats (Darienko *et al.* 2015; Malavasi *et al.* 2016). Being so flexible and adaptable, *Coccomyxa* may have an advantage when conditions become extreme (Barcytė and Nedbalová 2017). Extreme pH conditions influence photosynthesis, growth, and nutrient assimilation in algae (Lane and Burris 1981; Gensemer *et al.* 1993; Gerloff-elias *et al.* 2005). For several algal species, a range of external pH values at which optimal growth is realized correlates with the pH conditions of the habitat from which the species was isolated (Gerloff-elias *et al.* 2005). *Coccomyxa melkonianii* SCCA 048 is characterized for being able to live in extreme environment with high concentrations of heavy metals (Malavasi *et al.* 2016). The strain represents a lineage associated to a mine discharge habitat and for this reason, we felt that was important to study optimal pH of *Coccomyxa melkonianii* SCCA 048, since no data are available in the literature in this regards. Our results showed that the external pH induced changes in the morphology of the alga cells, which might help its adaptation to harsh environments (Barcytė and Nedbalová 2017). Phenotypic changes of *Coccomyxa* can be induced by direct environmental factors as was also demonstrated by Darienko *et al.* (2015) and Barcyte & Nedbalova (2017). Hence, ecological data

can represent a very useful tool to delimit morphologically often undistinguishable species (Malavasi *et al.* 2016). *Coccomyxa melkonianii* does not show significant differences in the growth rate when cultured at the pH range of 4.0 – 8.0, as result of its high adaptability to grow in laboratory at higher and lower pH values with respect to its optimum. It is known that the extreme external values of pH theoretically forces extremophile microalgae to expend energy in order to maintain neutral pH into the cytosol (Gross 2000; Vaquero *et al*. 2014). Extra energy maintenance costs are expected to make growth rate and productivity lower if compared to 'common' microalgae due to less energy fraction available for anabolism (Gross 2000; Vaquero *et al.* 2014). However, *C. melkonianii* did not lower the growth rate at sub-optimal pH and reached quite good biomass productivity rates, close to those reported for the non-extremophile strain *Chlamydomonas reinhardtii* (Talebi *et al.* 2013). It is worth to remark that the ability of *C. melkonianii* to grow well under all the three pH treatments tested implies the high adaptability of this strain to a quite broad range of pH values (Soru *et al.* 2018). Furthermore, our results showed that, within the pH range investigated, *C. melkonianii* was also characterized by quite high lipid productivities close to those ones observed in literature for other oleaginous microalgae such as *Dunaliella salina* (Talebi *et al.* 2013) and *Nannochloropsis oceanica* (Slocombe *et al*. 2015).

Identification of a certain species with desirable characteristics is a key component for achieving economic viability of the process (Sahu *et al.* 2013). It is known that environmental factors may influence the degree of fatty acids saturation (Yun *et al.* 2014). In fact, it should be noted that the FAMEs composition greatly varied depending on the pH of the cultures. In particular, neutral and alkaline pHs (i.e. 6.8 and 8.0) yield a good content of C16:0, C18:0, and C18:1. The latter one is the best candidate for biodiesel production due to its ideal viscosity (Yun *et al.* 2014) and the low percentage of polyunsaturated FAs (C18:2 and C18:3) could improve the oxidative stability of biodiesel. Furthermore, only at pH 4.0 experiment, a fair amount of C17:1 was found, which has been previously identified in lower concentrations in some microalgae strains isolated and cultivated under low pH conditions (Yun *et al.* 2014). This outcome suggests that this species is an attractive and versatile metal- and pH-resistant microalga suitable for the outdoor cultivation aimed to the production of valuable compounds (Soru et al. 2019).

5 Concluding remarks

The ability of *Coccomyxa melkonianii* SCCA 048 to actively grow and operate the lipid biosynthesis at different pH values might become a selective competitive advantage for this strain in continuous production processes, compared to non-extremophile microalgae. Furthermore, the ability to change the morphology may help the cells of such alga to survive harsh conditions. Such a strong adaptation response enables this extremophile microalga to reach biomass productivities similar to those of control cultures, which might be of biotechnological interest. It will, therefore, be essential to study not only the influence of pH, but also of heavy metals, on the laboratory growth of this organism. In fact, the capability to tolerate either high heavy metal concentrations and different pH values might be viably exploited for performing cultivation of *C. melkonianii* in open raceways.

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