Microalgae for Industrial Purposes

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Chapter 6
Microalgae for Industrial Purposes

Mario Giordano and Qiang Wang

Abstract The use of microalgae for the production of compounds of commercial relevance has received substantial interest in recent years, mostly because these organisms contain a plethora of valuable compounds and their high turnover rate and functional plasticity make them relatively easy to cultivate for the production of biomass and added-value molecules. The metabolic flexibility of algae allows using them for many commercial applications, but it also makes it easy for cultures to diverge from the intended biomass quality. A thorough comprehension of the principles that control growth and carbon allocation is therefore of paramount importance for effective production of algal biomass and derived chemicals. In this review, we intend to provide basic but exhaustive information on how algae grow and on their biotechnological potential. In addition to this primary goal, we also give the reader a succinct panorama of culturing systems and possible applications.

Keywords Algae • Carbon allocation • Genetics • Culture • Cell composition • Stoichiometry • Biofuel • Food • Feed

6.1 Introduction

The use of microalgal biomass for capturing CO_2, for the production of biofuel and pharmaceutical and nutraceutical products, as food and animal feed, and for wastewater treatment has recently become a focal point for research and the object of public interest (e.g., Rasala et al. 2014). Part of this growing popularity is due to the

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fact that microalgae are very versatile in their utilization. Furthermore, they represent an excellent alternative to land crops: with their fast turnover rates, microalgae can produce large amounts of biomass in a relatively small volume and within a relatively short time. Furthermore, microalgae cultivation can be carried out on land of low agricultural value, thus avoiding competition with traditional crops. Because of the unique metabolic plasticity of microalgae, it is relatively easy (and certainly easier than for most terrestrial embryophytes) to optimize resource allocation in the cell to favor the production of selected compounds, by controlling culture conditions. Their high photosynthetic yield and efficiency make microalgae potentially capable of capturing substantial amounts of CO_2, thereby contributing to the mitigation of global climate change, especially if microalgae cultures can be fed with flue gases from fossil fuel combustion.

In spite of the foregoing considerations and although the commercial production of microalgae has a rather long history (e.g., Ben-Amotz 2004), progress in the design of production plants and, in general, in the technologies (both biological and engineering) used to commercially exploit algae (Table 6.1) has been rather slow. The causes of this are various, with a great contribution attributable to the fact that players on the market tend not to share information (Grobbelaar 2009). Furthermore, algal cultivation is often conducted by operators who lack a thorough understanding of the physiology of these organisms, thus having limited ability to identify and solve the problems that may arise from their functional complexity. This chapter intends to highlight the crucial aspects of algal physiology and to suggest ways to avoid difficulties and facilitate the attainment of biomass of a defined quality or of specific compounds. To do so, we provide some basic information on how algae fix and allocate carbon and on how culture conditions can influence the organic composition of cells. We also discuss how the chemical composition of culture media affects algal growth and biomass quality. Finally, we provide examples of applications in which physiological knowledge may bring obvious advantages.

6.2 What Are Algae?

In the words of Raven and Giordano (2014), "algae frequently get a bad press. Pond slime is a problem in garden pools, algal blooms can produce toxins that incapacitate or kill animals and humans and even the term seaweed is pejorative, a weed being a plant growing in what humans consider to be the wrong place. Positive aspects of algae are generally less newsworthy." Giving a scientific sound definition of "algae" is not an easy task: algae are organisms that produce O_2 as a waste product of their photosynthesis, but are not "higher plants" (embryophytes) (Raven and Giordano 2014). According to this definition, prokaryotic (cyanobacteria) and eukaryotic photosynthetic organisms are algae. In the group of organisms included in this definition, we find organisms in an approximate size range from 1 μm to 1 mm,

Table 6.1 Main algal species used in biotechnological applications (the information reported in this table was mainly obtained from Enzing et al. 2014 and Borowitzka 2016)

Species	Phylum and class	Product	Production status
Artrospira platensis	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Mass production
Chaetoceros muellerii	Bacillariophyta, Bacillariophyceae	Biomass as dietary supplement	Small scale
Chlorella vulgaris	Chlorophyta, Chlorophyceae	Canthaxanthin, astaxanthin, β-carotene, biomass as dietary supplement	Mass production
Crypthecodinium cohnii	Miozoa, Dinophyceae	Docosahexanoic acid	Mass production
Dunaliella salina	Chlorophyta, Chlorophyceae	β-Carotene, glycerol	Mass production
Hematococcus pluvialis	Chlorophyta, Chlorophyceae	Astaxanthin, cantaxanthin, lutein	Mass production
Isochrysis spp.	Chlorophta, Chrysophyceae	Biomass as dietary supplement	Mass production
Nannochloropsis spp.	Ochrophyta, Eustigmatophyceae	Eicosapentanoic acid	Small scale
Nitzschia closterium	Bacillariophyta, Bacillariophyceae	Eicosapentanoic acid	Research
Nostoc commune	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Nostoc flagelliforme	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Nostoc sphaeroids	Cyanophyta, Cyanophyceae	Biomass as dietary supplement	Collected, not cultivated
Odontella	Bacillariophyta, Mediophyceae	Eicosapentanoic acid, docosahexanoic acid	Small scale
Pavlova lutherii	Haptophyta, Pavlovophyceae	Biomass as dietary supplement	Research
Phaeodactylum tricornutum	Bacillariophyta, Bacillariophyta incertae sedis	Eicosapentanoic acid	Small scale
Porphyridium cruentum	Rhodophyta, Protoflorideae	Biomass as dietary supplement, arachidonic acid, triacylglycerols	Small scale
Scenedesmus almeriensis	Chlorophyta, Chlorophyceae	Lutein, β-carotene	Research
Skeletonema spp.	Bacillariophyta, Bacillariophyceae	Biomass as dietary supplement	Small scale
Tetraselmis spp.	Chlorophta, Prasinophyceae	Biomass as dietary supplement	Research

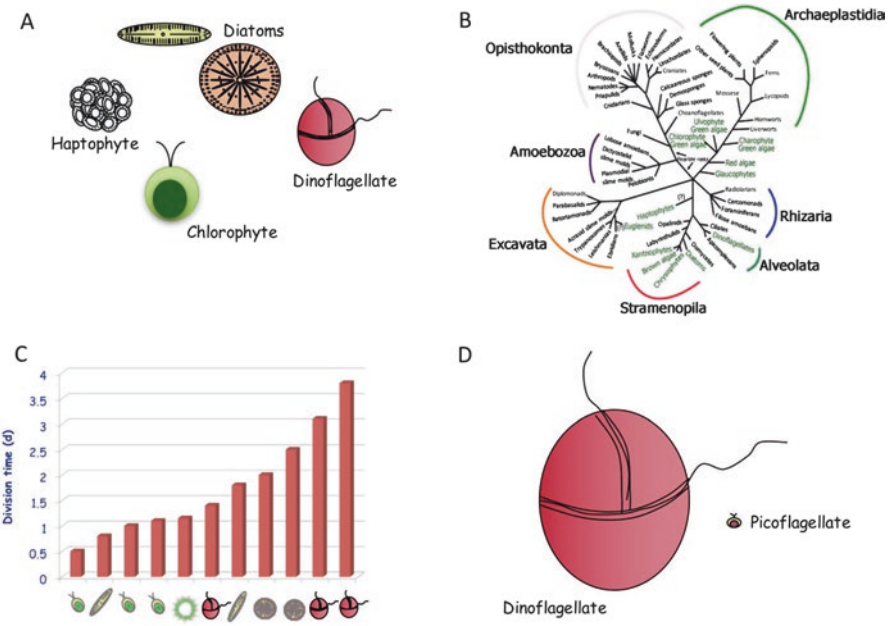

Fig. 6.1 The algae are extremely diverse in morphology (**a**), phylogeny (**b**), growth potential (**c**), and size (**d**). The information in **b** refers to eukaryotic algae and was derived from Knoll (2003) and Brodie et al. (2017). The growth rates in **c** are unpublished data; the species are not identified because of non-disclosure agreements

highly phylogenetically and morphologically diverse (and this diversity has only been minimally explored; de Vargas et al. 2015), with an extremely broad range of growth rates (Fig. 6.1).

