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Algal wastewater treatment and biomass producing potential: nutrient removal efficiency and cell physiological responses

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1. Aim of the thesis

The concept of microalgae cultivation as an integrated system in wastewater treatment has optimized the potential of the microalgae - based biofuel production. Microalgal biomass contains lipids, polysaccharides, proteins, pigments and other cell compounds, and it can provide different kinds of biofuels such as biodiesel, biomethane and ethanol; these microorganisms can also be used to produce bioplastic materials and the residual biomass can potentially be applied as fertilizer and soil amendment. Their application strongly depends on the cell composition and the production of biofuels and bioproducts from microalgae biomass appears not to be economically convenient (Brennan and Owende, 2010). The use of wastewater can reduce the cost of algae production minimizing the use of nutrients; algae growth requires the availability of primary nutrients such as nitrogen, carbon and phosphorus, and micronutrients, which can be costly if they need to be added in large amounts. Lundquist et al. (2010) reviewed several different algae - based wastewater treatments in conjunction with biofuel production and concluded that only those scenarios that emphasized wastewater treatment were able to produce low-cost biofuels .The aim of this work is to study a biological wastewater system on a laboratory scale growing mainly a newly isolated freshwater microalgae, *Desmodesmus communis*, in effluents generated by the wastewater reclamation facility (WRF) of the city of Cesena (Italy). The dual role of the microalgae in nutrient removal and biomass production is investigated characterizing the algal growth rate, the biomass yield and the nutrient removal capacity of *Desmodesmus communis* under different culture conditions. In addition, the evaluation of the photosynthetic efficiency and the detailed biomass biochemical characterization is performed in order to investigate the physiological performance of this species under different environmental variables. The final purpose of the present work is: to isolate and select the proper algal strain, to investigate the best media and culture condition and to demonstrate the potential use of the autochthonous microalgae *Desmodesmus communis* for the realization of an alga - based pilot plant for wastewater treatment and biomass production using the local wastewater resources. This study aims at giving the fundamental information about cell adaptation and responses at different but controlled media and culture conditions to better understand the way to improve algal biomass productivity and to optimize its composition in view of its application in the renewable energy field.

2. Background

2.1 Wastewater treatment methods and microalgae application

Every community produces both liquid and solid wastes. Disposal of untreated wastewater in rivers, streams, lakes and oceans may cause human-health and environmental hazards. Untreated wastewater contains: numerous pathogenic and disease-causing microorganisms that are dangerous for human-health; a large amount of organic material that could be involved in the production of malodorous gases; toxic compounds and nutrients which could stimulate the growth of aquatic plants and microorganisms until eutrophication takes place. The ultimate goal of wastewater management is the protection of the environment in a manner commensurate with public health and socio-economic concerns (Rawat et al., 2011).

Methods of sewage treatment consist of the combination of physical forces and chemical or biological processes which are known as *unit operations* and *unit processes* respectively. Both units are grouped together to provide what is known as *preliminary*, *primary*, *advanced primary*, *secondary* and *tertiary* treatment.

Preliminary treatment includes the screening and removal of debris, grit and any large solids that could damage the wastewater treatment plant's equipment. In the primary treatment phase, physical operations such as sedimentation are used to remove settleable solid materials and the advanced primary treatment has the same purpose as the primary treatment but includes the use of chemicals to enhance the process. In the secondary treatment phase, biological and chemical processes are used to remove most of the dissolved organic matter and nutrients such as N and P compounds from the wastewater. Tertiary treatment consists of processes designed to remove suspended solids created during secondary treatment and other residual constituents of concern that remain in significant quantities after secondary treatment. Disinfection of microorganisms such as bacteria, viruses and amoebic cysts usually belongs to the tertiary unit and it is commonly accomplished by the use of chemical and/or physical agents, mechanical means and radiation (Metcalf & Eddy, 2006).

Biological unit processes can be considered the most environmentally compatible and the least expensive methods, in particular when compared with chemical treatments which are additive processes and operatively more expensive. Meanwhile biological treatment uses microorganisms to reduce the organic content (carbonaceous BOD) and, in many cases, the nutrients. The majority of wastewater contains very high concentrations of nutrients, particularly total N and total P concentration as well as toxic metals, so there is no requirement for costly chemical-based treatments (Gasperi et al., 2008). For industrial wastewater, the main objective is to remove or reduce the concentration of organic and inorganic compounds. Because many of these compounds are toxic to microorganisms, pre-treatment may be required. Moreover domestic wastewater typically contains sufficient amount of inorganic and organic nutrients to support the microbial activity for the removal of carbonaceous BOD. In industrial wastewaters nutrients may not be sufficient and their addition is necessary for the proper growth of the bacteria (Metcalf & Eddy, 2006). The major biological processes used for wastewater treatment are identified in five groups: aerobic processes, anoxic processes, anaerobic processes, combined processed and pond processes. These are further divided in suspended-growth systems, attached growth systems and combinations.

2.1.1 Conventional Biological Treatment

In many cases the most common and suitable biological treatment process is activated sludge which is a suspended-growth system where aerobic microorganisms are used for oxidizing organic matter. In addition nutrient removal can be included in the processes. Two bacteria genera are responsible for nitrogen removal, *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* oxidizes ammonia to intermediate product nitrite. Nitrite is converted to nitrate by *Nitrobacter*. Approximate equations for the reactions that occur can be written as follows.

For *Nitrosomonas* the equation is

$$55\ NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7O_2N + 54NO_2^- + 57H_2O + 104H_2CO_3$$

For *Nitrobacter* the equation is

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + HCO_3^- + 195O_2 \rightarrow C_5H_7O_2N + 3H_2O + 400NO_3^-$$

Approximately 4.3 mg O_2 per mg of ammonia-nitrogen oxidized to nitrate-nitrogen is needed and 8.64 mg HCO_3^- per mg of ammonia-nitrogen oxidized is consumed (Metcalf & Eddy, 2006). It should be noted that changing ammonia-nitrogen in nitrate-nitrogen does not facilitate nitrogen removal but does eliminate its oxygen demand.

The aerobic environment in the reactor is achieved by the use of diffused or mechanical aeration, which also serves to maintain the mixed liquor in a completely mixed regime. The oxygen demand of aerobic bacteria accounts for the largest portion, roughly half, of the electricity demand for a common secondary wastewater treatment facility utilizing the activated sludge process (EPRI, 1994).

Denitrification is the second step in the removal of nitrogen by the nitrification-denitrification process. The removal of nitrogen in the form of nitrate by conversion to nitrogen gas can be accomplished biologically under anoxic conditions (without oxygen). Several genera of bacteria are responsible for this process such as *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Lactobacillus*, *Micrococcus*, *Proteus*, *Pseudomonas* and *Spirillum*.

The reactions for nitrate reduction are

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow NO_2 \rightarrow N_2$$

The last three compounds are gaseous products that can be released into the atmosphere. These anaerobic bacteria obtain energy for growth from the conversion of nitrate to nitrogen gas but they also requires a source of carbon for cell synthesis. Some biological denitrification systems use incoming wastewater, cell tissue or methanol as the carbon source.

Activated sludge systems also promote the formation of flocs which consist of bacteria, inorganic material and extracellular polymeric substances (EPS). EPS are also known as exocellular biopolymers which are involved with the flocs' settlable capacity. Because of their high density, these flocs are removed by gravity settling when they move from the mixed zone of the aeration basin to a clarifier settling unit. A portion of the sludge is collected and used in the anaerobic digestion process and a portion, known as return activated sludge (RAS), is recycled to the influent of the activated sludge basins to maintain the optimal population of organisms.

According to Metcalf & Eddy (2006) the treatment mechanisms of activated sludge have advantages such as:

- Minimal land requirement
- Proven process
- Effluent quality that meets discharge requirements
- Low odor emissions
- High loading capacity
- Different types of wastewater treatability including shocks and toxic loads

On the other hand the two main disadvantages that affect this treatment are the high costs (construction, equipment, operators and energy requirements due to pumping and aeration) and the high sludge production (Metcalf & Eddy, 2006).

The other mechanical wastewater treatment process is the attached-growth system. This treatment belongs to the biological processes in which the microorganisms responsible for the conversion of the organic matter or other constituents in the wastewater to gases and cell tissue are attached to some inert medium such as rocks, slag, or specially designed ceramic or plastic materials. Attached growth treatment processes are also known as fixed-film processes and they can be used for nitrifying systems in conjunction with the sludge activated system. The attached-growth systems include the roughing filter, rotating biological contactor and fixed-film nitrification reactor but the most commonly used is the trickling filter.

The trickling filter consists of a bed of highly permeable medium to which microorganisms are attached and through which wastewater is percolated or trickled. The filter media consist of rock or a variety of plastic packing materials and they are constructed with an underdrain system for collecting the treated wastewater and any biological solids that have become detached from the media. This underdrain system has a double role both as a collector and as a porous structure through which air can circulate. The liquid and the settled solid are separated and recycled to dilute the incoming wastewater and to maintain the biological slime layer. The organic material is degraded by a population of aerobic, anaerobic and facultative microorganisms such as bacteria,

fungi and algae, which are attached to the filter media; the hydraulic loading rate is adjusted to maintain a lime layer of uniform thickness. Only some algal strains such as *Phormidium*, *Chlorella* and *Ulothrix* grow in this system and they can survive in the upper reaches of the filters where sunlight is available. Generally, algae do not take a direct and effective part in waste degradation and they can clog the filter surface, but during the daylight hours they add oxygen to the percolating wastewater.

2.1.2 Natural biological treatment and the depuration role of microalgae

Natural wastewater treatment systems, such as ponds and wetlands, represent an alternative method to the mechanical systems described above. In natural systems bacteria and/or algae are grown in natural water bodies or also in artificial ponds and they rely on solar energy rather than electrical energy. While the construction and operation costs of these systems are much lower compared to that of the conventional systems, they are not able to achieve similar treatment levels. They are limited by higher hydraulic residence time (HRT) which is 50-500 times greater than mechanical systems (Oswald, 1995) and due to their shallow designs they require more land area.

Many species of microalgae are able to grow in wastewater conditions through their ability to metabolize even high concentration of nutrients. These microorganisms can play an important role in the wastewater treatment: they absorb excess nutrients leading to enhanced biomass production; they release oxygen through the photosynthetic process; in the industrial wastewater, especially, they are able to remove metals. Photosynthetic aeration is interesting as it reduces operation costs and limits the risk of pollutant volatilization under mechanical aeration. The release of free oxygen from photosynthetic microalgae is essential to allow efficient bioremediation of organic compounds by heterotrophic aerobic bacteria, responsible for oxidizing the influent BOD (Fig. 2.1). According to the photosynthetic reaction shown in the equation below, algal photosynthesis provides approximately 1.6 g O_2/g algae (Oswald and Gotaas, 1957); aerobic bacteria use dissolved oxygen as their electron acceptor and produce water and carbon dioxide (Metcalf & Eddy, 2006); water, CO_2 from cellular respiration, solar energy are then used in the chloroplast of algae cells to promote the photosynthetic process. Moreover microalgae absorb nitrogen and phosphorous present in the wastewater and they convert these nutrients inside the cell in protein, nucleic acid and phospholipid.

Photosythesis equation

$6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$

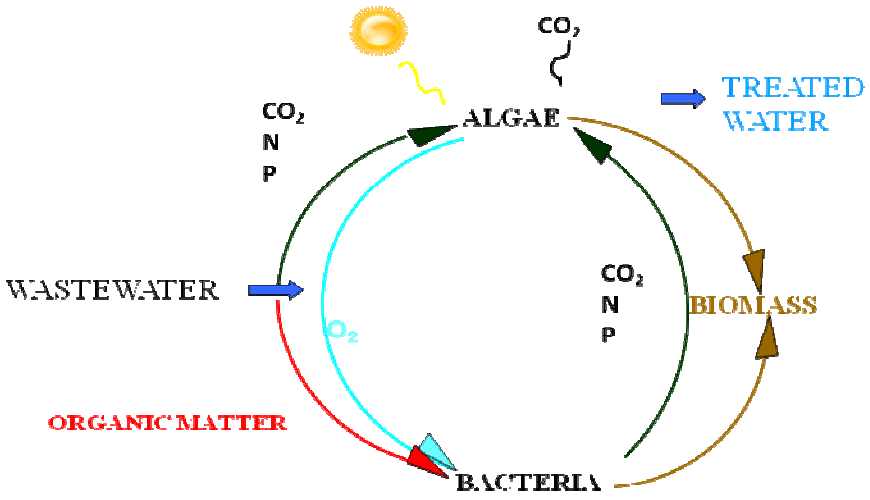

Fig. 2.1 Diagram demonstrating algal-bacterial interactions in natural treatment systems.

According to Metcalf & Eddy (2006) natural systems have advantages such as:

- Low costs and minimal energy input
- Reduced sludge production
- Natural UV disinfection of pathogens because of the long HRTs
- Biomass production for energy or fertilizing applications
- Providing habitat for wildlife

However some natural systems need to be supplemented by mechanical or chemical processes in order to produce an effluent quality that meets low requirements.

Stabilization ponds (lagoon or oxidation pond) and high rate ponds are two examples of natural wastewater treatment systems based on algae application.

Ponds can be defined as designed reactors constructed through excavation and compacting of earth to create reservoirs capable of holding water or wastewater (Oswald, 1995). A pond system, correctly designed and managed to cultivate anaerobic and aerobic bacteria and microalgae, can decompose waterborne organic wastes and synthesize protein- and energy-rich algal biomass from the products of decomposition (Oswald, 1995). Waste stabilization ponds are low-cost, low energy consuming and efficient systems for wastewater treatment, producing high-quality effluent which can be re-used as irrigation water (Mara, 2003). This technology, based on the capture of solar energy, is ideal for small communities when only low-level financial investment is available and where the climate and local environmental conditions permit the utilization of open ponds.

Aerobic stabilization ponds are large, shallow basins that contain bacteria and algae in suspension, and aerobic conditions prevail throughout its depth. This system is used primarily for the treatment of soluble organic waste and effluents from wastewater treatment plant (Metcalf & Eddy, 1991). In aerobic photosynthetic ponds, the oxygen is supplied by natural surface aeration and by algal photosynthesis. Except for the algal population, the biological community present in the ponds is similar to that present in the activated-sludge system. Algae and bacteria treat the wastewater according to a cyclic-symbiotic relationship as described for the HRPs' operation.

Facultative (aerobic-anaerobic) stabilization ponds are basins filled with raw wastewater or primary effluent. Three zones exist in a facultative pond: a surface zone where aerobic bacteria and algae exist in a symbiotic relationship, as previously discussed; an intermediate zone that is partly aerobic and partly anaerobic, in which decomposition of organic wastes is carried out by facultative bacteria; an anaerobic bottom zone in which accumulated solids are decomposed by anaerobic bacteria and gases such as CO_2, H_2S and CH_4 are produced as by-products, which are either oxidized by the aerobic bacteria or vented into the atmosphere.