6.3 Algae Produce Biomass Through Photolithotrophy, Heterotrophy, or Mixotrophy

Although the ability to carry out photosynthesis is certainly a key characteristic of algae, they are also capable of various degrees and various modes of heterotrophy. For some application, the ability of algae to combine autotrophy and heterotrophy can be advantageous, offering the ability to stimulate growth by supplying organic nutrients when light is not present or insufficient and when CO_2 is subsaturating. The ability to grow on organic substrates can also be exploited in phytodepuration processes that require the treatment of dissolved or particulate organic matter, in addition to that of inorganic nutrients. A brief description of algal trophisms may help the comprehension of the ensuing topics.

6.3.1 Photolitothrophy

Photosynthesis is a mode of nutrition that uses inorganic carbon in a light-dependent process to generate organic compounds (photolithotrophy). In photosynthesis the generation of energy in the form of reductants and ATP is conducted on the photosynthetic membranes, which in eukaryotes are located in the chloroplast (Chen et al. 2015a). The photosynthetic generation of reductants is the consequence of the excitation of specialized chlorophyll *a* molecules, which initiate an electron transfer along a redox gradient. This redox energy is employed chemo-osmotically (Mitchell 1961) to produce ATP, whereas the electrons are finally allocated onto soluble molecules (e.g., ferredoxins and NADPH) that can then be employed in metabolism (Schmollinger and Merchant 2014). The production of ATP and reductants is thus a function of the amount of photons captured by the so-called antenna systems, partially modulable ensembles of pigment–protein complexes. The energy made available by the processes occurring on photosynthetic membranes is then employed for the assimilation of primarily inorganic carbon. Appreciable proportions of reducing power and ATP are also used to acquire and assimilate other nutrients (N, P, S, etc.) and for various metabolic processes. CO_2 assimilation is carried out through the carboxylation of pentose ribulose bisphosphate (RuBP) with the catalysis of the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) (Marcus et al. 2011). The enzyme rubisco can also catalyze the oxygenation or RuBP, and O_2 and CO_2 compete with each other at the active sites of rubisco (Bowes et al. 1971). The presence of O_2 in the gas phase decreases the efficiency and yield of photosynthesis and leads to a conspicuous increase in the energetic cost of CO_2 fixation. Although in the course of evolution the ability of rubisco to favor carboxylation versus oxygenation has increased, no rubisco present today on the planet is capable of conducting carboxylation in the absence of oxygenation (Giordano et al. 2005). Most algae (Raven et al. 2005, 2008, 2012) overcome the difficulties associated to the double activity of rubisco (carboxylation and oxygenation) and the low CO_2:O_2 ratio in the extant atmosphere by expressing CO_2-concentrating mechanisms (CCMs) that pump CO_2 toward rubisco in an energy-dependent matter (Giordano et al. 2005; Raven et al. 2014). The CCMs are of various sorts and their modulation is strongly responsive to the CO_2:O_2 ratio in the environment. The activity of CCMs may also depend on factors that are distinct from the availability of CO_2 and O_2: the presence of CCMs allows algae to decrease the amount of rubisco in the cell and this leads to substantial savings in N, S, and also to an enhanced Fe and light use efficiency (Beardall and Giordano 2002; Raven et al. 2014); the availability of these resources can therefore have a role in the modulation of CCMs (Beardall and Giordano 2002; Raven et al. 2008, 2012, 2014). Elevated CO_2 leads to the downregulation of CCM (Giordano et al. 2005). CCM downregulation allows cells to save the energy they would otherwise have invested into pumping inorganic carbon toward rubisco. However, this does not necessarily create a higher growth rate, because the energy saved is not always invested in growth, or growth limitation (before the CO_2 increase) may not reside in CO_2 (Giordano and Ratti 2013).

6.3.2 *Mixotrophy*

Mixotrophy can be defined as the concomitant occurrence of photolithotrophy and chemo-organotrophy (use of exogenous organic C as the source of both energy and C for metabolism; also see Raven and Beardall 2016). The uptake of organic C can be carried out on a molecule-by-molecule basis (osmotrophy) or through the acquisition of particles (phagotrophy) (Flynn et al. 2010; Raven et al. 2013). Algae are, for the most part, primarily photolithotrophs and recur to mixotrophy only when photolithotrophy is hindered by the scarcity of inorganic nutrients or light. There are, however, protists that, although they fall within the definition of "algae" sensu Raven and Giordano (2014), are primarily phagotrophic, but can photosynthesize under prey limitation. Finally, some organisms are obviously derived from photosynthetic organisms but are obligate chemo-organitrophs (Mitra et al. 2016). The ancestral condition in algae is probably that of obligate photolithotrophs (Beardall and Raven 2016), which is confirmed by the fact that basal cyanobacteria such as *Glaeobacter violaceous* (Blank and Sanchez-Baracaldo 2010) are incapable of chemo-organotrophy (Beardall and Raven 2016, and references therein). On the other hand, Raven et al. (2009) wrote that "without phagotrophy at the cell level there would be no photosynthesis in eukaryotes"; in other words, as the acquisition of the chloroplast is the consequence of a phagotrophic event, phagotrophy/mixotrophy appears to be an inherent property of eukaryotic photosynthetic organisms. It must be considered that primary endosymbiotic events, that is, those in which a heterotrophic prokaryote engulfed a photosynthetic prokaryote, have most likely been relatively rare, whereas subsequent secondary and tertiary endosymbiotic events, in which an eukaryote engulfed a photosynthetic eukaryote, may have occurred more readily (McFadden 2001; Gentil et al. 2017; Lane 2017). Among extant algae, clades deriving from primary endosymbiosis are rarely mixotrophic, whereas mixotrophy is much more frequent in algae originated from secondary and tertiary endosymbiotic events (Beardall and Raven 2016). According to Raven (1995, 1997), the cost of the photosynthetic apparatus and the uptake systems for nutrients different from C sums up to about 50% of C, N, P, Fe, and of the energy cost to make a cell in a photolithotroph. The corresponding cost for the heterotrophic (phagotrophic) apparatus is less than 10% (also see Jones 2000 for further discussion on these matters). The advantage of mixotrophy over obligate photolithotrophy and obligate photo-organotrophy emerges especially in the light (Jones 2000), although the large number of possible nutritional conditions makes it hard to provide an univocal outcome of competition between organisms with different trophisms. It should also be considered that chemo-organotrophy leads to greater C loss through respiration and thus can lead to an elemental stoichiometry with lower C relative to N, P, and Fe. The concomitant use of photosynthesis can compensate for such unbalance, to a variable degree (Beardall and Raven 2016). Osmomixotrophy may also be important to recapture leaked dissolved organic carbon (Raven and Beardall 2016).

6.4 Cell Composition Results from a Combination of Genotypic and Environmental Constraints

The composition of cells is the result of the interaction of the genome with the environment leading to the best suited structural and functional cell organization. The composition of the cell is thus strongly dependent on the condition in which cells live or are cultured. At the same time, because it depends on the genotype, cell composition is strongly species specific. Different genotypes will respond differently to the same kind of environmental perturbation, with different degrees of compositional and functional homeostasis (Giordano 2013). The attitude to homeostatically retain cell composition is possibly also a function of growth rate: Fanesi et al. (2014) showed that, other things being equal, fast-growing microalgae have a lower tendency to compositional homeostasis than slow-growing ones, because they have a higher probability of competitively taking advantage of the investment in reproduction, regardless of the duration of the perturbation (Giordano 2013). Depending on both the species and the type of environmental perturbations, cells can adjust their growth performance through (a) regulatory processes that do not require changes in the expressed proteome (Giordano 2013; Raven and Geider 2003); (b) the production of new protein and the degradation of protein present before the perturbation was applied (acclimation); and (c) by changes in the genotype (adaptation) (Giordano 2013; Raven and Geider 2003). Most species, if maintained for a prolonged time in the same conditions, will tend to change their expressed proteome and acclimate to the environmental condition (Giordano 2013), and if a sufficient genetic heterogeneity is present in the population, genotype selection is also likely (Venuleo et al. 2017).