The Advanced Integrated Wastewater Pond Systems (AIWSP®) consist of a series of at least four ponds in series which are designed to best perform one or more of the basic wastewater treatment processes (Oswald, 1990). The first pond is the Facultative Pond with a pit in which the overflow velocity is maintained so low that suspended solids removal approaches 100% and BOD removal approaches 70% (Oswald, 1990). This

treatment step gives rise to the fermentation reaction of solids accumulated in the bottom zone of the basin. The second pond is an High Rate Pond in which algae provides the oxygen needed by bacteria to oxidize most of soluble and biodegradable BOD remaining in the effluent from the facultative pond. Moreover the re-circulation of algae-bearing water from the High Rate Pond to the Facultative Pond provides an oxygen rich cap on the facultative pond which mitigates the odors emerging from the fermentation pit (Oswald, 1990). The third pond is used to promote the sedimentation of the algae produced in the step before within 1-2 days HRT (Green et al., 1996). The final pond, called the Maturation Pond, provides 10 to 15 days of additional retention time and has the dual purpose of added disinfection and storage for irrigation. The AIWSP® require much less capital, energy, operation and maintenance than mechanical systems, require less land and produce less odor than ordinary ponds (Oswald, 1990).

2.2 Technologies for microalgae biomass production

Currently, photoautotrophic production is the only method which is technically and economically feasible for the large-scale production of algae biomass for non-energy production (Borowitzka, 1997). Photoautotrophs microalgae use light as the energy source and inorganic carbon as the carbon source and their production is based on autotrophic photosynthesis. Under natural growth conditions algae absorb sunlight and assimilate carbon dioxide from the air and nutrients such as nitrogen and phosphorous from the aquatic habitats. Therefore, as far as possible, artificial production should attempt to replicate and enhance the optimum natural growth conditions such as temperature, nutrients incoming, light-dark cycles, mixing, CO_2 supply, pH and contaminants. Two systems have been developed for microalgae production and they are based on open-pond and photobioreactor (PBR) technologies (Fig. 2.2 - 2.3).

Fig. 2.2 Open raceway for algae production. *www.seambiotic.com*.

Fig. 2.3 Photobioreactor for algae production. *www.sogepisrl.it*.

2.2.1 Open ponds

Algae cultivation in open pond production systems has been used since the 1950s (Borowitzka, 1999). These systems can be categorized into artificial ponds and natural water bodies such as lakes, lagoons and ponds. Three major artificial pond designs have been developed and operated on a large scale: firstly circular ponds with agitation provided by a rotating arm; secondly raceway ponds, also known as HRPs (High Rate Ponds), made of a closed loop, oval shaped re-circulation channels characterized by a

paddlewheel system; thirdly inclined systems where mixing is achieved through pumping and gravity flow.

The Inclined Systems can obtain high cell concentrations (up to 1 g L^{-1}) and a high surface-volume ratio but they are also limited by several problems such as sedimentation of cell culture where the turbulence flow is lower; strong evaporative losses and CO_2 outflow because of the thin layer; high energy consumption due to the need to pump continuously the culture to the head of the system (Borowitzka, 1999). According to the literature the inclined system can achieve a biomass productivity of 25 g m^{-2} per day of *Chlorella* (Šetlík et al., 1970).

The Circular Ponds, which are normally up to 45 m in diameter and 30 to 70 cm in depth with a centrally pivoted agitator, are considered the less convenient biomass production open system because they require high construction costs and energy input for mixing. Size is also a limiting factor because of poor mixing efficiency when the rotating arm gets too long (e.g. >50 m in diameter). Algal circular ponds can also be combined with the wastewater treatment. *Oscillatoria* was cultured in circular ponds using diluted wastewater, and the biomass productivity achieved was around 15 g m^{-2} d^{-1} along with the reduction of more than 80% of ammonia and 50% of total organic carbon in the wastewater (Sheehan et al., 1998).

The High Rate Ponds are the most commonly used artificial system because of their comparatively low construction and maintenance costs. They are designed to be shallow, generally 0.2–1 m deep, in order to allow maximum light penetration. The raceway shape includes a large paddlewheel vane pump to create the continuous circulation with a velocity of approximately 0.15-0.3 m s^{-1} and a gentle mixing to promote algae growth and prevent sedimentation, keeping the cells in suspension and exposing them periodically to light. A circulation velocity greater than 0.3 m s^{-1} is preferred but it can consume too much energy to be viable (Sheehan et al., 1998). Other types of mixing systems, such as pumps and airlifts, can also be used, but these are less popular than paddlewheels (Shen et al., 2009). Algae broth and nutrients are introduced in front of the paddlewheel and circulated through the loop to the harvest extraction point. The microalgae's CO_2 requirement is usually satisfied from the surface air, but submerged aerators may be installed to keep the optimum value of pH by diffusing air/CO_2 mixture. By using a 0.30

m deep high rate pond system, a cell concentration of up to 1 g L^{-1} can be achieved, and productivities of about 10 to 25 g m^{-2} d^{-1} have been reported (Becker, 1994; Lee, 2001).

Compared to closed photobioreactors, open pond is the cheaper method of large-scale biomass production. It has lower energy input requirements (Rodolfi et al., 2008), regular maintenance and cleaning are easier (Ugwu et al., 2008) and their production does not compete for land with existing agricultural crops (Chisti, 2008).

On the other hand open pond systems are influenced by environmental conditions, specifically by seasonal variations in temperature and irradiance that are not controlled and that cause a lower efficiency in biomass productivity compared to PBR especially during the winter. The low productivity is also due to poor mixing and water-gas transfer in open ponds that limit the photosynthetic efficiency.

In addition to this cultures can be easily contaminated by bacteria, virus, fungi, other algae species and protozoa. Monoculture cultivation is possible by maintenance of extreme culture environment, although only a small number of algae strains are grown successfully. Only three species are at present cultivated in open pond system: *Chlorella* which is adaptable to nutrient-reach media; *D. salina* which is adaptable to very high salinity and *Arthrospira* which is adaptable to high alkalinity.

Harvesting is another problem associated with open-pond systems. It is costly to separate algae from water because algae concentration in open ponds is usually very low, even less than 0.1% by weight (Becker, 1994), and algae size is very small. Concerning the potential application of these systems to the wastewater treatment, despite HRPs providing a promising wastewater treatment method and optimizing microalgae growth in a open reactor, the effluent is still rich in small microalgae (less than 10 μm) due to the slow separation in the liquid phase. The lack of efficient algal removal systems is the major reason why algal-based wastewater treatment is not used extensively by the wastewater industry. The selection of the harvesting method is important to the economics of biomass production and its future application as harvesting can make up 20-30% of the total cost of production (Rawat et al., 2011). Currently algal biomass is harvested by centrifugation or gravity sedimentation or by filtration, all of which may be preceded and eased by a flocculation step.

Finally the large amount of water evaporation is also a problem in the use of open ponds, especially in tropical or desert areas.

Open ponds, in particular HRPs, are actually employed in: biomass production, CO_2 biofixation from flue-gas and wastewater treatment. The efficiency of microalgal growth in the wastewaters depends on a variety of variables as in any growth medium such as pH, temperature, nutrients, O_2, CO_2 and light, as previously described. The major difference between wastewater media and artificial growth media is the high concentration of nutrients naturally present in the wastewater, such as N and P. In the primary effluent much of N is often in the form of ammonia which at high concentration and elevated pH level can cause the inhibition of the photosynthetic process in microalgae (Aharon and Azov, 1976). Also the presence of toxic compounds such as cadmium, mercury and organic chemical compounds represents a critical factor in the growth of the algae, especially in industrial wastewater. Unicellular chlorophytic microalgae that belong to the genera *Chlorella* and *Scenedesmus* have been shown to be able to survive in wastewater (Aslan and Kapdan, 2006), but there is a variation in nutrient removal efficiency and biomass production between species. Park et al. (2011) have recorded the following attributes of microalgae species for use in HRPs:

- High biomass productivity when grow in wastewater
- Tolerances to seasonal and diurnal variation on outdoor conditions
- Capacity to form aggregates to enhance ease of harvesting
- Accumulation of high lipid or other valuable products

2.2.2 Closed photobioreactors

Photobioreactors can be defined as a culture system for phototrophs in which a great proportion of the light (> 90%) does not impinge directly on the culture surface, but has to pass through the transparent reactor's walls to reach the cultivated cells. Closed microalgae photobioreactors are designed to overcome some of the major problems associated with the open pond systems in terms of avoiding contamination, achieving higher culture densities and providing closer control over physical-chemical conditions. PBR permit monoculture for prolonged duration and a high quality biomass production

whose high-value products are utilized in the pharmaceutical and cosmetics industry. Reported productivities generally range from 20 to 40 g m^{-2} per day (Shen et al., 2009).

Closed systems include mainly the tubular, flat plate and column photobioreactors of various shapes, sizes, and lengths constructed in various transparent materials such as glass and plastic. Algae can circulate inside the system with a mechanical pump or airlift system which also provide CO_2 and O_2.

The Serpentine Tubular Photobioreactors consist of several transparent tubes connected in series by a U-bend to form a flat loop that can be arranged in various configurations: straight vertical, horizontal, inclined or helical. Gas exchange and nutrient addition take place in a separate vessel. Manifold tubular photobioreactors are a series of tubes connected at the end by two manifolds, one for distribution and one for the collection of the culture suspension. Tubular PBRs are the most commonly used in the commercial algae cultivations because of their ease of construction, improved control of gas transfer, large surface area volume ratio, and fairly good biomass productivities (Ugwu et al., 2008). Carlozzi (2003) reported a productivity of 47.7 g m^{-2} d^{-1} of *Arthrospira* in a tubular undulating row photobioreactor (TURP-10r) under outdoor conditions with south-north orientation.

The Flat-plate Systems consist of a transparent rectangular container with the light path usually between 0.1 and 0.3 m for the maximum solar energy capture. This type of PBR is usually inclined or vertically aligned. An example of flat-plate system is the 110 L Green Wall Panel PBRs located in Livorno, Italy. The system uses 0.3 mm thick flexible low-density polyethylene films instead of high-cost transparent tubes. *Nannochloropsis* sp. was cultured in this system in two phases: nutrient sufficient and nitrogen (N) starving. With about 30% lipid content, biomass productivity was around 30 g m^{-2} d^{-1}. The system was estimated to have a lipid yield of 20 tons ha^{-1} $year^{-1}$ in the Mediterranean climate and about 30 tons ha^{-1} $year^{-1}$ in sunny tropical areas (Rodolfi et al., 2008).

The Column Photobioreactors are made of vertical columns aerated from the bottom and illuminated through transparent walls or internally. This type of closed system is compact, low-cost, and easy to operate monoseptically. Moreover, they are very promising for large-scale cultivation of algae (Ugwu et al., 2008).

The evident advantages that characterize the PBR systems are firstly the higher productivity and cell density which can reduce algae harvesting and drying costs; secondly the reduced contamination risks; thirdly the better control of culture conditions such as temperature, light, pH, and nutrients for prolonged duration and finally the reduced CO_2 losses. Despite these advantages photobioreactors may suffer from some limitations such as overheating, toxic accumulation of oxygen, bio-fouling, deterioration of material, cell damage by shear stress and a great difficulty in scaling-up the system. Oxygen removal is considered one of the most difficult problems to overcome, especially when considering scale up (Carvalho et al., 2006). As a consequence of these limitations, PBR's technology is still too expensive to achieve the production of a low-cost biomass with the aim of obtaining economic products such as biofuels. Moreover the application in the wastewater treatment processes is not convenient.

2.3 Algae biofuels

Biofuels are defined as liquid transport fuels derived from biomass resources (Murphy et al., 2011). In recent years there has been a global interest in the use of biofuels due to the continuous increase of the energy demand and the necessity to mitigate the emissions of greenhouse gases (GHG). Nowadays there is still a plentiful supply of fossil fuels at a reasonably low cost, although this is likely to change in the future because of the increasing oil prices and climate concerns. Governments have become active with the aim of securing the supply of raw materials and limiting the environmental impact and many innovative proposals have been made. In March 2007 the EU's leaders endorsed an integrated approach to climate and energy policy that aims to combat climate change and increase the EU's energy security. They set a series of demanding climate and energy targets to be met by 2020, known as the "20-20-20" targets. These are:

- a reduction in EU greenhouse gas emissions of at least 20% below 1990 levels
- 20% of EU energy consumption to come from renewable resources

- a 20% reduction in primary energy use compared with projected levels, to be achieved by improving energy efficiency.

Development projects have been started and potential candidate fuels have been studied in the energy field but there is still a debate as to which fuels from biomass appear the most attractive depending on their yield potentials and other aspects correlated to their production.

First generation biofuels have been mainly extracted from food and oil crops including rapeseed oil, sugarcane, sugar beet, maize and vegetable oils and animal fats via sugar fermentation or oil processing techniques like trans-esterification, exploiting sugars, starch and vegetable oils (Murphy et al., 2011). The use of these types of biofuel has generated a lot of controversy due to the impact on global food markets and on food security. The demand for biofuel could cause, in this way, a lack of well-managed agricultural practices in emerging economies, a damage in bio-diversity conservation and the fact that it also requires a large portion of arable area with high water and fertilizer consumption. Furthermore, since about 1% (14 milllion hectares) of the world's available arable land is used for the production of biofuels, providing only 1% of global transport fuels (Brennan and Owende, 2010) it is obviously quite impractical to increase that share to 100%.

Second generation biofuels derive from the whole plant matter of dedicated energy crops or agricultural residues, forest harvesting residues or wood processing waste. The fuel is produced from the recalcitrant biomass components, such as lignocellulosic material via pre-treatments and fermentations or thermo-chemical routes, including pyrolisis and gasification and fuel synthesis (Murphy et al., 2011). However, the technology for conversion in the most part has not reached the scales necessary for commercial exploitation (Brennan and Owende, 2010).