The consequences of these considerations for the commercial cultivation of microalgae are that (a) a tight match between genotype and cultural conditions must be ensured to obtain the desired end product; (b) stability of culture conditions is required to ensure constancy in the quality of the product and in productivity; and (c) a control of the genetic stability of the population is important to prevent a shift in dominance that may lead to the prevalence of a strain with non optimal characteristics (from the commercial perspective).

6.4.1 Elemental Stoichiometry and Organic Cell Composition

Cell composition results from the availability of the various elements and their metabolic and structural requirements. Quigg et al. (2003, 2011) and Ho et al. (2010) reported the elemental composition for a large number of species cultured under presumably resource-replete conditions. The species-specificity of these cell stoichiometries emerges clearly; macronutrients (i.e., C, N, P, S) generally show a higher degree of similarity than micronutrients across species (Giordano 2013 and references therein). An obvious reciprocal relationship exists between cell

stoichiometry and environmental chemistry; the oceanic "Redfield ratio" (Redfield 1934) is a typical example of this. A mechanistic basis for the Redfield ratio was proposed by Loladze and Elser (2011), who suggested that the fairly constant elemental stoichiometry of phytoplankton in the ocean, especially with respect to the N:P ratio, is imposed by the fact that rapid growth constrains the ratio between cell protein (main sink for N) and RNA (main sink for P) (e.g., Norici et al. 2011; Raven et al. 2012; Geider and La Roche 2002). The relative content of N:P can be used as a proxy for the protein to RNA ratio and this can be related to growth through the capacity for protein synthesis (growth rate hypothesis: Elser et al. 2011; Flynn et al. 2010; Loladze and Elser 2011; Giordano et al. 2015b) It is worthwhile noticing, however, that the growth rate hypothesis does not always apply to microalgae (Flynn et al. 2010; Nicklisch and Steinberg 2009; but also see Giordano et al. (2015b).

In a commercial cultivation system, most likely, growth conditions are resource replete, which may lead to the fact that cell elemental composition does not reflect the requirement to achieve the maximum possible growth rate but is influenced by luxury accumulation of some elements (Giordano 2013).

Elemental composition is connected to the organic cell composition also because an imbalance between C and N (or C and P, or C and S) can lead to different C allocation patterns. When, for instance, the C:N ratio is higher than the C:N ratio in protein and nucleic acids and the excess of C surpasses the requirement for structural non-N/P-containing pools, two options exist: the C in excess (relative to other nutrients) is not acquired (Kaffes et al. 2010) or C is assimilated beyond strict growth requirements with a consequent increase in the size of pools that do not contain N and P, such as carbohydrates and lipids (Giordano et al. 2015a; Giordano and Ratti 2013; Montechiaro and Giordano 2010; Palmucci et al. 2011). Whether the excess C is allocated to carbohydrates or lipids depends on genotypic, energetic, and size constraints (Palmucci et al. 2011). The genotypic constraints are associated with the preference for some metabolic pathways by a species/strain (Palmucci and Giordano 2012). The energetic constrains are associated with the different cost of allocating C to carbohydrate or lipid and mobilizing it (Montechiaro and Giordano 2010; Palmucci et al. 2011); this of course becomes relevant only when energy availability (i.e., light; as is often the case in dense commercial cultures) limits growth (Ruan and Giordano 2017; Ruan et al. 2017). The spatial constraints occur because the lower hydration of lipids makes it easier for them to accumulate when space is limited (Palmucci et al. 2011). The outcome of an imbalance between C and N is thus not easily forecasted and should be assessed case by case.

6.5 Genetic Modification of Algae: Tools and Aims

The enormous pool of metabolic possibilities constituted by microalgae translates into a very large and mostly unexplored potential for applications. It also minimizes the need for genetic manipulations, because many functional variants are present in nature (although they may not all have been discovered yet). Many applicative

problems may be solved through the search of "natural" species/strains with the required metabolic capabilities. This notwithstanding, genetic manipulation is possible and, where allowed, it may offer the best solutions for specific problems (Gressel 2008). The generation of mutants is important for strain improvement for biotechnological applications, but it can also be used, in the secure space of a laboratory, for functional analyses of genes and proteins. Few genetically modified strains of microalgae are used commercially nowadays, partially because molecular tools (e.g., efficient nuclear transformation, availability of promoter and selectable maker genes, and stable expression of transgenes) are not available for some commercially important species (Amaro et al. 2011). However, recent developments of high-throughput technologies have enabled the profiling of mRNA, proteins, and metabolites, giving rise to the fields of transcriptomics, proteomics, and metabolomics, respectively (Lee et al. 2010); these methodologies allow the comprehension of the consequences of genetic manipulation at the whole cell level, thus facilitating their application in a productive context.

Despite the increasing number of sequenced microalgae genomes, precise and programmable genome editing has been reported for only a few eukaryotic microalgae, such as the eustigmatophyte *Nannochloropsis* sp. (Kilian et al. 2011), the green alga *Chlamydomonas reinhardtii* (Sizova et al. 2013), the diatoms *Phaeodactylum tricornutum* (Nymark et al. 2016) and *Thalassiosira pseudonana* (Poulsen et al. 2006). The genome editing methods used for these studies (Daboussi et al. 2014; Hopes et al. 2016; Nymark et al. 2016; Shin et al. 2016; Wang et al. 2016) (see following), together with the ever-growing number of tools for transgene expression, cloning, and transformation (e.g., Rasala et al. 2014; Scaife et al. 2015), open up very promising perspectives for the future of algal genetic manipulation.

The methods for targeted gene knockout and gene replacement based on homologous recombination (HR) have driven rapid progress in understanding many of the complex metabolic and regulatory networks in eukaryotic cells (Weeks 2011). The main obstacle for direct gene targeting is the low frequency of HR between nuclear genes and donor DNA. Recombination efficiency may be increased by the use of zinc-finger nucleases (ZFNs), which cut the genome at specific sites to facilitate HR. Sizova et al. (2013) published a nuclear gene targeting strategy for the green alga *Chlamydomonas reinhardtii* that is based on the application of ZFNs. In the case of *C. reinhardtii*, insertional mutagenesis to disrupt a gene of interest is commonly employed. For instance, by exploiting a collection of *C. reinhardtii* insertional mutants originally isolated for their insensitivity to ammonium, Emanuel et al. (2016) found a strain that, in addition to its ammonium-insensitive (AI) phenotype, was unable to correctly express nitrogen assimilation genes in response to NO signals. The difficulty of extending this approach to more species resides in the fact that it cannot prescind from the existence of large collections of mutants and from the screening of many thousands of clones.

MicroRNAs (miRNA) are 21- to 24-nucleotide RNAs present in many eukaryotes that guide the silencing effector Argonaute (AGO) protein to target mRNAs via a base-pairing process (Bartel 2009). Chung et al. (2016) showed that miRNAs in

C. reinhardtii regulate gene expression primarily by destabilizing mRNAs, using target sites that lie predominantly within coding regions.

Protein-based systems involving mega nucleases and "transcription activator-like effector nucleases" (TALENs) allow precisely targeted genome editing of eukaryotic microalgae genomes (Daboussi et al. 2014; Weyman et al. 2015). These systems bear great potential for research and generation of tailored strains, although they are labor intensive and rather costly. Recently, a much simpler and inexpensive method for genome editing in algae, CRISPR/Cas9, came about (Nymark et al. 2016). It was developed to generate stable targeted gene knockouts in the marine diatom Phaeodactylum *tricornutum*, but it should be easily adaptable for use in other microalgae. Shin et al. (2016) applied this system to *C. reinhardtii*; they directly delivered the Cas9 protein and the "single-chain guide RNAs" (sgRNAs) to three different genes, obtaining mutations at the Cas9 cut sites with a significantly improved targeted mutagenic efficiency. Wang et al. (2016) established a precise CRISPR/Cas9-based genome editing approach for the industrial oleaginous microalga *Nannochloropsis oceanica*, using the gene encoding nitrate reductase (NR; g7988) as an example. The isolated mutants, in which precise deletion of five bases caused a frameshift in NR translation, grew normally in the presence of NH_4^+ but failed to grow when N was supplied as NO_3^-. This demonstration of CRISPR/Cas9-based genome editing in industrial microalgae is very promising for microalgae-based biotechnological applications. Also, editing of the chloroplast genome is of interest for biotechnological applications, because it may allow transgene insertion via HR with expression that is not subject to nuclear gene-silencing mechanisms; furthermore, plastidial transformation may take advantage of the prokaryotic organization of chloroplast genomes to co-express multiple genes in operons (Wannathong et al. 2016). New simple and inexpensive protocols have recently been developed to this end (Wannathong et al. 2016), but their effectiveness for species different from *C. reinhardtii* is still to be demonstrated as is their applicability for biotechnological purposes.

Many cyanobacterial strains are amenable to transformation and homologous recombination. Cis genetic modification (through genome editing) is the most common approach for engineering cyanobacteria (Berla et al. 2013). Typically, chromosomal mutations are generated through the insertion of a plasmid that contains the gene(s) of interest, a selectable marker gene, and flanking sequences homologous to the targeted chromosomal sequence (homology arms). Numerous heterologous genes have been inserted through these methods in the model cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 (Savakis and Hellingwerf 2015). However, genome editing of cyanobacteria is more challenging than in model heterotrophic prokaryotes such as *Escherichia coli*, primarily because cyanobacteria often contain multiple genome copies per cell and long-term instability of the genes introduced (Kusakabe et al. 2013; Ramey et al. 2015). CRISPR interference is emerging as a promising method to repress expression of specific genes, with no need for gene knockout also for prokaryotes (Huang et al. 2016).

6.6 How Are Algae Cultured?

Large-scale microalgal cultivation can be attained by a number of culturing systems, the choice of which depends on cost, available technology, and desired quality of the biomass or added-value product. Consequently, no set recipe for a successful cultivation exists and physiological, engineering, and economic analyses must be conducted to ensure good results.

Algal commercial cultivation is often conducted empirically without a full understanding of the physiology behind it, which can lead to unsatisfactory results or to products that are highly variable in quality because of the lack of control on the biological processes controlling biomass production.

From a trophic point of view, microalgae can be grown photolithotrophically (phototrophy), chemo-organitrophically (heterotrophy), or mixotrophically (Chojnacka and Marquez-Rocha 2004; see above). Photolithotrophic growth is advantageous because it can use natural sunlight as the energy source and mineral media, which are relatively inexpensive; it is unavoidable when obligate photolithotrophs are used. Light must be in large supply to sustain photolithotrophic growth (Perez-Garcia et al. 2011), which makes this mode of mass cultivation economically convenient in area with high insolation. In intensive cultures, light penetration can be substantially attenuated and light can become the limiting factor for growth. This problem is usually addressed through careful design of culturing systems. In recent years, a molecular approach to the problem of light availability in intensive culture systems has also been taken, through the production of genetically modified strains that have antennae of smaller size and thus a decreased light attenuation across the culture (Mooij et al. 2015). These strains, however, have not yet found commercial application and still need to be tested for productivity at a usefully large scale.

In some cases, higher productivity can be attained through heterotrophic or mixotrophic cultivation methods (Chen et al. 2016). Heterotrophy can be maintained in total darkness by supplying organic compounds (e.g., glucose, acetate, glycerol) as both energy and carbon source, eliminating the need for illumination but adding a cost for the organic substrates. The cultivation of *Chlorella protothecoides* (now *Auxenochlorella protothecoides*) under mixotrophic condition was reported to increase the yield of biomass and lipid (Wang et al. 2013). The mixotrophic cultivation of the green alga *Chlorella* sp. C2 in a 5-L bioreactor resulted in a maximum biomass productivity of 9.87 g L^{-1} day^{-1}; this productivity declined to 7.93 g L^{-1} day^{-1} when the culture size was scaled up to 50 L (Chen et al. 2016), which is still a very small volume for commercial application. The change in productivity with increasing cultural volume warns us about the extrapolation of data obtained from small-scale tests to larger-scale cultivation. Cultivation on organic substrates currently is rarely utilized in the commercial production of algal biomass because the number of heterotrophic or mixotrophic algal strains that can be used is limited, the presence of organic carbon makes it very difficult to control bacterial proliferation, cases of growth inhibition by soluble organic compounds were reported, and because of the higher cost of growth media (Zhang et al. 2014a). There are however

applications for which mixotrophy can be useful and almost unavoidable. One such application, for instance, is the use of algae in wastewater treatment, where the water affluent cannot be fully deprived of organic components and a concomitant utilization of the inorganic and organic components is desirable or necessary (Cai et al. 2013).

In terms of the engineering of the cultivation systems, both open ponds and photobioreactors are used for algal cultivation. When very large quantities of product must be generated at low cost, open ponds are the most common solution (Borowitzka 1999). However, not all species are amenable to effective cultivation in open ponds, and the susceptibility to weather (especially rain and temperature variations) and light availability makes an open pond suited mostly for tropical and subtropical regimes with low precipitation and cloud cover (Richmond 1986). Algae cultivation in open-pond production systems has been used since the 1950s (Chojnacka et al. 2004), in both natural (lakes, lagoons, ponds) and artificial basins. Shallow raceway ponds, in which the algal suspension is mixed with paddle wheels, are the most widely used systems because they are relatively easy and cheap to construct and operate (Doucha and Lívanský 2006). Currently, more than 90% of world microalgae biomass production is obtained in raceway ponds. Some ponds are built on non-arable lands adjacent to power plants to have access to CO_2 from flue gases or near wastewater treatment plants to easily access nutrient supplies. Although widespread, open ponds have their drawbacks such as relatively low culture density and biomass productivity, high evaporative losses, and susceptibility to weather and to contamination by bacteria or undesired algal strains (Chen et al. 2013; Richardson et al. 2012).

When the environmental conditions are not suitable for open ponds, or a high and verifiable quality of biomass is required or biomass is used for the production of added-value compounds, algae can be cultured in closed or nearly closed systems, the photobioreactors (PBR). The engineering of PBRs is very diverse (Behrebs 2005 and references therein), and there are various designs of PBRs for different uses and of different cost. We refer to the numerous reviews and research papers on these topics published in recent years for more details (e.g., Zittelli et al. 2013; Pires et al. 2017).

In a continuous culture system, such as most PBRs, the carrying capacity (i.e., maximum biomass that can be obtained) is determined by the concentration of the factor(s) that limit growth. If light is sufficient, the composition of the medium is therefore a crucial aspect in the planning of a successful production system. In most cases, care is taken to provide an excess of macronutrients but little attention is given to micronutrients and elemental stoichiometry. The consequence often is an imbalance in nutrient availability, resulting in unnecessary costs and lack of control on limiting factors (Giordano 2013). The rate of biomass production, instead, is a function of the rate by which limiting nutrients are supplied to the culture (dilution rate). A dilution rate that surpasses the genotypically fixed maximum growth potential of a strain will lead to the decrease of the cell number per unit of volume of culture. Dilution rates that are lower than this higher limit are sustainable. It should

be considered that a suboptimal growth rate may impact cell metabolism and thus affect biomass quality (Fanesi et al. 2014).

6.6.1 Harvesting and Dehydration of Algal Biomass

After cultivation, biomass has to be separated from the growth medium and recovered for downstream processing. Harvesting usually involves two steps: (1) bulk harvesting (or primary harvesting), to separate the microalgae from their growth medium, usually done by sedimentation, flocculation, or flotation; (2) thickening (or secondary dewatering), to concentrate the microalgal slurry after bulk harvesting, typically by centrifugation or filtration (Lam and Lee 2012; Zhang et al. 2014b). Thickening by centrifugation and filtration use up considerable energy and, although often employed, represents one of the main costs for commercial algae (and algal products) production. Flocculation is used to increase the efficiency of gravity sedimentation (Brennan and Owende 2010). However, conventional flocculants are often toxic, whereas non toxic flocculants (e.g., organic polymers) are presently too expensive for large-scale applications (Lee et al. 2013). Autoflocculation, which can be induced by increasing the H^+ concentration in the medium, and electrolytic flocculation may be used to separate algae from the medium without the addition of chemicals; estimates suggest that these methods would be significantly more economical than other harvesting techniques (Beuckels et al. 2013; Coons et al. 2014; Lee et al. 2013). Bioflocculation is the process of flocculation induced by microorganisms or by compounds they produce; it is possibly the most environmentally friendly among the flocculation methods (Wan et al. 2015). In a study by Wang et al. (2015), co-culturing of *Chlorella* and bioflocculant-producing bacteria was optimized to decrease adverse effect of co-culturing and proved to be effective in facilitating harvesting; such an approach may be a good option for the collection of algal biomass in wastewater treatment plants, where the bacterial component is unavoidable. Electroflocculation is another option: Coons et al. (2014) reported that, in the production of algal lipids, the cost of electroflocculation with inert electrodes was appreciably lower than that of membrane filtration, which, in turn, was less costly than centrifugation. The same authors suggested that ultrasonic harvesting, which operates through a standing wave created by forward and reverse propagating pressure waves in the water, could afford substantial economic advantages in comparison to other harvesting methods.