Finally the term "third generation" biofuel is used to denote fuels derived from algae. Microalgae, especially, are defined as all the unicellular and simple multi-cellular microorganisms, including both prokaryotic microalgae, i.e. cyanobacteria, and eukaryotic microalgae, e.g. green algae, red algae and diatoms. These microorganisms are actually considered a novel source for potential future biofuels in comparison with the

terrestrial biomass. The advent of third generation biofuel seems to satisfy all the conditions for a technically and economically viable fuel resource (Schenk et al., 2008):

- microalgae are already reported to produce more oil for biodiesel production than traditional crops on an area basis. An algae open pond system with a productivity of 10 g m^{-2} d^{-1} at 30% of total fatty acid is capable of producing a biodiesel yield of 12000 L ha^{-1} compared with 1190 L ha^{-1} for rapeseed;
- microalgae have the potential to generate significant quantities of biomass due to their rapid growth potential and shorter harvesting cycle (~1-10 days depending on the process) compared with conventional crop plants which are usually harvested once or twice a year;
- microalgae grow in aqueous media, but still need less water than crop plants. Moreover nutrients for their cultivation (especially nitrogen and phosphorous) can be obtained from wastewater therefore reducing the load on freshwater sources and nutrients purchased. Greater light capture and conversion efficiencies ultimately lead also to reduce fertilizer inputs and as a consequence to limit waste and pollution production;
- microalgae can be cultivated on non-arable land minimizing associated environmental impacts without compromising the production of food;
- the biochemical composition of the algal biomass can be modulated by varying growth conditions enhancing the oil yield or C/N ratio.

2.3.1 Algae biofuels: a brief history

The idea of using microalgae as a source of transportation was first proposed in the 1950s (Oswald and Golueke, 1960). An initial techno-economic analysis was developed of a conceptual process that used wastewater for make-up water and nutrients, and they concluded that algae power was competitive with nuclear power (Oswald and Golueke, 1960). This concept attracted little interest or support at the time. However, after the energy crisis of 1973, all alternative sources of energy returned in vogue and research on anaerobic digestion of algae for methane production was restarted (Lundquist et al., 2010). From 1978 to 1996 the U.S. Department of Energy invested more than US$ 25 million in the Aquatic Species Program to develop renewable transportation fuels from

microalgae (Sheehan et al., 1998). Many techno-economic studies relating to algae biofuels and oil extraction processes have been carried out. An "International Network for Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae" was formed in 2002 and continued until 2007, as part of the IEA Greenhouse Gas R&D Programme, with support from DOE NETL and the Italian oil company Eni. A "Technology R&D Roadmap" was developed for this initiative (Benemann et al., 2003) and a resource assessment of algae biofuels was performed with emphasis on synergies with municipal wastewater treatment. However, as commercial interest in algae biofuels increased, concerns about safeguarding intellectual property became an impediment to this cooperative effort, and the Network suspended activities in 2008 (Lundquist et al., 2010).

Nowadays algae fuel research is becoming more and more a commercial business that involves corporate investments from US$ 10 million (BP, Shell, Eni) to US$ 100 million (ExxonMobil). Two US trade organizations (the ABO, Algal Biomass Organization, and the NAA, National Algae Association) and an European one (EABA, European Algae Biomass Association) are active in the field.

Unfortunately, much of the current interest in algae oil production is based on a lack of understanding of the science that aims to improve this technology, and on a misreading, or lack of reading, of the prior technical reports (Lundquist et al., 2010).

2.3.2 Biomass conversion

As Posten and Schaub (2009) described, the selection of conversion processes depends on the kind of biomass used and on the desired product. The conversion technologies for utilizing microalgae biomass can be separated into two basic categories of thermochemical and biochemical conversion depending on the type and quantity of biomass feedstock; the desired form of the energy (Fig. 2.4); economic considerations; the specific project; and the desired end form of the product (Peter, 2002).

Thermochemical conversion covers the thermal decomposition of organic components in biomass to yield fuel products, and is achievable by different processes such as direct combustion, gasification, thermochemical liquefaction and pyrolisis.

The biological process of energy conversion of biomass into other fuels includes anaerobic digestion, alcoholic fermentation and photobiological hydrogen production.

The main biofuels in use today are ethanol from carbohydrate fermentation and biodiesel/green diesel based on plant lipid fractions (Greenwell et al., 2010). If plant-derived oils are used as raw materials, only minor chemical modifications are required to achieve a fuel. Today most commonly methyl esters are produced from the raw oil (fatty acid methyl ester, FAME). There are several differences between the lipid composition of microalgae and higher plants. For example, the relative proportion of polar lipids (triglycerides) is significantly higher in microalgae. The other notable difference is that long-chain polyunsaturated fatty acids (greater than C18), common in microalgae, are not produced in significant quantities in higher plants. Both of these aspects will affect the efficiency of biodiesel synthesis, as well as the fuel properties. Fermentation of carbohydrates (sugar, starch, cellulose after hydrolysis) leads to ethanol as its product. Anaerobic fermentation also leads to methane as its final product. Chemical synthesis starting from synthesis gas (CO/H_2) may lead to different kinds of synthetic fuels (synthesis hydrocarbons, methanol, dimethylether). Synthesis gas is produced via gasification.

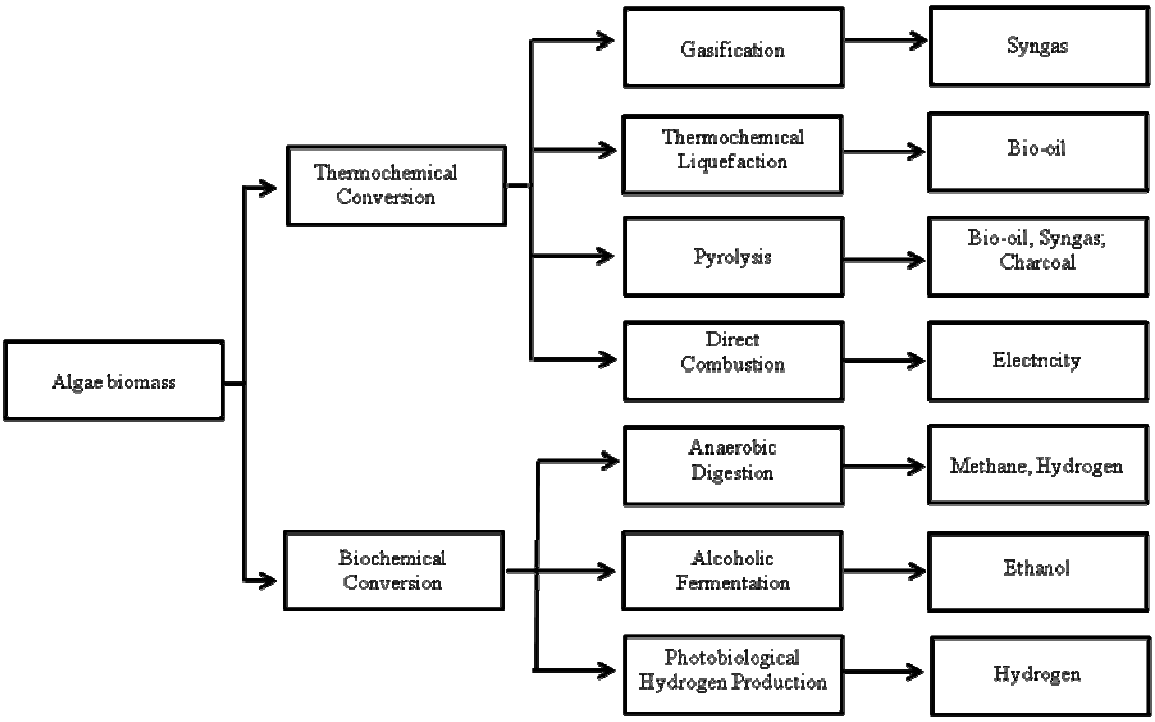

Fig. 2.4 Potential algal biomass conversion process (adapted from Tsukahara and Sawayama (2005)).

2.3.3 Other applications of microalgae biomass extracts

Microalgae biomass also has applications for human nutrition, especially strains such as *Chlorella*, *Spirulina* and *Dunaliella* which dominate the market (Brennan and Owende, 2010). They can provide many important extracts including β-carotene, astaxanthin, and C-phycocyanin that have a wide range of applications in the nutraceuticals, cosmetics, food and feed industries. Microalgae are also a primary source of polyunsaturated fatty acids (PUFAs), and supply whole food chains with these vital components; they have many other applications such as additives for infant milk formula. Specific algal species are suitable for the preparation of animal feed supplements and they also represent a natural food source of many important aquaculture species like mollusks, shrimps and fish. The main applications for algal biomass in this field are: fish feed; the coloring for farmed salmonids; the stabilization and improvement of the quality of the culture medium; and the enhancement of the immune systems of fish.

Microalgae biomass application can also be extended to biofertilization. Some conversion technologies such as the pyrolisis result in the formation of the solid charcoal residue "biochar", that has potential agricultural applications as a biofertilizer and for carbon sequestration. As an example, the algal biomass obtained in a wastewater treatment process can be converted into energy obtaining a residue that can be utilized as fertilizer.

2.4 Biochemistry and Physiology of Algae

2.4.1 Photosynthesis in Algae

The photosynthetic process involves a series of reactions that start with light absorption, involves synthesis of NADPH and ATP as intermediate energy-conserving compounds, and leads to CO_2 fixation in the Calvin cycle. It be represented by the reaction:

$$CO_2 + H_2O + 8 \text{ photons} \rightarrow O_2 + 1/6\ C_6H_{12}O_6$$

Photosynthesis is the fundamental driving force that supports all biofuel synthetic process, converting solar energy into biomass, carbon storage products (e.g. carbohydrates and lipids), and H_2 (Beer et al., 2009).

The integration of metabolic pathways is coordinated through complex mechanisms that regulate photosynthetic output to the distribution of reductant for the synthesis of proteins, nucleic acids, carbohydrates, lipids, and H_2.

In green algae (Fig. 2.5), the light harvesting complex bound to chlorophyll and carotenoids capture light energy as photons. This energy is used by photosystem II in the catalytic oxidation of water, forming protons, electrons, and molecular O_2. Low-potential electrons are transferred through the photosynthetic electron transport chain leading to the reduction of ferredoxin. Reduced ferredoxin subsequently reduces ferredoxin-NADPH oxidoreductase for the production of NADPH used in CO_2 fixation, leading to starch and lipid synthesis. An electrochemical gradient is formed because of the release of protons after water oxidation into the thylakoid lumen, which is used to drive ATP production via

ATP synthase. The photosynthetic products NADPH and ATP are substrates for the Calvin-Benson cycle where inorganic CO_2 is fixed into 3-C molecules that are assimilated into sugars, starch, lipids, or other molecules required for cellular growth. The substrates for hydrogenases, H^+ and e^- are supplied via either the photosynthetic electron transport chain or from fermentation of stored carbohydrates (starch). In the glycolitic pathways the oxidation of pyruvate during glycolysis, catalyzed by either the pyruvate dehydrogenase complex (PDH) under aerobic conditions or pyruvate-ferredoxin oxidoreductase (PFR) under anaerobic conditions, can be used to generate acetyl-CoA for lipid biosynthesis. Reduced ferredoxin resulting from the activity of PFR can also be used to reduce H_2ase.

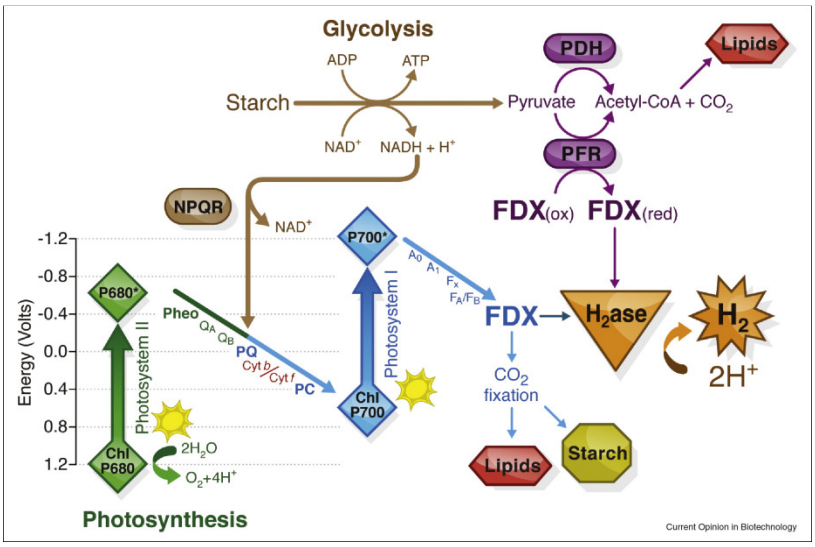

Fig. 2.5 Photosynthetic and glycolytic pathways in green algae related to biofuel and biohydrogen production (from Beer et al. (2009) adapted from Posewitz et al. (2004)).

2.4.2 CO_2 fixation and the photosynthetic carbon reduction (Calvin) cycle

Eukaryotic microalgae acquire dissolved inorganic carbon from the surrounding aqueous medium to support photosynthesis. The dominant means of carbon incorporation in photosynthetic organisms is through carboxylation in the Calvin cycle. Carbon enters the cycle as CO_2 and leaves in the form of sugar phosphate. At the heart of the Calvin cycle is the carboxylation of ribulose-1,5-biphosphate (RuBP) to form two molecules of

glycerate-3-phosphate (G3P). G3P is reduced to glyceraldehydes-3-phosphate (GA3P) until the regeneration of the RuBP substrate (Fig. 2.6). The cycle is autocatalytic, with net incoporation of CO_2 leading to a build-up of intermediates.

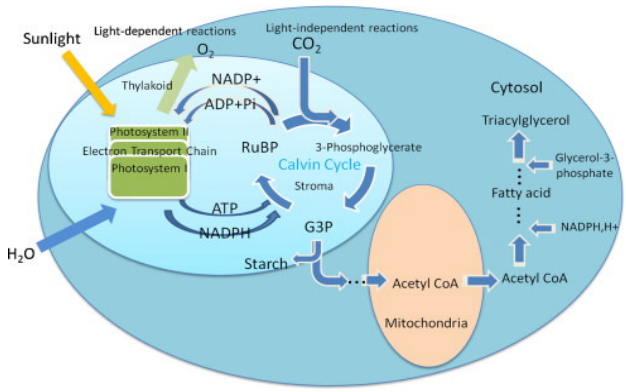

Fig. 2.6 Schematics of photosynthesis, CO_2 fixation and carbon accumulation in microalgae cells.

The two molecules of 3-carbon organic acids which are synthesized inside the cell chloroplast, are substrates for starch and oil production. However, O_2 competes with CO_2 in a process known as photorespiration because the enzyme that fixes CO_2 ribulose-1,5-biphosphate carboxylase, is also an oxygenase (RUBISCO). The phytoplankton usually suppresses the oxygenase activity of RUBISCO by actively concentrating CO_2 within the chloroplast (Badger et al., 1998). The ability to take up and accumulate inorganic carbon internally appears to have developed in order to overcome the deficiencies in the principal carboxylase of these microalgae, and the limitation in the supply of CO_2 from an external medium (Colman et al., 2002). Products of the oxygenase reaction are glycerate-3-phosphate and glycolate-2-phosphate; especially this last product is subsequently metabolized to glycine, which, when condensed with another glycine molecule to produce serine, results in the loss of CO_2 (Zeng et al., 2011). This process reduces the photosynthetic carbon fixation efficiency by 20-30% (Zhu et al., 2008).

One of the most attractive applications of microalgae biomass production is the potential to fix CO_2 from the atmosphere or combustion gas flue. Between 1.6 and 2 g of CO_2 is captured per gram of microalgal biomass produced (Herzog and Golomb, 2004). Moreover it has been demonstrated how high CO_2 levels (30-50%) are favorable for high

accumulation of total lipids and polyunsaturated fatty acids in *S. obliquus* and *C. pyrenoidosa* (Tang et al., 2011).

2.4.3 Algae lipids and biosynthesis

Microalgae have long been known to be rich in lipids and especially nowadays the renewed interest in the use of algal lipid-derived biofuels, biodiesel in particular, has increased and encouraged the research on algal lipids and lipid metabolism. Since the non-polar triacylclycerols (TAGs) are the best substrate to produce biodiesel, it is important to define the lipid fraction that is determined and reported in a pool of research results.

Lipids were defined as the biochemical compounds not soluble in water but soluble in non-polar organic solvents (Ohlrogge and Browse, 1995). This definition has been the basis for the quantification of the "total lipid" fraction of algae, as the total quantity of compounds soluble in a chloroform - methanol mixture (Bligh and Dyer, 1959). This class of compound is extremely diverse in structure and actually constitutes the products of several distinct biosynthetic pathways.

Lipids are traditionally subdivided in two main classes, polar and neutral lipids based on their chemical characteristics. Neutral lipids include the tri-, di-, and monoglycerides, waxes and isoprenoid-type lipids, like carotenoids. Polar lipids include phospholipids and glycolipids. An important subcategory of polar lipids is the glycolipids, esters of fatty acids and glycerol in which one of the hydroxyl groups of the glycerol is combined with a sugar molecule (in this case galactose) to form ester linkages with fatty acids (Greenwell et al., 2010). The relative composition of algal lipids depends on the species used, the nutrient and environmental conditions in which the cells are cultured and harvested.

Algae synthesize fatty acids as building blocks for the formation of various types of lipids. The most commonly synthesized fatty acids have chain lengths that range from C16 to C18 (Hu et al., 2008). Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone. Polyunsaturated fatty acids (PUFAs) contain two or more double bonds. As clearly described by Greenwell et al. (2010), the enzyme acetyl-CoA

carboxylase (ACCase) is generally considered to catalyze the first reaction of the fatty acid biosynthetic pathway, it combines acetyl-CoA, CO_2 (dissolved as HCO_3^-) and ATP to form malonyl-CoA. The central carbon donor for all subsequent elongation reactions is the malonyl-CoA; however before entering the fatty acid synthesis pathway, the malonyl group is transferred from CoA to a protein cofactor, acyl carrier protein (ACP). ACP is a small acidic protein that contains a phosphopantethein proshetic group to which the growing acyl chain is attached as thioester (Ohlrogge and Browse, 1995). After transfer to ACP, the malonyl-thioester enters into a series of condensation reactions with acyl-ACP acceptors. Whereas the enzyme complex malonyl-CoA/ACP is highly specific, acetyl-CoA/ACP is less so and can react with other low-molecular-weight acyl-CoA compounds and give rise to branching fatty acids with an uneven carbon number which are rare in microalgae (Greenwell et al., 2010). The fatty acids produced are incorporated into lipid components in either the chloroplast or the endoplasmic reticulum (Fig. 2.7).

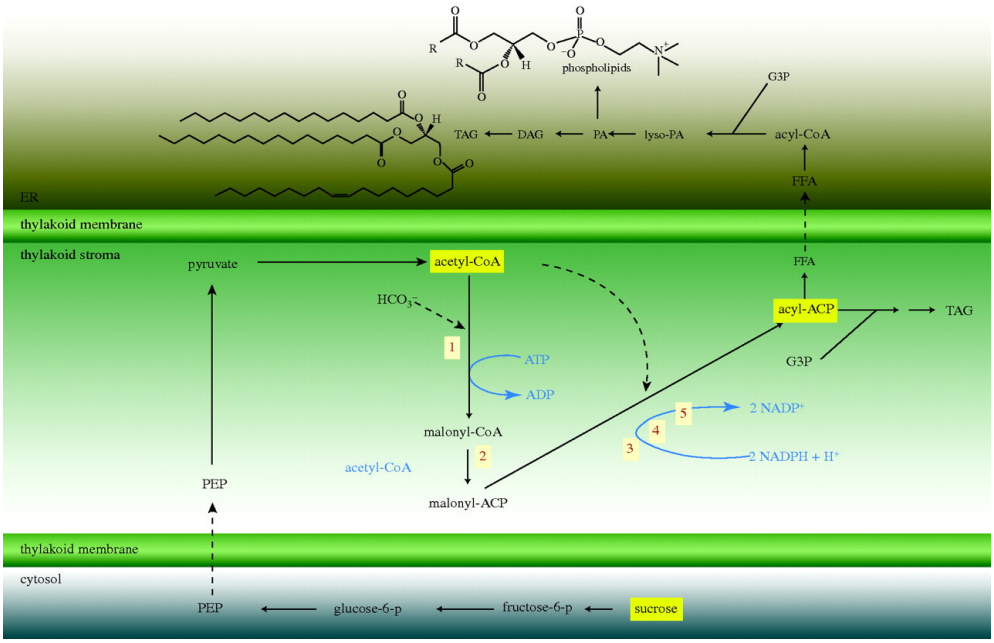

Fig. 2.7 Overview of the fatty acid synthesis pathway (Kennedy pathway) from acetyl-CoA via ACCase. Enzymatic reactions involved: (1) ACCase; (2) malonyl-CoA : ACP transferase; steps 3–5, subsequent condensation reactions catalysed by (3) 3-ketoacyl ACP reductase; (4) 3-hydroxyacyl ACP dehydrase and (5) enoyl ACP reductase. ACP, acyl carrier protein; FFA, free fatty acid; G3P, glycerol-3-phosphate; lyso-PA, lyso-phosphatidic acid; TAG, triacylglycerides; DAG, diacylglycerides (from Greenwell et al. (2010) adapted from Ohlrogge & Browse (1995)).

The origin of acetyl-CoA for this reaction is an important point of regulation for this part of the lipid synthesis pathway. It can be derived from either cytosolic or plastidial glycolysis, or directly from dihydroxyacetone phosphate from the Calvin cycle in the light.

Another metabolic pathway for lipid accumulation in cells is through the recycling of existing fatty acids from other cell components like the membranes.

The decision on whether lipids are synthesized is strongly dependent on the metabolic status of the cell and on different stress conditions. Many microalgae do not produce a large amount of lipids during exponential growth. Instead, when they are subjected to environmental stress, such as lack of nitrogen, they slow down their rate of proliferation and start producing energy storage products, such as lipids and starch (Hu et al., 2008).

The properties of biodiesel, produced by trans-esterification of triglycerides with methanol, yielding the corresponding mono-alkyl fatty acid esters, depend on its component fatty acid esters (Knothe, 2005). The most important characteristics include ignition quality (i.e. cetane number), cold-flow properties and oxidative stability. While saturation and fatty acid profile do not appear to have much of an impact on the production of biodiesel by trans-esterification process, they do affect the properties of the fuel product (Hu et al., 2008). Saturated fats produce a biodiesel with superior oxidative stability and a higher cetane number, but rather poor low-temperature properties. The fuel product produced using these saturated fats has a gelatinous stiffness. Biodiesel extracted from a biomass enriched in PUFAs has good cold-flow properties. On the other hand these fatty acids are particularly sensitive to oxidation and the biodiesel have also instability problems during prolonged storage.

2.4.4 Uptake of inorganic nutrients

The essential nutrients required by all algae for the production of organic matter are the elements: C, H, O, N, S, P, K, Na, Ca, Mg and Cl. In addition to these elements, photoautotrophs need various trace elements such as Fe, Mn, Cu, Zn, Co and Mo as well as a few essential vitamins. Among these nutrients, only N and P can really limit the biomass production, therefore the research on phytoplankton-nutrient dynamics has been focused mostly on these two elements. Inorganic carbon can also be considered a limiting

nutrient to the phytoplankton growth: its biological availability can become especially rate-limiting under certain conditions due to the rather slow conversion between HCO_3^- and CO_2 (Williams et al., 2002).

The uptake of nutrients can occur in three ways:

- by diffusive transport from the bulk medium to the cell surface
- by advective transport due to water motion in the microenvironment of the cell
- by chemical reactions in the diffusive boundary layer when the system is out of equilibrium for a given nutrient species.

The uptake may not require an expenditure of energy. For example, nonelectrolyte elements such as CO_2, NH_3, O_2 and N_2 can be taken up through absorption by an algal cell wall or by passive transport. Active transport, on the other hand, results when ions such as NO_3^- and PO_4^{3-} are transported across the cell membrane against an electrochemical gradient, through an ion pump for example. This is more rapid but requires energy and demonstrates Michaelis-Menten saturation kinetics:

$$U = \frac{U_{max} R}{K_R + R}$$

with U_{max} which represents the maximum uptake rate, R the extracellular concentration of the rate-limiting nutrient, and K_R the half-saturation constant for uptake (also called Michaelis constant).

The rectangular hyperbolic function used in the Michaelis-Menten model captures typical features of nutrient uptake:

1) U vanishes at R = 0
2) U increases monotonously with increasing R
3) increase of U is maximal at small values of R
4) at very high value of R the function U goes into saturation.

In the process, phosphate energy bonds in ATP are used by ATPase to move ions across the cell membrane using proton (H^+) pumps.

Nitrogen, usually in the form of nitrate, is taken up and stored by a cell or converted to cellular nitrite (via nitrate reductase) and ultimately to ammonia (via nitrite reductase). The conversion does not require ATP as the process is a decrease in free energy. Nitrate reductase includes iron and molybdenum as cofactors, and iron concentrations may be low in neutral to alkaline natural waters where iron is bound in low solubility compounds, such as $Fe(OH_3)$.

Ammonia, which is also the most abundant compound in the wastewater, is often taken up easily by the microalgae because it can be used in a more direct way than nitrate in the biosynthesis of amino acids and chlorophyll. Its uptake is also independent of iron availability. However ammonia is usually toxic to microalgae at high concentration (Aharon and Azov, 1976). Ammonia is rapidly incorporated, and this is done by the enzyme glutamine synthetase in the plastid and cytoplasm. It is assimilated into the amide position (via ATP) of glutamate to form glutamine via the enzyme glutamine synthetase. The second step is the transfer of the amide nitrogen to 2-oxoglutarate to produce glutamate. Ammonia assimilation takes place in the chloroplast, where ATP and reduced ferrodoxin are available. Thus, also ammonia assimilation is actually part of the photosynthetic process in algae cells, with a major source of ammonia coming from respiration, where two molecules of glycerine are converted to one molecule each of serine, ammonia and CO_2.

Phosphorus is acquired primarily as orthophosphate ions and also via inorganic polyphosphates and organo-phosphor compounds. Phosphomonoesterases on the cell surface are required to cleave the ester linkages joining phosphate groups to organic compounds. Once in the cell, inorganic phosphate is stored in vacuoles or polyphosphate compounds vescicles or incorporated into phosphorlated compounds.

3. The Chlorophyceae genera *Scenedesmus* and *Desmodesmus*

The newly isolated algal strain (Fig. 3.1), which is the main microorganism investigated within the present research thesis, is a green algae, which from a first morphological identification carried out by an optical microscope, appeared to belong to the genus *Desmodesmus* or *Scenedesmus* (Order Sphaeropleales). This algal strain was isolated from an artificial freshwater pond in the province of Forlì-Cesena (Emilia-Romagna, Italy) in February 2009. The freshwater algal genera *Desmodesmus* and *Scenedesmus* are commonly found in water bodies and different species of this genus have been studied worldwide *in vitro* due to their rapid growth and ease of handling (Lürling, 2003). In addition, more recently species belonging to these genera have been used for industrial purposes due to their ease of cultivation and adaptation to the environmental conditions moreover they showed to be versatile organisms for the use in domestic and industrial wastewater treatment (Martínez et al., 2000; Mayeli et al., 2005; Voltolina et al., 2005; Hodaifa et al., 2008).

Algal species belonging to the genera *Desmodesmus* and *Scenedesmus* vary in their phenotype (Lürling, 1998). These microorganisms exist as unicells or as multiples of two, with four or eight celled coenobia. The species differ mostly in the number and type of spines on the cells and the texture of the wall. The morphology of the colony can be altered by varying the initial cell density (Egan and Trainor, 1989), nutrients, pH, and temperature moreover, chemicals released from grazers also induce morphological changes (Mayeli et al., 2005). The algal adaptation capacity and the development of various defense mechanisms in response to zooplankton grazing, such as spines and colonies, make the strains belonging to these genera attractive for their application in a large scale cultivation system, although they are not known as an algal genera rich in lipids and therefore attractive for biofuel production.

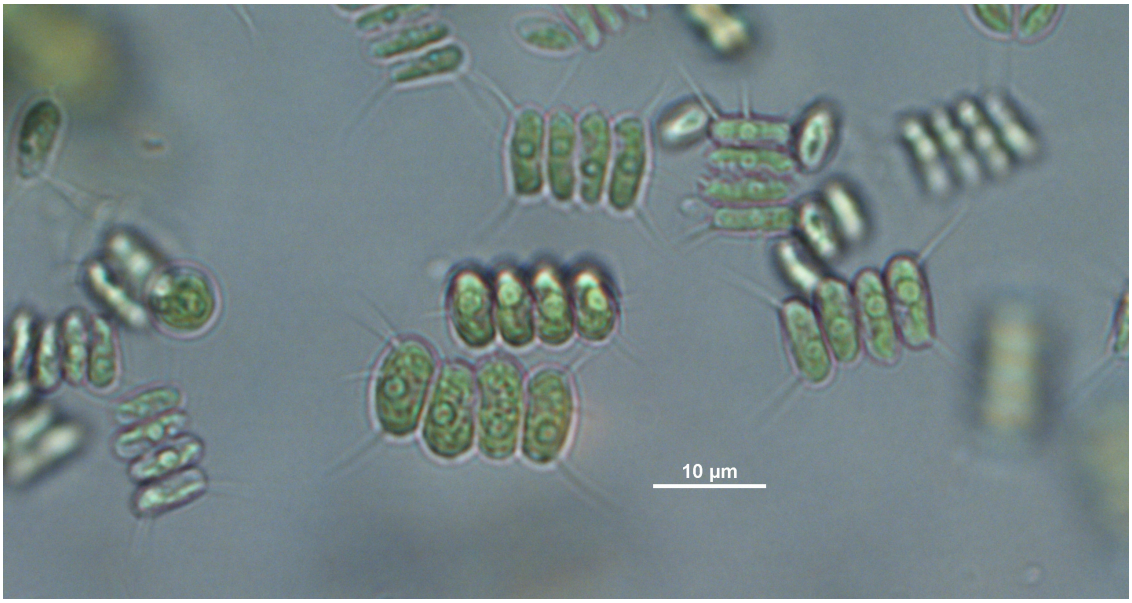

Fig. 3.1 Micrograph (100X) of *Desmodesmus communis* isolated from an artificial freshwater pond in the province of Forlì-Cesena (Emilia-Romagna, Italy) in February 2009.