Drying of biomass is among the most expensive steps in microalgal production, because the evaporation of large volumes of water drains large amounts of energy; yet, it is usually a necessary step, because the presence of water interferes with transport and processing (Kumar et al. 2010). Spray, drum, freeze, and solar drying are commonly applied methods. Solar drying is economical, but it requires large extensions of land and is not feasible in temperate climates, where sunlight is not always sufficient (Zhang et al. 2014a).

Another common problem in algal commercial cultures is the identification of the appropriate time for harvesting. A method that appears especially suited for such task is Fourier-transform infrared spectroscopy (FTIR) (Domenighini and Giordano 2009; Giordano et al. 2001). A number of papers have proved the reliability, rapidity, and low cost of this methodology, which affords a snapshot of cell composition (Jebsen et al. 2012; Montechiaro and Giordano 2010; Palmucci and Giordano 2012; Palmucci et al. 2011) and allows reliably following changes in biomass quality over time (Giordano et al. 2017; Giordano and Ratti 2013; Memmola et al. 2014). The advantage of FTIR is that various organic pools can be determined concomitantly, with no need for extractive procedures and in quasi-real time. The disadvantage is that, in complex whole-cell spectra, the identification of specific compounds or small pools may not be easy (and sometimes is not possible). In these cases, other methodologies may be better suited. For lipids, fluorescent probes such as the lipophilic Nile red and BODIPY 505/515 can be used; these compounds can detect neutral lipids in intact cells (Cooper et al. 2010). However, the relatively long time required for staining and detecting the fluorescent probe, the relatively high cost of the probe, and the potential errors caused by the different permeability of the probes into different microalgae cells represent drawbacks of the use of these molecular probes. Qiao et al. (2015) developed a method to determine the optimal harvest time in oil-producing microalgal cultivations by measuring maximal photosystem II quantum yield (Fv/Fm); although this method afforded good results, it must be considered that Fv/Fm is associated with a number of events occurring within the cells and is not highly specific.

6.7 Products and Applications

6.7.1 Biofuels from Algae

Microalgae can, in principle, be used for the production of several different types of biofuels: biodiesel can be obtained from algal oil (Ahmad et al. 2011), biomethane, also known as biogas, can be produced through anaerobic digestion of algal biomass (Frigon et al. 2013), hydrogen can be generated photobiologically (Zhang et al. 2012), and bioethanol can be produced in the dark by anaerobic fermentation (Bigelow et al. 2014). The production of these biofuels can be combined in the same process, because the residue of the oil extraction for biodiesel production can be further processed into ethanol, methane, or H_2 (Mata et al. 2010; Singh and Cu 2010) (Fig. 6.2). The literature on algal biofuels is vast (e.g., Demirbas 2009; Kapdan and Kargi 2006; Mata et al. 2010; Spolaore et al. 2006). We shall therefore not linger on details in this section of our review. In spite of the broad interest in algal biofuels, the actual commercial production of such forms of renewable energy is, to say the least, limited: the very large volumes of cultures required to obtain a meaningful quantity of biofuels, together with the still relatively low price of fuels

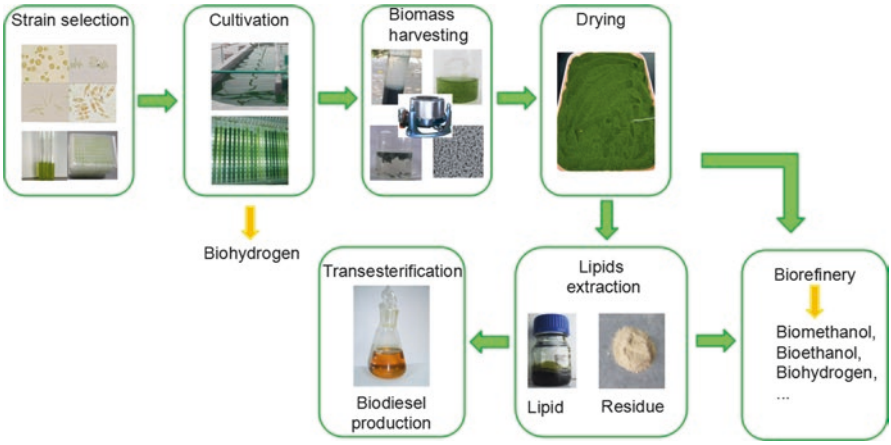

Fig. 6.2 The overall process flow for microalgal biofuel production

derived from fossil oil and other sources, is still in the way of a significant exploitation of algal fuels (Borowitzka 2016 and references therein).

6.7.1.1 Biodiesel

Biodiesel production comprises six steps: (i) strain selection; (ii) cultivation; (iii) biomass harvesting; (iv) biomass drying; (v) lipids extraction; and (vi) transesterification (Zhang et al. 2014b). We have already provided information on the first four steps; thus, the ensuing paragraphs focus on lipid extraction and transesterification.

Before lipid extraction, cells are usually lysed to facilitate access of the solvents to lipids within the cells. Various lysis procedures can be used: high pressure homogenization (HPH) (Samarasinghe et al. 2012), bead mills (Doucha and Lívanský 2008), ultrasonic disruption (Adam et al. 2012; Bigelow et al. 2014), and electroporation (Sheng et al. 2011). Ultrasonic disruption is possibly the procedure with the lowest energy requirement (Coons et al. 2014). Subsequently, lipids and fatty acids are extracted from the microalgal slurry mainly by two methods: the hexane Soxhlet method and the Bligh–Dyer method (Demirbas 2009; Kanda et al. 2013). Hexane-based oil extraction is more energy efficient and is therefore preferred for scaling-up efforts (Peralta-Ruiz et al. 2013). However, the use of chemical solvents has intrinsic problems associated with the toxicity of these compounds to humans and environment. Several supercritical fluids, especially supercritical CO_2, have been used for microalgal lipid extraction for the production of biodiesel. Although supercritical extraction is nontoxic and provides a nonoxidizing environment that avoids degradation of the extracts (Mouahid et al. 2013), it is expensive.

After lipid extraction, fatty acids transesterification is generally used to produce biodiesel (Lam et al. 2010). Lipid extraction and transesterification can be carried out simultaneously, simplifying the process and reducing the overall cost of

microalgal biodiesel production (Lam and Lee 2012). Biodiesel recovery in in situ transesterification is negatively affected by excessive biomass moisture (>20% m/m) (Sathish et al. 2014). Given the aforementioned high cost of biomass dehydration, this excessive moisture has a nontrivial effect on the economic performance of the production system. An improved *in situ* transesterification process that directly converts wet oil-bearing microalgal biomass into biodiesel was recently proposed (Dang-Thuan et al. 2013).

6.7.1.2 Other Microalgal Biofuels

Bioethanol

Most microalgae do not contain lignin; this property of algal biomass facilitates the enzymatic hydrolysis necessary for bioethanol production (Sun and Cheng 2002). Furthermore, in appropriate culture conditions, many algal species can accumulate high amounts of ethanol if substrates can be fermented (Bibia et al. 2017; Farias Silva and Bertucco 2016). Green algae of the genera *Scenedesmus*, *Chlorella*, *Chlorococcum*, and *Tetraselmis* and cyanobacteria of the genus *Synechococcus* have been reported to be potentially good sources of bioethanol (Farias Silva and Bertucco 2016). Typically, bioethanol is produced by the hydrolysis of sugars and their subsequent fermentation in microaerobic or anaerobic conditions using yeasts (Farias Silva and Bertucco 2016). Algae can generate ethanol directly in the dark, by fermentative metabolism (Ueno et al. 1998); however, this process does not seem to be sufficiently efficient for commercial exploitation. Algae can also produce ethanol directly via photofermentation (photanol) (Hellingwerf and Mattos 2009). In photofermentation, the glyceraldehyde-3-phosphate generated in the Calvin cycle is converted to phosphoenolpyruvate and then to pyruvate; pyruvate is decarboxylated to acetaldehyde (by pyruvate decarboxylase), which is finally converted to ethanol by alcohol dehydrogenase. Some engineered cyanobacteria have been made able to directly produce ethanol (and other compounds) through photofermentation in amounts and rates that appear to be compatible with their commercial exploitation (Farias Silva and Bertucco 2016 and references therein).