3.1 Microalgae identification

The taxonomy of the species belonging to the genus *Scenedesmus* has been the subject of debate in numerous studies. Since 1828 *Scenedesmus* was studied and firstly placed in the diatoms genus *Achnanthes* (Lürling, 2003). After a century the genus was divided into four subgenera followed by further new subdivisions until the introduction of the molecular techniques such as DNA/DNA hybridization and nucleotide sequence analysis that were introduced to assist the reclassification of *Scenedesmus*. Because of the phenotypic plasticity that characterizes these microorganisms only the sequence analysis of the 18S-rDNA gene clearly supported the designation of just two subgenera *Desmodesmus* and *Scenedesmus*, a division which was also confirmed by the phylogenetic analysis based on the secondary structure model for ITS-2 region (Van Hannen et al., 2002).

A first step in the molecular identification of the algal strain studied in the present work was carried out during an International Training Course for microalgal identification at the Spanish Bank of Algae Marine Biotechnology Center at the University of Las Palmas

de Gran Canaria (Spain) in 2011. A synthetic description of the molecular technique performed and the result obtained are described below.

Total genonimic DNA (gDNA) extraction from algal cells were performed by cycles of cells frozen with liquid nitrogen and ground with glass beads and vortex. For gDNA purification NucleoSpin® Plant II Genomic DNA kit extraction (Macherey-Nagel) was chosen. The 5.8S, ITS2 and 28S D1-D3 rDNA fragments were used for the molecular identification; the specific region was amplified using the primers ITS03F (Lenka et al., 2011) and T24UR (Saunders and McDevit) and internal primers, T16NF (Saunders and McDevit) and ITS055R (Marin et al., 2003). This region was amplified by a polymerase chain reaction (PCR), using an initial step of 3 minutes at 95ºC, followed by 30 cycles of 95ºC (1 min), 55 (2 min) and 72ºC (3 min) and a final step of 5 min at 72 ºC. PCR products purification were performed using the NucleoSpin Extract II_PCR clean up kit (Macherey-Nagel) and sequenced by the sequencing service of the University Hospital of Gran Canaria. The query was aligned to sequences of *Scenedesmus obliquus*, using the FinchTV1_4_0 and the Mesquite software and the Nucleotide BLAST to compare the query with the the GenBank DNA database. The whole fragment matched with the species *Scenedesmus obliquus* although comparing the first 192 nt of the sequence, which code for the ITS2 region, the fragment matched 100% with the species *Desmodesmus communis*. Since there was a difference of 11nt in the 28S rDNA gene between the studied strain and *Scenedesmus obliquus* but there was none between the studied strain and *Desmodesmus communis* in the ITS2 region, the newly isolated strain probably belongs to the latter species. Further analysis will be carried out by aligning the sequence to the secondary structure (ITS2+28S) which will allow the exact division of the sequence into 5.8S, ITS2 and 28S regions.

According to the preliminary molecular identification results, the newly isolated algae studied in this research thesis has been recognized as *Desmodesmus communis*. In the present work, the result discussion regarding this algal strain investigation has frequently considered interesting comparable results achieved in other works relating to species belonging to the genus *Scenedesmus*. This comparison is possible owing to the similarity between these genera, as outlined above, and the strong possibility that many studies purporting to investigate *Scenedesmus* may well in fact be investigating *Desmodesmus*. Moreover the relative low number of studies with *Desmodesmus* can also be due to the taxonomic chaos which currently exists in the genera *Scenedesmus* and *Desmodesmus.*

4. Experimental section

4.1 Materials and Methods

4.1.1 Microalgae isolation and community composition

Desmodesmus communis was isolated from a water sample collected from an artificial freshwater pond in the province of Forlì-Cesena (Emilia-Romagna, Italy) in February 2009. The isolation of the green freshwater microalgal strain was performed by using a capillary pipette method (Hoshaw and Rosowski, 1973). After an initial growth in microplates, the culture has been preserved in a Chu13 modified medium (Largeau et al., 1980) at 20°C under illumination with cool white lamps (90 μE m^{-2} s^{-1}, light/dark cycle 16/8 h).

The consortium of algae derived from treated wastewater samples which were collected from the tertiary treatment sedimentation pond of the WRF of Ravenna (Italy) just before the beginning of the experiment during the summer 2011.

Identification of algal taxa in the algal consortium was carried out using an Optical Microscope (Axiovert S 100) at 32x and 40x and algae were identified to the genus level using information in Standard Methods and other identification materials. Algae enumeration was performed following the Utermöhl method (Hasle, 1978).

The biovolume of *Scenedesmus* (*Desmodesmus)* sp., *Monoraphidium* sp., and other unidentified nanoplankton strains, which resulted as the most abundant in the consortium at the end of the experiment, was calculated with the assumption of prolate spheroid (1),for the first two genera, and sphere shape (2), for the unidentified nanoplankton, using the following equations (Sun and Liu, 2003):

1) $V = \pi/6\ b^2\ a$

2) $V = \pi/6\ a^3$

where a = length, b = width.

These measurements were performed on 90 cells sample^{-1} of the algal consortium in the stationary phase (day 14) in the experiment carried out in section 4.2.

Stock cultures of *Desmodesmus communis* and algal consortium were cultivated, after acclimatization, in 1L Ilmabor® bottles at room temperature (18-25°C) and light intensity which was defined for each experiment condition with light/dark cycle 16/8 h. The bottles were aerated and stirred with a magnetic system to keep the cells in suspension and in completely mixed conditions. Cultures were aerated continuously with filtered (0.22 µm) mixtures via bubbling from the bottom of the bottles with an aeration rate of 142 mL min^{-1} (Fig. 4.1).

For all the experiments, the initial cell concentration in the bottles was approximately of ~ 0.1 g/L DW (dry weight) and each condition duplicated.

Fig. 4.1 Experimental culture conditions.

4.1.2 Culture medium

The wastewater used in these experiments was obtained from the WRF of the city of Cesena (Italy). Two effluents were collected: the primary and the secondary effluent after the primary and the secondary sewage treatment phase respectively. Both effluents were filtered through a nylon mesh of 200 µm porosity. Sterilized Chu13 modified medium (Largeau et al., 1980) was also used to test the growth efficiency of *Desmodesmus communis* in axenic conditions and the effect of the CO_2 enrichment on the growth rate in the experiment carried out in section 4.2.

Primary and secondary effluents received from the local utility company in Cesena were periodically analyzed for physical-chemical characteristics to monitor the change in nutrient concentration throughout the year. Biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), total volatile solids (VSS), total nitrogen (TN) and total phosphorous (TP) showed a consistent reduction in the secondary effluent (data not shown). Laboratory analysis were carried out to determine the nutrients' initial concentration at each condition in all the experiments. N/P ratio was calculated in terms of atoms.

4.1.3 Measurement of cell growth

The dry cell weight (g L^{-1}) was represented as volatile suspended solids (VSS) and was measured according to the APHA Methods 2540 E. A total suspended solids (TSS) measurement occurred before the VSS determination and was also performed according to the APHA Methods 2540 B. The ashes quantification was obtained calculating the fixed residual after the VSS determination, due to the strong correlation observed between this value and the value obtained at 550°C for 4 hours. The filters used for solids tests were Whatman GF/C and they were prewashed and ashed. The filtrate from the GF/C filtrations was used for nutrient determination.

Because the correlation between absorbance and VSS was strong, the growth rate of the culture was also monitored daily by optical density measurement at a wavelength of 750 nm using a spectrophotometer (model 7800, Jasco) after appropriate dilution with the same medium used in the culture. The growth curves have been followed until the beginning of the stationary phase. The biomass was determined every three days from the

beginning of the experiments and its biochemical composition was analyze suddenly after the beginning of the stationary phase for each conditions.

Biomass productivity (g L^{-1} d^{-1}) was calculated in the batch cultures from the variation in biomass concentration (g L^{-1}) within a cultivation time (d) according to the following equation:

$P = [X_1 - X_0] / [t_1 - t_0]$

where X_1 and X_0 were the biomass concentration (g L^{-1}) on days t_1 and t_0, respectively.

Biomass productivity (g L^{-1} d^{-1}) was calculated in the semi - continuous cultures from the variation in biomass concentration (g L^{-1}) within a cultivation time (d) according to the following equation:

$P = [X_t * V_{in}] / V_{tot}$

where X_t was the biomass concentration (g L^{-1}) on day t, V_{in} was the volume (L) of the primary effluent which inflow in the system in 1 day and V_{tot} was the total volume of the system.

Specific growth rate μ (d^{-1}) was calculated from the following equation:

$\mu = \ln(OD_1/OD_0) / [t_1 - t_0]$

where OD_1 and OD_0 were the optical absorbance at a wavelength of 750 nm on days t_1 and t_0, respectively.

4.1.4 Determination of ammonia, nitrate and nitrite concentration

Ammonia, nitrate and nitrite were measured by colorimetric analysis using tests kits (DR/2010; Hach, Colorado, USA) using the Nessler Method, Cadmium Reduction Method and Diazotization Method (Accu Vac Amplus) respectively. The culture samples were filtrated using Whatman GF/C filters and the filtrate was used for nutrient determination.

Each analysis was repeated in duplicate.

4.1.5 Determination of reactive phosphorus

The Ascorbic Acid Method was used to measure dissolved reactive phosphorus (APHA Method 4500 E) using a K_2HPO_4 stock solution as standard. The culture samples were filtrated using Whatman GF/C filters and the filtrate was used for phosphorus determination.

Each analysis was repeated in duplicate.

4.1.6 Extraction and fractionation of total lipids

The extraction of the total lipid fraction from the algal pellets was performed through a modified procedure from the literature (Bligh and Dyer, 1959). The culture samples were filtered through Whatman GF/C filters and the pellet collected has been washed with DI water several times to remove any salts or dissolved nutrient present in the media. The algal pellets were preserved at -20°C and were lyophilized in a freeze drier for dry weight measurement before the lipid extraction. Lyophilized samples (~ 100 mg) were homogenized in 15 mL of chloroform - methanol (2:1, v/v) and the extraction was carried out in a Soxhlet extractor apparatus at 80°C for 2 hours. The supernatant homogenate was filtered through Whatman GF/F filters. The residue was extracted again with the procedure described above three times. The solvent layer was evaporated to dryness and the total lipids were measured gravimetrically.

According with Yamaguchi et al. (1987), the total lipids were fractionated on a column (1x15 cm) packed with silica gel using 150 mL of hexane, 150 mL of chloroform, and 150 mL of methanol in order to isolate hydrocarbons, non - polar lipids, and polar lipids, respectively. The isolated lipids in each eluate were measured gravimetrically after evaporation of the solvent.

4.1.7 Determination of total fatty acids

The determination of total fatty acids (TFAs, sum of free fatty acids, FFAs, and bounded fatty acids, BFAs) in algal pellets was performed through a slightly modified procedure from the literature (Griffiths et al., 2011). The algal pellets were obtained by

centrifugation at 5000 rpm at 4°C for 20 min; the pellets were preserved at -25°C and were dried at 80°C before the determination of TFA. Dried samples (5 mg) were dissolved in dimethylcarbonate (0.4 mL) containing tridecanoic acid (0.02 mg). 2,2-Dimethoxypropane (0.07 mL) and potassium methoxide (0.2 mL) were then added; the samples were placed in an incubator at 90°C for 30 min. After cooling for 5 min to room temperature, BF_3-methanol reagent (0.2 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (0.02 mg) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-dimethylcarbonate layer, containing TFAs, was transferred to vials for GC-FID.

Each analysis was repeated in quadruplicate.

4.1.8 Determination of protein content

The protein concentration of microalgae was estimated with the Folin Phenol reagent (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. The protein determination was carried out using the algal pellets obtained by centrifugation at 5000 rpm at 4°C for 20 min and preserved at -80°C.

Each analysis was repeated in duplicate.

4.1.9 Determination of carbohydrate content

Polysaccharides were extracted from cultures following the Myklestad & Haug protocol. (1972). Two volumes of absolute ethanol were added to one volume of culture and stored at -20°C for 24h. The solution was centrifuge 25 min at 12000 rpm at 4°C, the pellet was used to measure the polysaccharides content. The carbohydrate digestion was carried out using solfuric acid 80% at room temperature for 20h, and the total carbohydrate content of microalgae was determined by the Phenol Sulfuric Method (DuBois et al., 1956; Hellebust and Craigie, 1973) using glucose as standard.

Each analysis was repeated in duplicate.

4.1.10 Determination of chlorophyll *a* content

Chlorophyll *a* content was measured by a spectrophotometric method slightly modified from the literature (Ritchie, 2006; Xue et al., 2011). The algal pellets were obtained by centrifugation of 5 ml of algal culture at 5000 rpm at 20°C for 20 min; the pellets were preserved at -25°C. The cell pellets were then mixed with 5 ml of pure ethanol, heated in a 80°C water bath for 10 min, cool down for 30 min and centrifuged at 5000 rpm for 10 min. The supernatant was collected and the procedure has been repeated three times. The total supernatant absorbance was measured at wavelength of 665 nm and 750 nm. Each analysis was repeated in duplicate. Chlorophyll *a* content was then calculated using the following equation (Ritchie, 2006):

$$\text{Chl}a\ (\text{mg L}^{-1}) = 11.9035*(\text{Abs}_{665} - \text{Abs}_{750})$$

Each analysis was repeated in duplicate.

4.1.11 Biomass elemental composition

Biomass characterization was supported by the measurement of organic carbon and nitrogen through elemental analysis. The culture samples were filtered through Whatman GF/C filters and the pellet collected has been washed with DI water several times to remove any salts or dissolved nutrient present in the media. The algal pellets were preserved at -20°C and were lyophilized in a freeze drier for dry weight measurement before the CHN determination. Elemental analysis was conducted on 2-3 mg of lyophilized biomass using a ThermoFisher organic elemental analyzer (Flash 2000) configured for CHNS-O determination using a copper/copper oxide column. The standard 2,5-Bis-(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT) was used for calibration. C/N ratio was calculated in terms of atoms.

4.1.12 Induction curves

Kinetics and parameters of photosystem II (PSII) can be measured by means of pulse - amplitude modulated fluorometry (PAM) (Ralph and Gademann, 2005). Mini - PAM (H.

Walz, Effeltrich, Germany) was used to follow the growth of *Desmodesmus communis* and algal consortium cultures. The model used is: 101 - PAM connected to a PDA - 100 data acquisition system, high power LED Lamp Control unit HPL - C and LED - Array - Cone HPL - 470 to supply saturated pulses, US - L665 and 102 - FR to supply far red light and measuring light respectively. Algal sample analyzed in cuvettes (10x10 mm) mounted on an optical unit ED - 101US/M. Measurements were performed at room temperature (23 - 25°C) and the cell density of the culture samples was kept constant through dilutions. Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (Φ_{PSII}) and effective quantum yield of PSII (Φ'_{PSII}), calculated as :

$\Phi_{PSII} = F_v/F_m = [F_m - F_0]/ F_m$ (Bolhar-Nordenkampf and Oquist, 1993)

$\Phi'_{PSII} = \Delta F/F'_m = [F'_m - F]/ F'_m$ (Genty et al., 1989)

Φ'_{PSII} provides an indication of the amount of energy used in photochemistry (Ralph and Gademann, 2005).

The photochemical quenching (qP), showing the proportion of light excitation energy converted to photochemical act by the active PSII reaction centers, was evaluated as:

$qP = [F'_m - F]/ [F'_m - F_0']$ (Schreiber et al., 1986)

The non - photochemical quenching (NPQ), representing all quenching processes of the PSII chlorophyll fluorescence not directly related to photochemistry, was calculated by the ratio:

$NPQ = [F_m - F'_m]/ [F_m - F_0']$ (Schreiber et al., 1986)

Photon energy captured by a chlorophyll a molecule can either drive photosynthesis (qP) be emitted as fluorescence, or be converted to heat (NPQ) (Ralph and Gademann, 2005).

The minimal fluorescence (F_0) was measured on dark adapted cultures for 20 min, by using modulated light (ML) of low intensity (2 µmol m^{-2} s^{-1}) which induces a fluorescence emission without inducing photosynthesis. As Juneau et al. (2001) explained in his study, F_0 fluorescence level represents the fluorescence yield when all PSII reaction centers are open and consequently all Q_A (PSII primary electron acceptor) is fully oxidize (Fig. 4.2). Maximal fluorescence yield (Fm) is induced by a short saturating pulse (SP) of

3000 µmol m^{-2} s^{-1} for 0.8 s, this pulse triggers the reduction of all Q_A closing all PSII reaction centers. The change of the fluorescence yield (F) during the following illumination by actinic light (AL) which induces photosynthesis and the typical Kautsky effect (Kautsky and Hirsch, 1931). Simultaneously, the change of the maximal fluorescence yield (F_m') is induced by saturating pulses (SP) given periodically every 60s. At the steady state of electron transport, AL is turned off and a far - red light (FR) is applied in order to ensure rapid and complete oxidation of Q_A. Under this conditions, the fluorescence level obtained, F_0', represents the fluorescence yield when all PSII reaction centers are in a open state for a light - adapted sample.

During measurements, the AL intensity was similar to the one used for the growth of the algal cells in order to avoid photoinhibitory effects and to provide optimal conditions for photosynthetic activity.

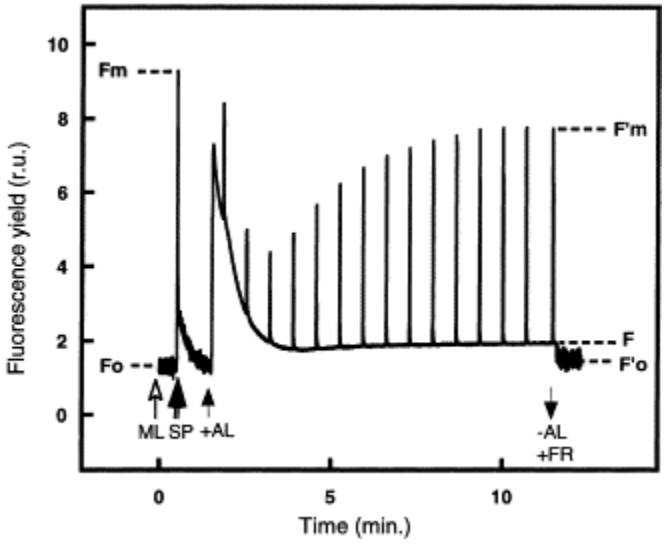

Fig. 4.2 Schematic representation of the fluorescence induction kinetic obtained by using the PAM-fluorometric method. The different types of light used during the measurement are indicated (ML=modulated light; SP=saturating pulse; AL=actinic light; FR=far-red light). The fluorescence yields needed to calculate the fluorescence parameters are also indicated (F_o=constant fluorescence; F_m=dark-adapted maximal fluorescence; F'_m=light-adapted fluorescence maximal; F=fluorescence level at steady state of electron transport; F'_o=minimal fluorescence in a light-adapted state). From Juneau et al. (2001).

4.1.13 Rapid light curves and photosynthetic parameters measurements (α, E_k and $rETR_{max}$)

Pulse - amplitude modulated fluorometry (PAM) described above provides light curves which can assess not only the present photosynthetic capacity, but the algae's potential activity over a wide range of photosynthetically available radiations (PAR). PAR has been defined in reference to the 400 - 700 nm spectral interval according to the SCOR/UNESCO Working Group 15 (Tyler, 1966). This type of measurement is known as a photosynthesis - irradiance curve (Ralph and Gademann, 2005). The culture samples are dark adapted for 20 min. A rapid light curve uses 60 s of actinic light at each of nine light steps and measures the photosynthetic performance as a function of irradiance. This curve has three distinct regions: the light limited, the light - saturated and the photoinhibited region (supra - optimal irradiance). The rise of the curve in the light - limiting region (α) is proportional to efficiency of light capture. The maximum quantum yield (Φ_{PSII}) for photosynthesis is implicit in α; thus Φ_{PSII} is defined in terms of light absorbed by the phytoplancton, whereas α is defined in terms of irradiance (Sakshaug et al., 1997).

To quantitatively compare rapid light curves, they need to be described by several characteristic parameters such as α, minimum saturating irradiance (E_k) and maximum photosynthetic rate ($rETR_{max}$) (Ralph et al., 2002). Because the absorption factor which is a factor representing the fraction of irradiance absorbed by the photosynthetic pigments was not possible to be determined, the rETR, on the y - axis was calculated as (Saroussi and Beer, 2007) :

$$rETR = PAR * Yield$$

To determine α, E_k, and $rETR_{max}$ the rapid light curve was fitted to a curve using the Platt et al. (1980) equation. Data were exported from WinControl software . Empirical data was mathematically fitted to a double exponential decay function (Platt et al., 1980) :

$$P = Ps\ (1 - e^{-\alpha Ed/Ps}) * e^{-\beta Ed/Ps}$$

where Ps is a scaling factor defined as the maximum potential rETR, α the initial slope of the rapid light curve before onset of saturation, Ed the PAR, and β characterized the slope of the rapid light curve where PSII declines (Ralph and Gademann, 2005). The following parameters $rETR_{max}$ and E_k were estimated using the following equations:

$rETR_{max} = Ps(\alpha/[\alpha + \beta]) (\beta/[\alpha + \beta])^{\beta/\alpha}$

$E_k = rETRmax/\alpha$

As Saroussi and Beer (2007) suggested the first value of the rapid light curve was used for determining the maximal quantum yield.

No weighting of the curve fitting was applied as Platt et al. (1980) suggested. The regression model had the following settings to ensure convergence: iterations = 100, stepsize = 100, tolerance = 0.0001, initial seed value for P = 5, α = 0.05 and β = 0 as reported also by Ralph and Gademann (2005).

The samples used for the rapid light curves estimation were diluted and the optical densities didn't vary much by treatments and by time.

Under moderate irradiance, the capacity of the electron transport chain limits photosynthesis and the curve reaches a plateu, where maximum photosynthetic capacity occurs ($rETR_{max}$). With even higher irradiance (supra - saturating), the curve often tends to decline.

4.1.14 Statistical analysis

For all statistical analyses, STATISTICA 6.1 software was used. The Cochran's C Test was used to check the homogeneity of variances and the Tukey Test was applied when results showed significant difference.

4.2 Effects of municipal wastewater on cell growth and cell composition of an autochthonous strain of *Desmodesmus communis* and of a natural microalgae consortium

4.2.1 Introduction

Microalgae have high potential to remove inorganic nutrients from the wastewater and to produce a biomass useful to produce biofuels, fertilizers or other bioproducts. It is known that the growth rate and biomass composition of microalgae can vary under different medium compositions and cultivation conditions (Chen et al., 2010). To realize coupled wastewater alga - based treatment and biomass production, one of the main key point in this research is selecting the proper microalgae species able to grow in the wastewater and to compete with other organisms in view of its potential application in a large scale system; it is also important characterizing the algal biomass in view of its future commercial application. Wastewater generated by the wastewater reclamation facility (WRF) of the city of Cesena (Italy) has been used in this study as the growth medium for algae cultivation. A monoculture of *Desmodesmus communis*, isolated in an area closed to Cesena and an indigenous algae consortium have been studied and compared to test the feasibility of an algae - based technology for nutrient removal and biomass production. Two different kinds of effluent from the wastewater treatment plant have been collected: one, which is rich in nutrients, from the primary treatment phase, and the other one from the secondary treatment phase, in which biological and chemical processes have been already used to remove most of the dissolved organic matter and nutrients, such as N and P compounds, from the wastewater. A third medium, Chu13 modified (Largeau et al., 1980) has been used as a control condition to determine which medium offers the best substrate for the cell growth and how the biomass composition changes under different nutrient concentrations. The Chu13 modified medium has been also tested with air (0.03% CO_2) and air enriched with 2% CO_2 to evaluate how the carbon limitation condition inhibits the cell growth and influences the biomass composition. The autochthonous isolate microalgae *Desmodesmus communis* was examined as a monoculture with regards to its ability to grow and produce biomass under different wastewater effluent and cultivation conditions. The algae consortium was grown in the primary effluent and its biomass has been characterized with attention to the species

succession. This study evaluated the nutrient - removal capacity of both cultures and determined the nitrogen incorporation efficiency as protein, as well as polysaccharides and the total fatty acid (TFA) content which is the best substrate to produce biodiesel (Rodolfi et al., 2008). Determining the composition of microalgae is also a way to estimate their anaerobic digestion potential. These microorganisms have proportions of proteins (6-52%), lipids (7-23%) and carbohydrates (5-23%) that are strongly species dependent and they can be deeply affected by environmental conditions (Sialve et al., 2009). Therefore, the C/N ratio of the species was analyzed because its variations may affect the performance of the anaerobic digestion process.

4.2.2 Overview of experiments

Different sets of experiments were run in batch cultures either using *Desmodesmus communis* or the algal consortium under different growth conditions.

The following configurations were set up (Tab. 4.1): *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPE); *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 88 µE m^{-2} s^{-1} (SPELL); *Desmodesmus communis* in secondary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SSE); *Desmodesmus communis* in Chu13 modified medium insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SCC); *Desmodesmus communis* in Chu13 modified medium insufflated with air (SCA); *Desmodesmus communis* in primary effluent wastewater enriched with $(NH_4)_2SO_4$ and insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPEM and SPEH); algal consortium in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (ACPE).

Media compositions are reported in Tab. 4.2.

Systems' setting, temperature, light/dark cycle and air - CO2 flow were set as described in section 4.1.1.

Condition	Strain	Medium	Light	Air-CO_2 mixture
SPE	*Desmodesmus communis*	Primary Effluent	High	2% CO_2
SPELL	*Desmodesmus communis*	Primary Effluent	Low	2% CO_2
SSE	*Desmodesmus communis*	Secondary Effluent	High	2% CO_2
SCC	*Desmodesmus communis*	Chu13mod	High	2% CO_2
SCA	*Desmodesmus communis*	Chu13mod	High	Air
SPEM	*Desmodesmus communis*	Primary Effluent ammonium Medium concentration	High	2% CO_2
SPEH	*Desmodesmus communis*	Primary Effluent ammonium High concentration	High	2% CO_2
ACPE	Algal Consortium	Primary Effluent	High	2% CO_2

Tab. 4.1 Experimental set up conditions.

Condition	NO_3-N (mg/L)	NO_2-N (mg/L)	NH_3-N (mg/L)	P (mg/L)	N/P
SPE	0.60 ± 0.01	0.60 ± 0.01	33.62 ± 3.36	1.54 ± 0.12	50:1
SPELL	0.03 ± 0.02	< 0.01	30.12 ± 0.07	2.00 ± 0.28	33:1
SSE	4.94 ± 0.21	< 0.01	0.24 ± 0.02	< 0.01	nd
SCC/SCA	22.49 ± 2.12	< 0.01	< 0.01	4.48 ± 0.12	11:1
SPEM	0.64 ± 0.18	0.03 ± 0.00	55.92 ± 0.25	1.72 ± 0.02	73:1
SPEH	0.70 ± 0.23	0.05 ± 0.02	84.62 ± 0.53	1.73 ± 0.05	109:1
ACPE	0.61 ± 0.09	0.09 ± 0.01	23.30 ± 1.95	1.70 ± 0.01	31:1

Tab. 4.2 Initial concentration of nutrients in the studied configurations (Tab -1).

nd = not detectable

4.2.3 Results and Discussion

The effects of media and cultivation conditions on the growth rate

Fig. 4.3, 4.4, 4.5, 4.6 and 4.7 illustrate the growth pattern as optical density over the batch cycle and Tab. 4.3 summarizes the growth rate values.

The CO_2 effect on *Desmodesmus communis* growth capacity was studied comparing two culture conditions, SCC and SCA, using the same culture media, Chu 13 modified, with and without CO_2 enrichment, respectively, (Fig. 4.3). SCA growth curve showed a μ of 0.43 d^{-1} with the culture entering the stationary phase in only 4 days. Such a low rate compared with SCC μ of 0.63 d^{-1} supported the necessity to enrich the aeration system with CO_2 to increase the algal growth rate and final yield in the following experiments.

To test the possibility to use wastewater as an algal growth medium, *Desmodesmus communis* was grown in the primary effluent (SPE) and in the secondary effluent (SSE), both added with 2% CO_2 (Fig. 4.4) while SCC culture was used as a control condition.