Molecular Hydrogen

Some green microalgae are capable of H_2 generation, a clean fuel with H_2O as the only major by-product. As opposed to non biological production processes, bio-H_2 can be produced at ambient temperature and pressure and has no demand for metal catalysts. The matter has been excellently summarized by Eroglu and Melis (2016). We shall therefore not overly linger on this theme and simply mention that two light-dependent electron transport pathways leading to H_2 production have been identified in *Chlamydomonas reinhardtii*: one draws electrons from water lysis at photosystem II, and the other uses the reducing power allocated on quinones through their reduction by hydrogenase. Also, a light-independent fermentative pathway leading to H_2 production has been identified in *C. reinhardtii* (Eroglu and Melis 2016). Incompatibility of simultaneous O_2 and H_2 evolution from microalgae has so

far hindered the development of large-scale bio-H_2 production (Rajvanshi and Sharma 2012). By using artificial miRNA (amiRNA) technology, a transgenic knockdown *C. reinhardtii* strain for the oxygen-evolving center (*OEE2* gene) was obtained; in this strain, O_2 is not released and the H_2 yield is about twofold higher than that of the wild type under similar growth conditions (Ngan et al. 2015).

Biogas

Microalgal biomass represents a potential alternative to biogas production from terrestrial crops (Dębowski et al. 2013). Photoautotrophically grown *Scenedesmus obliquus*, when used as biogas substrate, proved to produce more methane than maize silage (Wirth et al. 2015). However, a number of difficulties are associated with the use of algae for biogas production. For instance, the cell walls of some algae are resistant to anaerobic digestion and some algal strains generate compounds that are toxic to the bacteria that carry out anaerobic digestion; furthermore, in some cases, the C:N ratio of algae is unfavorable to anaerobic digestion (Dębowski et al. 2013 and references therein). This notwithstanding, the high turnover rate of algae and the possibility of selecting strains with suitable elemental stoichiometry make algae very interesting candidates for biogas production (Mussgnug et al. 2010). Some authors have also reported that when algae are mixed with traditional feedstocks they improve the efficiency of biogas production (Mussgnug et al. 2010; Zhong et al. 2012). Miao et al. (2014) showed that co-digestion of cyanobacteria with swine manure leads to an improved efficiency of both biodegradation and methane production as compared to the same processes without the addition of the algae. Zhao and Ruan (2013) also demonstrated the feasibility of adjusting the C/N ratio to increase biogas production by the addition of algae (mostly *Microcystis*) to kitchen wastes.

6.7.1.3 Challenges and Solutions for Algal Biofuel Production

Microalgal biofuel production is presently not conducted on a large scale because overwhelming investments in capital and operation are required (Chen et al. 2015b; Zhu 2015). In the case of biodiesel, for instance, the species that are known to be highly oleaginous often grow slowly. In such case, genetic manipulation may be advantageous and possibly necessary to obtain strains that can ensure sufficiently high productivity to make biodiesel production economically viable (Anandarajah et al. 2012; Iwai et al. 2014). It should also be considered that monocultures are susceptible to contamination, especially in conditions that intrinsically do not allow a tight control of the microbiota (e.g., wastewater); strains that grow slowly, such as the oleaginous ones, are especially likely to be outperformed by faster-growing competitors (Chen et al. 2015b). Mixed cultures of algae have been reported to persist in wastewater treatment systems and to be more stable and more resistant to exogenous invasion than monocultures (Chen et al. 2015b). This report would however need to be confirmed under a wider range of conditions; also, in-culture evolution (Borowitzka 2016) may have a stronger role in mixed cultures than in

monospecific cultures because of the selective pressure exerted by interspecific interaction (Venuleo et al. 2017). Some algal species used in large-scale open-pond commercial production are restricted to geographic locations with warm climates and would be unable to grow at acceptable rates during the hot or cold seasons of certain geographic regions (Holbrook et al. 2014). One solution to this problem is to identify indigenous algae that are adapted to the local environment (Holbrook et al. 2014).

Lipid accumulation occurs within microalgal cells according to the general principles outlined in previous publications (Giordano 2013; Palmucci et al. 2011; Raven and Giordano 2016). Zhang et al. (2013) suggested a possible connection between the oxidative stress induced by N-shortage and neutral lipid accumulation; applications of N-limitation or starvation, however, are inefficient methods to increase lipid accumulation because they can also significantly lower biomass and lipid productivity (Borowitzka 2016). A two-stage cultivation strategy has often been proposed for the production of stress-induced algal compounds (Borowitzka (2016) and references therein), in which a full-strength medium is used to promote biomass buildup, followed by a stress treatment (e.g., N-starvation; Zhu et al. 2014) to trigger the accumulation of the target compound. Also, "mid-point" approaches (i.e., compromises between best condition for growth and production of the compound of interest) have been suggested to simplify processes and decrease production costs (Borowitzka 2016). Zhu et al. (2016) proposed a single-step approach for boosting lipid production: these authors showed that the addition of trace amounts of urea to the growth medium significantly stimulated the accumulation of neutral lipids without affecting growth rates.

6.8 Microalgae for Bioremediation

6.8.1 CO_2 Fixation and Flue Gas Treatment

Carbon is the main nutrient in microalgal cells (36–65% of dry matter). It is therefore extremely alluring to use algae to sequester CO_2, at least temporarily (Singh and Ahluwalia 2013). The frequent suggestions to utilize algae for this purpose have rarely considered the physiological nuances of the responses of algal cells to elevated CO_2. As explained earlier, the excess CO_2 may be not taken up by the cells (thus conferring no advantage and only increasing the cost of new biomass production) or it is assimilated and subsequently elicits a nutritional unbalance leading to a change in biomass quality (Beardall and Giordano 2002; Giordano and Ratti 2013; Lynn et al. 2010; Palmucci et al. 2011; Raven et al. 2011, 2012). Also, the impact of elevated CO_2 on growth rates is variable, mostly species specific, and depending on energy availability (Beardall and Giordano 2002; Raven et al. 2011, 2012; Wu et al. 2012). Nevertheless, the utilization of algal biomass for the mitigation of CO_2 emission has its merits, provided that appropriate strains and suitable culture conditions

are selected and consideration is given to the fact that biomass will in the end release the CO_2 that it fixed. Liu et al. (2013) described a high-throughput screening method to rapidly identify microalgae strains that can tolerate high CO_2 condition or flue gases. Microalgae reported to tolerate high levels of CO_2 include *Chlorella* sp. (Qi et al. 2016), *Scenedesmus* sp. (Liu et al. 2013), and *Dunaliella tertiolecta* (Farrelly et al. 2013). Jacob et al. (2015) estimated that algal cultivation systems, whether they are tubular or flat photobioreactors or open ponds, can allow an effective and significant conversion of the CO_2 emitted by coal power plants into biomass. Once again, this work does not take into account the complex physiology associated with CO_2 fixation, but it does show that, at least in principle, algal cultivation can be coupled to industrial activity to minimize environmental impact. Experimental evidence (although in small-scale experiments) showed that the addition of flue gases to cultures of *Scenedesmus quadricauda* afforded a decrease by 85% v/v of CO_2, (and also 62% v/v of NO_x and 45% v/v of SO_x) in the flue gas; somewhat lower fixation capacities were obtained using *Botryococcus braunii* and *Chlorella vulgaris* (Kandimalla et al. 2016). Interestingly, the amount of fixed gases increased if the algae were cultured in mixotrophic conditions.