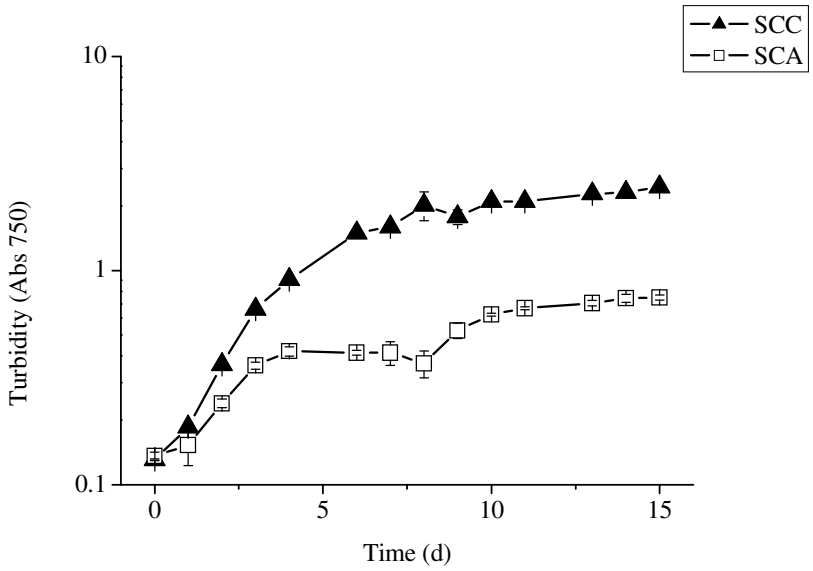

Fig. 4.3 Growth curves of *Desmodesmus communis* reported as optical density at 750 nm. A synthetic medium, Chu 13 mod, was used to grow the cultures under two aeration conditions, SCC, insufflated with air - CO_2 (98/2 v/v) and SCA, insufflated only with air.

SPE and SCC cultures were characterized by the same μ of 0.63 d^{-1} and they reached the stationary phase at the same moment, despite the complete difference between the media and the nitrogen form utilized (Fig. 4.4). In the SPE culture *Desmodesmus communis* was grown in the primary effluent with an initial concentration of NH_3-N of 33.62 mg L^{-1} while in SCC an initial concentration of NO_3-N of 22.49 mg L^{-1} was used (Tab. 4.2). On comparing the growth rate of SPE culture with SCC culture we deduced that, from a kinetic standpoint, the primary effluent behaved as a complete medium equivalent to the synthetic medium, and that the P limitation condition of the wastewater medium didn't affect the growth efficiency of the algae. In addition, the concordance between the μ values recorded for SPE and SCC cultures indicated that the organic carbon usually present in the primary effluent (SPE culture) didn't influence the algal growth as also observed by Martínez et al.(2000). The culture of *Desmodesmus communis* set up in the secondary effluent (SSE), in which all the nutrients and BOD (biochemical oxygen demand) have been already depleted during the secondary sewage treatment, showed a low μ of 0.48 d^{-1} and entered the stationary phase in a shorter time respect to the SPE and SCC cultures (Fig. 4.4). This condition has shown such a lower growth rate value

compared with those found in the primary effluent conditions or in the synthetic medium, presumably due to the nitrogen limitation.

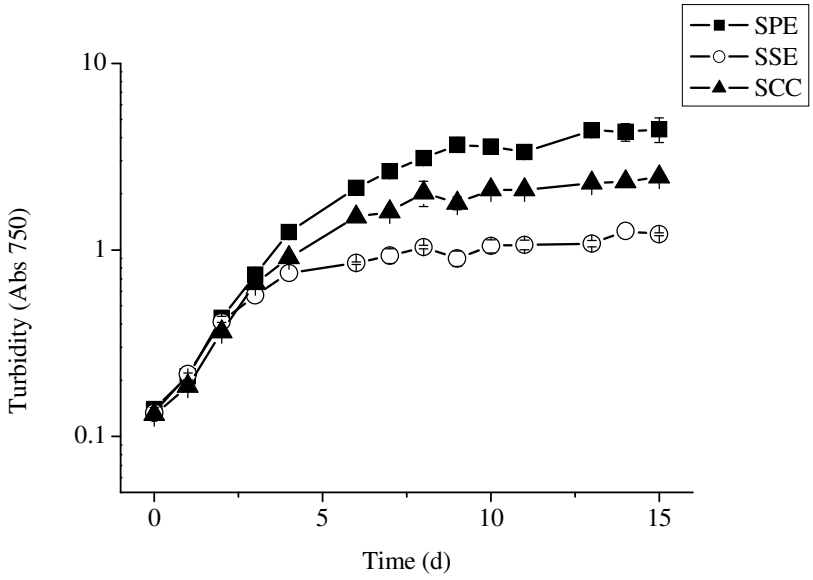

Fig. 4.4 Growth curves of *Desmodesmus communis* reported as optical density at 750 nm. Three different culture media were tested, SPE represents the primary effluent, SSE represents the secondary effluent and SCC the Chu13mod medium.

In a further study the primary effluent has been enriched with ammonium to test the ability of this microalgae to grow at high levels of ammonium, such as those that could be achieved in this effluent during the year; these cultures, grown in the primary modified effluent added with 2% CO_2 and NH_3 are represented by SPEM (final NH_3-N concentration of 55.92 mg L^{-1}) and SPEH (final NH_3-N concentration of 84.62 mg L^{-1}) growth curves (Fig. 4.5).

The exponential growth phase length varied under the different conditions; as shown in Fig. 4.5 the lower the initial concentration of nitrogen is, the shorter is the exponential phase. SPEM and SPEH cultures, which started with a NH_3-N concentration of 55.92 and 84.62 mg L^{-1} (Tab. 4.2), respectively, entered the stationary phase after a higher number of days with respect to the other culture condition and they achieved a growth rate (μ) of 0.68 and 0.66 d^{-1}, respectively. These results substantiated the idea to use the primary

effluent as culture media as the algae showed an good tolerance to high levels of ammonium as those that can be reached over the year, especially during the summer, at the WRF of Cesena (data not shown).

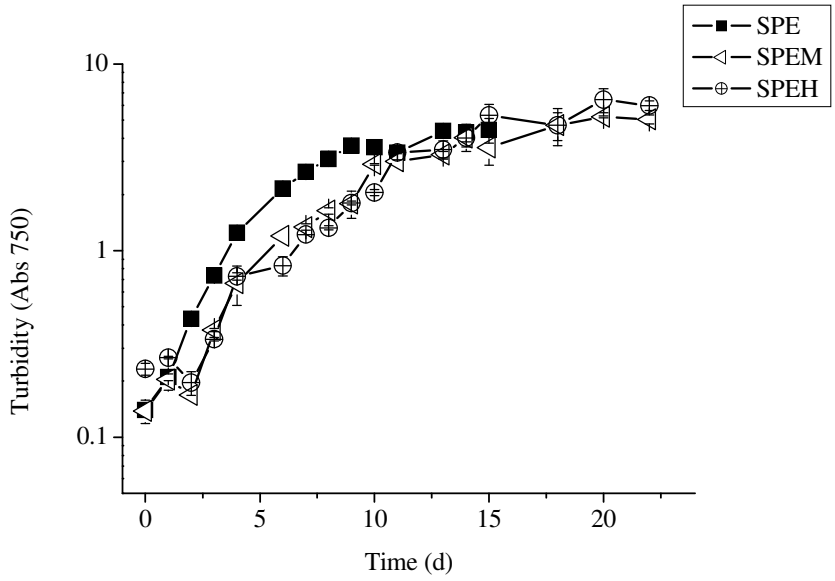

Fig. 4.5 Growth curves of *Desmodesmus communis* reported as optical density at 750 nm. Three different initial ammonia concentrations were tested using the primary effluent as growth medium. SPE had an initial concentration of NH_3-N of 33.62 ± 3.36; SPEM had an initial concentration of NH_3-N of 55.92 ± 0.25; SPEH had an initial concentration of NH_3-N of 84.62 ± 0.53.

A further investigation on the growth capacity of the microalgae *Desmodesmus communis* in the primary effluent was carried out growing it at low light exposure (SPELL) and comparing the results in terms of growth rate and biomass composition (see next paragraph) with those obtained at the same growth conditions but high light intensity (SPE) (Fig. 4.6). SPELL culture was characterized by a μ of 0.37 d^{-1}, which is 1.7 times lower than the SPE μ, and it also entered the stationary phase in short time. This condition, which differed from the SPE condition only in terms of light intensity, seemed to give results more similar to the ones related to the SSE condition, which was characterized by nutrient limitation. Comparing the growth rate of SPELL culture and SSE culture, whose μ was 0.48 d^{-1}, it is possible to hypothesize that the low light condition has been more influent than the nutrient starvation on the growth efficiency of *Desmodesmus communis*.

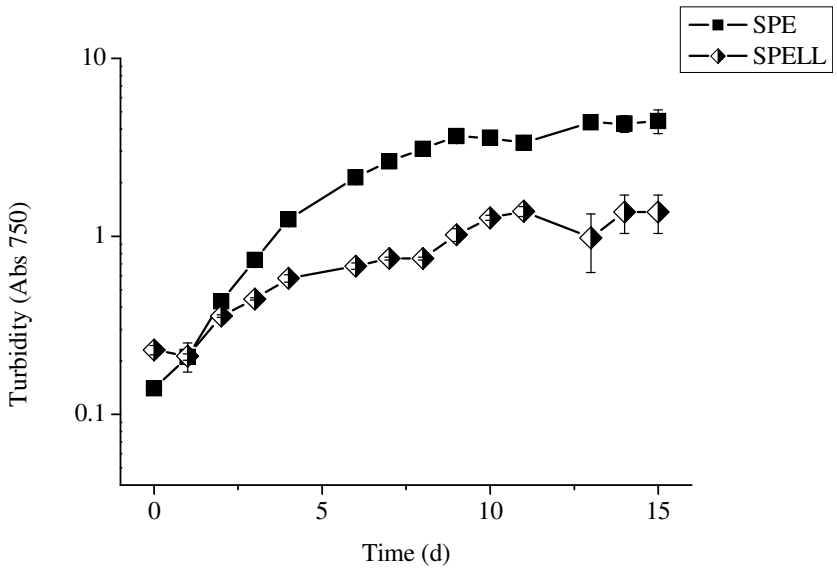

Fig. 4.6 Growth curves of *Desmodesmus communis* reported as optical density at 750 nm. The growth efficiency was studied using the primary effluent and keeping the cultures under a light intensity of 440 µE m^{-2} s^{-1} (SPE) and 88 µE m^{-2} s^{-1} (SPELL).

The primary effluent added with 2% CO_2 was used as medium to grow the natural algal consortium (ACPE) and its growth was compared with that of *Desmodesmus communis* under the same cultivation conditions (Fig. 4.7). The growth rate of the algal consortium (ACPE) was 0.53 d^{-1} and this culture entered the stationary phase in 8 days (Fig. 4.7), suggesting that *Desmodesmus communis* had a greater adaptation and affinity than the algal consortium in urban wastewater, as well as many other strains of the genus *Scenedesmus* showed in other works (Martínez et al., 2000; Ruiz-Marin et al., 2010).

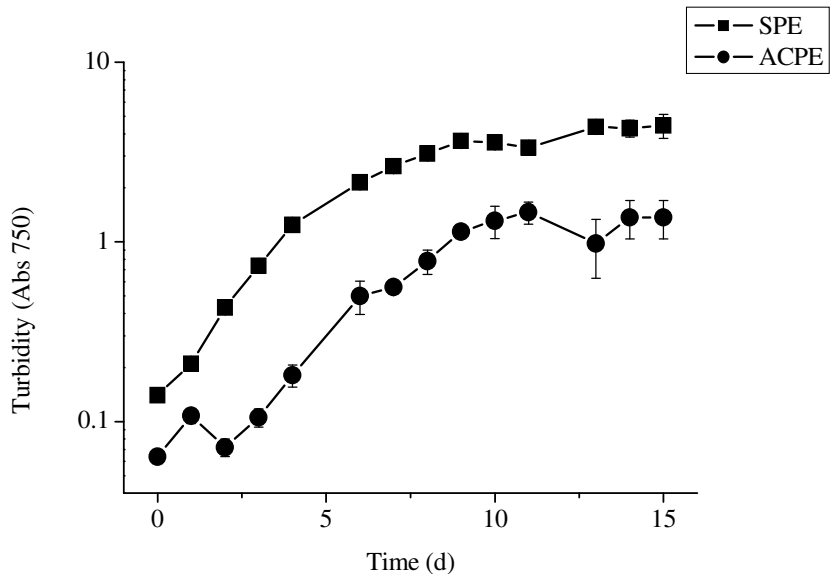

Fig. 4.7 Growth curves of *Desmodesmus communis* (SPE) and an algal consortium (ACPE) in the primary effluent, reported as optical density at 750 nm.

The effects of media and cultivation conditions on the biomass production

Tab. 4.4 reports the biomass yield, as VSS values, as well as its composition in terms of polysaccharides, proteins, total fatty acids (TFAs) and C/N ratio measured as the stationary phase reached the plateau (biomass characterization described in next paragraph and shown in Fig. 4.8). At this stage of growth, the biomass concentration of SPE, SCC, SPEM and SPEH didn't show any significant differences ($p < 0.05$, ANOVA). SPELL, SSE, SCA and ACPE cultures were characterized by lower biomass concentration values. Especially the *Desmodesmus communis* culture without any CO_2 supply had a VSS value of 0.26 g L^{-1}, which is 4.5-fold lower than the VSS value obtained in the *Desmodesmus communis* culture added with 2% CO_2.

The algal consortium reached a biomass concentration that is 1.4-fold lower than that of the *Desmodesmus communis* culture under the same cultivation conditions, as reported above for the growth rate.

The biomass concentration increased also during the stationary phase reaching the maximum values at the end of this phase for each culture condition, as shown on Tab. 4.3.

The results represented in Tab. 4.3, also show that the biomass productivity was strongly dependent on the medium used, especially on its nitrogen concentration and even more on

the light intensity. SPEH condition that was characterized by the highest value of ammonium and the highest light intensity, reached the maximum value of biomass productivity (0.227 g L^{-1} d^{-1}) and a maximum biomass concentration of 3.83 g L^{-1} in 22 days. No significant differences in biomass productivity were obtained between SPE and SPEM, SCC, ACPM culture conditions using the primary effluent ($p > 0.05$, ANOVA).

On the other hand, the algal biomass productivity achieved in the SPELL condition was only 0.018 g L^{-1} d^{-1} which is about 7 times lower compared with the SPE one. This reduction in terms of productivity, as well as the low growth rate measured for the SPELL culture and reported above, is due to the light limitation.

SCA culture biomass productivity achieved 0.022 g L^{-1} d^{-1} due to the carbon limitation which caused a reduction in the productivity of almost 80% compared with the SCC condition. In a previous study, *Scenedesmsus obliquus* SJTU-3 achieved a biomass productivity of 0.083 g L^{-1} d^{-1}, 0.158 g L^{-1} $d^{-1}$and 0.155 g L^{-1} d^{-1} at 0.03%, 5% and 10% CO_2 respectively (Tang et al., 2011). These results show a higher productivity rate compared with the *Desmodesmus communis* grown in Chu13 modified medium at 0.03% CO_2 (air). On the other hand using a primary effluent at 2% CO_2 as culture medium, *Desmodesmus communis* achieved high biomass productivity, similar to the study carried out by Tang et al. (Tang et al., 2011) which obtained the same results but only at higher CO_2 levels (5% and 10%). It would be interesting to further study how the increase in CO_2 levels could influence the biomass productivity of *Desmodesmus communis* grown in the primary effluent . In future applications the flue gas from power plants and other sources could also be used to supply CO_2.