Flue gases contain different NO_x species, most of which are restricted by legislation and therefore must be removed (Van Den Hende et al. 2012). NO_x may serve as a nitrogen source for microalgae cultivation (Chen et al. 2016; Raven and Giordano 2016; Zhang et al. 2014a). Thus, "denoxification" (DeNOx) by microalgae (biodenox) may be a worthy contribution to flue gas treatment. As the efficiency of NO_x removal by microalgae varies dramatically among species, it is necessary to select or genetically modify suitable algal candidates for this purpose. Some strains of the genera *Chlorella*, *Scenedesmus*, and *Dunaliella* have been reported to significantly remove NO_x (Jin et al. 2008; Nagase et al. 2001; Santiago et al. 2010; Kandimalla et al. 2016), although high levels of NO_x tend to depress photosynthesis. In a typical flue gas from incineration processes, about 90–95% of the NO_x is given by NO (Fritz and Pitchon 1997). When NO dissolves in water, it is oxidized to nitrite and nitrate (Niu and Leung 2010). Nitrite has an inhibitory effect on algal growth, which is exerted through a retardation of electron transfer from Q_A to Q_B (Q_A is a bound quinone; Q_B is a quinone that binds and unbinds to photosystem II), and by interference with the donor side of PSII (Zhang et al. 2017). The screening of nitrite-tolerant microalgae species is therefore crucial for the use of algae in $DeNO_x$ approaches. Li et al. (2016) analyzed numerous *Chlorella* strains in this respect and found that the degree of nitrite tolerance was a strain-specific feature, although most *Chlorella* strains showed the ability to withstand high concentrations of nitrite. The nitrate and nitrite generated by the dissolution of NO in water can be directly assimilated by algae (Giordano and Raven 2014; Raven and Giordano 2016); NO dissolution is however rather slow and often limits the rate of combined nitrogen assimilation. Zhang et al. (2014a) reported on a two-step microalgal bio-$DeNO_x$ roadmap, in which NO_x-rich flue gases were first fixed, mostly as nitrite, to flue gas fixed salts (FGFS), and then used as nitrogen source for *Chlorella* sp. cultures. By using FGFS with NO_2^- equivalent to 5-fold that in the common culture medium BG11 (Stanier et al. 1971), up to 60% v/v of the NO_x was removed from the medium with an

inoculated cell density of 0.07 g DW L^{-1}, together with the production of 33% algae lipids (Zhang et al. 2014a). The mixotrophic cultivation of *Chlorella* sp. with FGFSs and glucose achieved an overall $DeNO_x$ efficiency of 96%, demonstrating the feasibility and practicality of efficient biological $DeNO_x$ by microalgae (Chen et al. 2016).

In most incineration flue gases, SO_x are also present; they mainly consist of SO_2, with a minor contribution (2–4% v/v) by SO_3; both SO_2 and SO_3 are highly soluble in water; SO_2 tends to hydrate to H_2SO_3, which dissociates in protons and sulfite (at pH >6) and bisulfite (prominent between pH 2 and 6); SO_3 hydrates to H_2SO_4, which typically dissociates in protons and sulfate (SO_4^{2-}); SO_4^{2-} tends to prevail at pH >1.9; also the oxidation of H_2SO_3 can generate H_2SO_4 and SO_4^{2-} (Stumm and Morgan 1981; Van Den Hende et al. 2012 and references therein). The dissolution of SO_x, therefore, causes acidification of the medium, the extent of which depends on the SO_x content of the flue gas, which is a function of the combustion substrates from which it was generated. The consequence of SO_x dissolution in the growth medium can be such to limit the choice of algae to acidophilic and/or bisulfite-tolerant strains (see Van Den Hende et al. 2012 and references therein for details); in some cases, scrubbing SO_x from the flue gas may be a precondition for any microalgal treatment. If acidity and toxicity of SO_x-derived solutes do not prevent algal survival, algae can assimilate substantial amounts of SO_4^{2-} (Norici et al. 2005; Ratti et al. 2011; Giordano and Raven 2014; Prioretti and Giordano 2016), compatibly with elemental stoichiometry in the growth medium and stoichiometric constraints of cell growth (Giordano 2013).

6.8.2 Wastewater Treatment by Microalgae Cultivation

Large-scale microalgae culture may compete with crops and human activities with respect to water usage. Large amounts of nitrogen and phosphorus are also required, and their cost is high (Lardon et al. 2009). Both water and nutrients can be obtained from wastewaters; culturing algae in wastewaters also affords obvious environmental benefits. Microalgae are very effective at removing nitrogen, phosphorus, and toxic metals from wastewaters, producing cleaner effluents with high concentrations of dissolved oxygen (Gomez et al. 2013). Cabanelas et al. (2013) used *Chlorella vulgaris* for nitrogen and phosphorus removal from municipal wastewater with the highest removal rates of 9.8 (N) and 3.0 (P) mg l^{-1} $days^{-1}$. Some studies also reported on the cultivation of microalgae in sewage under mixotrophic conditions. Cheng et al. (2013) found that mixotrophic microalga–bacteria systems significantly promoted algal growth and nutrient removal efficiency; maximal biomass and lipid productivity was attained when the alga *Desmodesmus* sp. CHX1 was used to treat piggery wastewater. Moreover, the co-culture of microalgae and bacteria in wastewater was reported to obtain 50–60% and 68–81% dissolved organic carbon (DOC) removal efficiency from municipal and industrial wastewater mixtures,

respectively (Nielsen 2015). Zhou et al. (2012) developed an effective organo-photolithotrophic system for improved wastewater nutrient removal, wastewater recycling, and enhanced algal lipid accumulation with *Auxenochlorella protothecoides* UMN280. Carbohydrate-rich and nitrogen-deficient solid wastes and some food industry wastewaters, such as olive mill wastewater, can be also used for hydrogen production (Keskin et al. 2011). It was reported that photosynthetic H_2 evolution from *C. reinhardtii* grown in advanced solid-state fermentation wastewater was increased by more than 700% compared to the cells grown in TAP medium (Chen et al. 2014). A study was also carried out to evaluate the potential of the green alga *Scenedesmus obliquus* grown in different concentrations of wastewater to produce biomass rich in sugar to produce bioethanol by fermentation processes; it was found that the highest removal efficiency of biological oxygen demand (BOD) and chemical oxygen demand (COD) were 18% for *S. obliquus* grown under aeration conditions and that the highest ethanol efficiency of biomass hydrolysate was 20.33% (Hamouda et al. 2016). Also, biomethane production in digesters could be improved by the addition of microalgae biomass harvested from algae-based swine wastewater digestate (Perazzoli et al. 2016). Because of the complex nature of wastewaters, issues such as contamination, inconsistent wastewater components, and unstable biomass production hinder efforts to use wastewater for large-scale algal cultivation (Cai et al. 2013).

The combination of CO_2 and/or NO_x fixation from flue gases and nutrient removal from wastewaters may provide a very promising alternative to current bioremediation strategies; the concomitant supply of nutrients from the gas and the liquid phase synergistically increases the effectiveness of depuration by algae (Chen et al. 2015b) and also stimulates algal growth and accumulation of added-value metabolic products (e.g., lipids) within the cells (Devi and Mohan 2012). Chinnasamy et al. (2010) cultured *Chlamydomonas globosa*, *Chlorella minutissima*, and *Scenedesmus bijuga* in untreated wastewater from the carpet industry to which a gas stream containing 5–6% v/v CO_2 was added; biomass productivity reached 5.9–21.1 g m^{-2} day^{-1}. The cyanobacterium *Aphanothece microscopica* Nägeli cultivated in a photobioreactor using supplemented wastewater from an oil refinery was found to assimilate CO_2 when light was present; the capacity for CO_2 sequestration was lowered by one fourth when the algae were cultured in a light/dark photoperiod rather than under continuous light (Jacob-Lopes et al. 2010). The other important finding of this study was that only a small portion (about 3% v/v) of the CO_2 sequestered during cultivation was in the end effectively fixed in algal biomass, whereas the rest was probably released as biopolymers or volatile organic compounds. This finding is a warning about the direct extrapolation to commercial application of physiological studies that do not include a thorough analysis of biomass.

Recently, the use of microalgae for the concomitant remediation of environmental pollution and biofuel production has also been proposed; this would allow decreasing energy, nutrients, water cost, and also CO_2 emissions (Chen et al. 2015b; Sun et al. 2013), making biofuel production from microalgae more environmentally sustainable, cost-effective, and profitable (Chen et al. 2015b; Nayak et al. 2016) (Fig. 6.3).

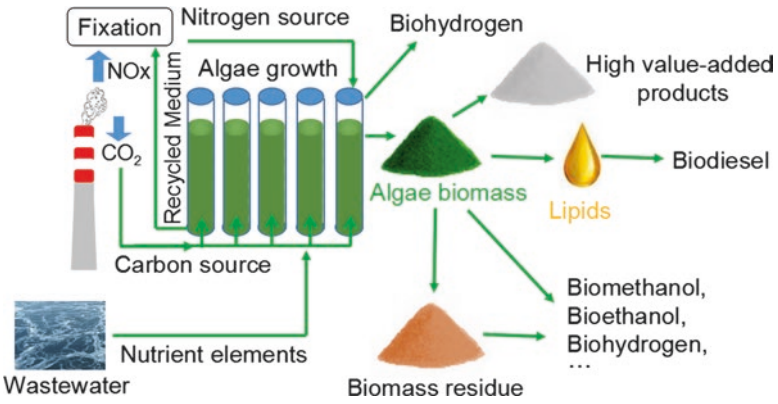

Fig. 6.3 Flowchart of the combination of environmental pollution control and biofuel production

Chlorogonium sp. showed good potential in the simultaneous purification of saline sewage effluent and CO_2 sequestration while delivering feedstock for potential biofuel production in a waste-recycling manner, achieving high removal efficiencies of NH_3-N, NO_3^--N, TN, and PO_4^{3-}-P, at a CO_2 consumption rate of 58.96 mg l^{-1} day^{-1}, and lipid content of 24.26% m/m of the algal biomass (Lee et al. 2015). An economically viable algal biofuel-based $DeNO_x$ process using *Chlorella* was evaluated and verified in actual industrial flue gas condition by Zhang et al. (2014a). To reduce the mismatch between the large amount of NO_x contained in flue gases and the relatively low capacity for its assimilation in photolithotrophic algal growth, the possibility of managing NO_x by culturing oil-producing *Chlorella* strains mixotrophically was tested (Chen et al. 2016). After a stepwise optimization of mixotrophic cultivation of *Chlorella* using FGFS, an impressive $DeNO_x$ efficiency of more than 96%, with a biomass productivity of 9.87 g l^{-1} day^{-1} and a high lipid productivity of 1.83 g l^{-1} day^{-1}, were obtained.

6.9 Microalgal Cultivation for Food or Feed Production

The large and increasing demand for animal feed exerts a tremendous pressure on food crops, because, on a purely economic basis, the conversion of land use from crops (for humans) to animal feed production is more profitable; this trend is, however, in obvious conflict with the need to support the increasing human population on our planet. Microalgae can be effectively and conveniently used as animal feed (Norambuena et al. 2015; Packer et al. 2016; Tibbetts et al. 2017; Vidyashankar et al. 2015); furthermore, their cultivation poses minimal or no threat to crop production (Vidyashankar et al. 2015). The nutritional and bioactive effects of microalgal biomass have been assessed in a variety of studies (Benemann 2013; Wells et al. 2017). The composition of algae, whose cells are rich in carotenoids and other

antioxidants, essential polyunsaturated fatty acids, minerals, and protein with a balanced amino acid profile, makes them an excellent alternative to conventional feedstocks such as corn, soya, barley, and skimmed milk (Shields and Lupatsch 2012). There are several examples of the utilization of algae for animal feed: *Chlorella vulgaris* has been used for the development of pet and fish feed (Groza et al. 1966; Li et al. 2015); *Dunaliella* can be used directly as a nutritional additive for fish or for secondary biological baits (such as rotifers and *Artemia*) and other aquacultured animals (Del Campo et al. 2007; Elbermawi 2009); *Spirulina maxima* has been used in swine feed (Saeid et al. 2013). Several studies have suggested that small amounts (2.5–10% of the diet) of algae in fish diets result in higher growth rates, feed utilization efficiency, carcass quality, physiological activity, intestinal microbiota, disease resistance, stress response, modulation of lipid metabolism, and protein retention during periods of reduced feed intake, and also lead to a higher palatability in sea urchin formulated feed (Cyrus et al. 2015; Valente et al. 2006; Nakagawa 1997; Norambuena et al. 2015; Wassef et al. 2005).

The widespread and growing interest in algae as food and food complements for humans emerges clearly in the recent literature (Cottin et al. 2011; Hafting et al. 2015; Harnedy and Fitzgerald 2011; Knies 2017; Packer et al. 2016; Pangestuti and Kim 2011; Sinéad et al. 2011; Wells et al. 2017). Limiting our *excursus* to microalgae (see Packer et al. 2016 for a panorama on macroalgae used for food), numerous species have been traditionally grown or have been collected as food: *Nostoc sphaeroides*, for instance, is a edible cyanobacteria widely cultivated in Hubei Province, China (Yi et al. 2016); there is evidence that in Central America the Aztecs were already eating cyanobacteria (*Spirulina*) collected from lakes in the fifteenth century; populations inhabiting the banks of Chad Lake, in Africa, have also traditionally used the cyanobacterium *Arthrospira* (formerly *Spirulina*) (Reed et al. 1985). *Arthrospira* was possibly the first microalga that spread widely across the shelves of supermarkets and "natural food" shops; this species encountered the favor of consumers for its rich protein, linolenic acid, and phycocyanin content. Nowadays, China is the main producer of *Spirulina* in the world (Lu et al. 2011). Also, *Dunaliella salina* encountered substantial success by its high content of β-carotene, an antioxidant in its own right and a precursor of vitamin A, with the first large production plants becoming operative in the 1980s in Israel, Australia, and the USA (Borowitzka 2016 and references therein). In more recent years, the fad of natural nutritional complements has facilitated the expansion of the market for nutritional products from algae, to which, with variably sound scientific bases, antioxidant, antibacterial, antiinflammatory, antiviral, and anti-cancer functions, for example, have been attributed (Wells et al. 2017). The consumption of microalgae as food has also, to some extent, been driven by the producers, who with the decline of profitability in algal biofuels have looked for alternative uses of their biomass (Packer et al. 2016). More species are now cultured in large-scale plants for the production and commercialization of β-carotene, astaxanthin, phycocyanin, some fatty acids (including Ω-3 and Ω-6), and other bioactive substances (Borowitzka 2016). *Chlorella* has been marketed as a health food because of its alleged ability to stimulate the human immune system; and its production is mainly distributed in

Japan, China, France, Portugal, and South Korea (An et al. 2008; Liu and Hu 2013; Saad et al. 2006). The green alga *Haematococcus pluvialis* can accumulate carotenoids, mostly astaxanthin and its ester derivatives, when subject to nutrient limitation, high temperature, or excessive light (Borowitzka 2016); these compounds have a high value on the market as antioxidants (Bagchi et al. 2001; Hagen and Grunewald 2001). The mass culture of *H. pluvialis* is mainly concentrated in Japan, Israel, and the USA (Gómez et al. 2013). Also, some heterotrophic species, such as the dinoflagellate *Crypthecodinium cohnii* and the labyrinthulid *Ulkenia*, have been used for production of docosahexaenoic acid (DHA), which has been proposed as a baby food additive (Ganuza et al. 2008; Lee Chang et al. 2014).

6.10 Conclusions

In the light of all these facts, it seems fair to conclude that, although some applications of microalgal cultivation appear not to be economically sustainable, at this point in time (e.g., the still fashionable use of these organisms for the sole production of biofuels), the use of microalgae in large-scale multifunctional plants is feasible and promising. Also, the direct use of algal biomass for human and animal nutrition or for the production of nutritional complements appears to have a positive outlook in terms of market demand and economic sustainability. However, further studies must be conducted, both on the engineering aspects of large-scale algal culturing systems and, possibly more importantly, on the specific challenges that industrial applications pose to algal physiology (e.g., responses to high CO_2, NO_x, and SO_x concentration, temperature, and low light penetration; C allocation under different growth regimes) and on the functional diversity of algae, which has been only marginally explored.

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