SSE productivity reached a low value of 0.029 g L^{-1} d^{-1} due to the nutrient limitation in the secondary effluent which proved to be the worst culture medium condition to apply to maximize the biomass production.

Condition	VSS_{max} ($g\ L^{-1}$)	μ (d^{-1})	P ($g\ L^{-1}\ d^{-1}$)
SPE	2.13 ± 0.93	0.63 ± 0.03	0.138 ± 0.018
SPELL	0.70 ± 0.00	0.37 ± 0.09	0.018 ± 0.002
SSE	0.79 ± 0.07	0.48 ± 0.00	0.023 ± 0.003
SCC	1.85 ± 0.27	0.63 ± 0.03	0.110 ± 0.002
SCA	0.44 ± 0.03	0.43 ± 0.08	0.022 ± 0.002
SPEM	3.34 ± 0.29	0.68 ± 0.12	0.177 ± 0.029
SPEH	3.83 ± 4.38	0.66 ± 0.09	0.227 ± 0.040
ACPE	0.96 ± 0.05	0.53 ± 0.21	0.078 ± 0.007

Tab. 4.3 Comparative analysis of algal growth and biomass yield in different culture conditions (Tab. 4.1). VSS represents the maximum biomass in terms of volatile suspended solids reached during the batch experiment. μ is the growth rate calculated during the exponential phase and P is the biomass productivity calculated during the stationary phase.

Condition	VSS ($g\ L^{-1}$)	Polysaccharides (% w/w)	Proteins (% w/w)	TFA (% w/w)	Ash (% w/w)	C/N
SPE	1.30 ±0.00	58.8 ± 3.6	16.9 ± 1.8	4.9 ± 0.9	6.2 ± 0.9	16.3
SPELL	0.70 ± 0.00	47.3 ± 2.0	15.8 ± 1.7	3.7 ± 0.5	9.7 ± 0.5	10.3
SSE	0.66 ± 0.02	61.9 ± 1.8	9.6 ± 0.3	9.3 ± 1.6	1.9 ± 0.5	39.3
SCC	1.19 ± 0.01	65.0 ± 0.7	9.5 ± 1.1	5.6 ± 0.3	4.0 ± 0.1	24.3
SCA	0.26 ± 0.01	23.7 ± 1.0	27.3 ± 5.9	1.4 ± 0.7	13.3 ± 2.1	7.1
SPEM	1.40 ± 0.03	50.6 ± 0.1	19.9 ± 0.7	3.6 ± 0.8	3.0 ± 0.8	10.9
SPEH	1.33 ± 0.08	34.7 ± 5.0	26.6 ± 1.9	2.1 ± 0.9	3.0 ± 0.8	7.6
ACPE	0.96 ± 0.05	49.7 ± 2.7	39.2 ± 3.2	2.4 ± 0.2	6.0 ± 1.0	15.7

Tab. 4.4 Biochemical and elemental characterization of the algal biomass (expressed in terms of VSS, volatile suspended solids) measured when each culture (Tab. 4.1) reached the stationary phase.

Biomass characterization

The biomass composition summarized in Tab. 4.4, is also reported in Fig. 4.8 as percentage over the dry biomass of polysaccharides, proteins, total fatty acids (TFAs) measured as the stationary phase reached the plateau, for each culture condition.

The limited nutrient condition (SSE) showed scarce effect on the increment of the biomass production, as shown above, but it improved the TFA content and consequently the C/N ratio determined in the algal biomass produced. The TFA concentration per VSS increased by 53% comparing with the TFA concentration in the biomass obtained growing *Desmodesmus communis* in the primary effluent. Despite that, the TFA relative composition didn't show any significant differences ($p > 0.05$, ANOVA) in each culture conditions as it is summarized in Tab. 4.5. The most abundant fatty acids are the palmitic acid, the oleic cis and oleic trans acids which achieved a relative percentage of 24.5%, 22.4% and 20.4%, respectively.

Fatty acids		Realtive composition
ESADECADIENOIC	16:2	14.3%
ESADECATRIENOIC	16:3	4.1%
PALMITIC	16:0	24.5%
LINOLEIC	18:2	12.3%
OLEIC CIS	18:1 (9c)	22.4%
OLEIC TRANS	.....18:1 (9t)	20.4%
STEARIC	18:0	2.0%

Tab. 4.5 Summary of the total fatty acid composition (%) representative for each culture conditions.

C/N ratio of SSE culture reached the value of 39.3 which is comparable with the ratio characteristic of terrestrial plants (Elser et al., 2000). Different C/N ratios in SPE, SPEM and SPEH were obtained ($p < 0.05$, ANOVA), confirming the correlation between the nitrogen concentration in the medium and the nitrogen concentration incorporated in the biomass as protein. The use of a medium rich in ammonium permitted the obtainment of the maximum biomass productivity but also the highest content of nitrogen per cell. High nitrogen content and low polysaccharide and lipid content over the dry biomass can be

consider a negative characteristic of the biomass for its application in energy recovery. When the cellular non polar lipid fraction does not exceed 40% over the dry biomass, anaerobic digestion of the whole biomass appears to be the best strategy. On the other hand high N content in the algae lead to a significant release of ammonia and a potential inhibition of the digestion process. (Sialve et al., 2009). For *Desmodesmus communis*, as well as for several species, the high proportion of proteins is reflected by a low C/N values especially if compared with those of terrestrial plants.

Comparing the biomass composition obtained from the analysis of SPE, SPEM and SPEH cultures, it is possible also to observe that the polysaccharides content and the C/N ratio decreased with the increasing of the initial nitrogen concentration in the medium but no consistent differences were observed in the TFA content, except between SPE and SPEH cultures which achieved a TFA percentage of 4.9 and 2.1% respectively, which are significantly different ($p < 0.05$, ANOVA).

The biochemical biomass composition of the ACPE culture, compared with those of the SPE condition, showed a higher protein percentage ($p < 0.05$, ANOVA), but a similarity in TFA, polysaccharides content and in the C/N ratio. Probably similar C/N ratio is due to a higher total lipid content, not determined, in the ACPE culture.

The main differences in the biomass composition between SPE and SPELL cultures consisted in the polysaccharides content and in the C/N ratio which achieved a lower level under low light exposure. On the other hand no significant differences were observed in the protein and TFA content ($p > 0.05$, ANOVA).

Finally, comparing the biomass composition of SCC and SCA cultures it is possible to deduce the effect of the CO_2 supply in the percentage of the biochemical compounds in the algal biomass. The culture added with 2% CO_2 was characterized by higher level of polysaccharides and TFAs, lower protein content and consequently by higher C/N ratio, as shown in Tab. 4.4.

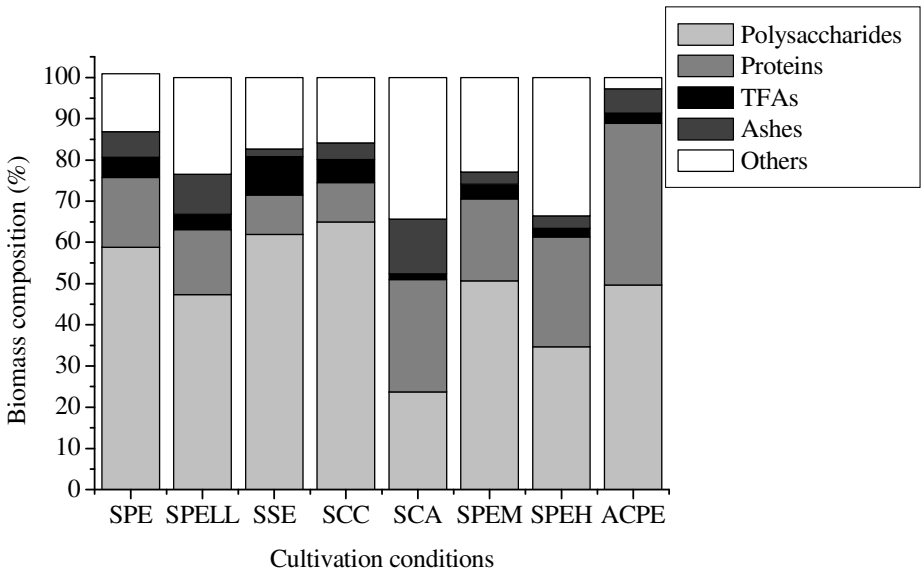

Fig. 4.8 Biochemical composition of the algal biomass obtained in each culture condition (Tab. 4.1) measured as the culture entered the stationary phase.

The effects of N/P ratio on nutrient removal

Tab. 4.2 represents the initial concentrations of nitrogen and phosphorus in the medium, and the initial N/P ratios, which show the P limitation condition of the wastewater utilized.

The nitrogen and phosphorus removal patterns of the algal consortium growing in the primary effluent and those of *Desmodesmus communis* growing at different initial nutrient concentrations are shown in Fig. 4.9, 4.10, 4.11, 4.12 and 4.13. SCC culture removed all the nitrate, the most abundant N form in the medium, in only 3 days (Fig. 4.9). The ammonia removal was complete in almost 11 days when the initial ammonia concentration was above 55.92 mg L^{-1} (SPEH culture), while SPE and SPEM cultures depleted all the ammonia content in almost 7 days (Fig. 4.10). Obviously, the 100% nutrient removal needs a longer algae - based treatment process as the initial concentration of nutrient to remove is higher. The treatment duration depends also on the algal strain and its specific growth rate. As Fig. 4.13 shows, the phosphorus was completely removed in 24h by *Desmodesmus communis* (μ = 0.63 d^{-1}) and in 7 days by the algal consortium (μ = 0.53 d^{-1}). On the other hand, the phosphorus was 100% removed by *Desmodesmus communis* in SPEM, SPEH and SCC cultures in 3 days, probably because the first two culture conditions were also characterized by a high

ammonia level and the third condition had a higher P initial concentration (Fig. 4.11). The ammonia nitrogen was initially removed more consistently in the monoculture of *Desmodesmus communis* with respect to the algal consortium culture, even if it was 100% depleted in 7 days in both culture conditions (Fig. 4.12). No data were collected on the nutrients removal in the SPELL culture over the batch cycle, but they were 100% depleted at the end of the experiment.

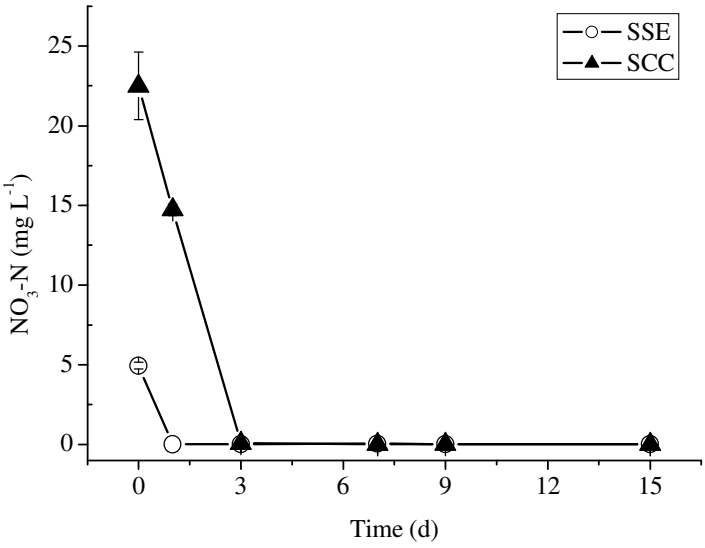

Fig. 4.9 Nitrogen removal in SSE and SCC cultures (Tab. 4.1).

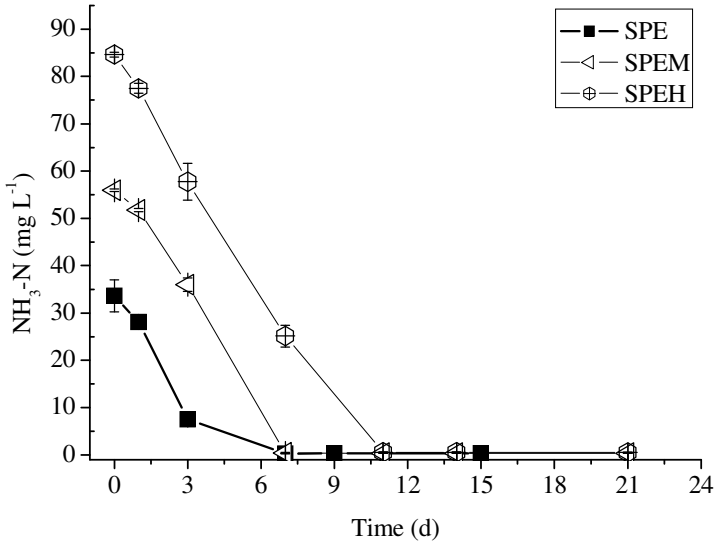

Fig. 4.10 Ammonia removal in SPE, SPEM, SPEH cultures (Tab. 4.1).

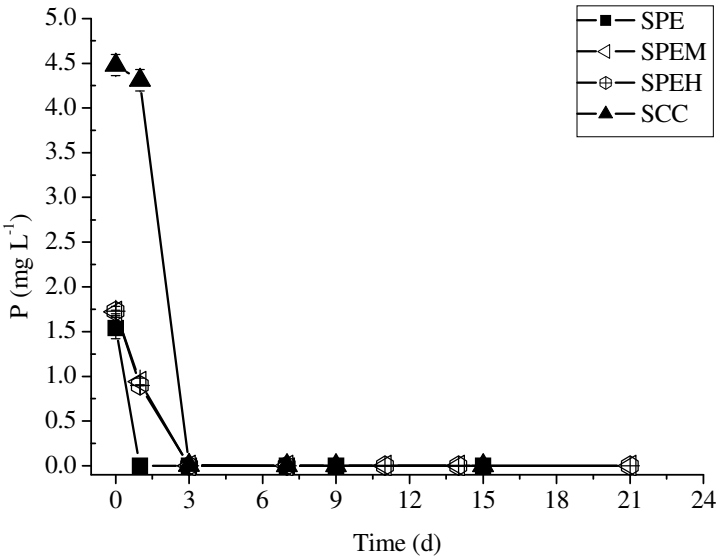

Fig. 4.11 Phosphorous removal in SPE, SPEM, SPEH and SCC cultures (Tab. 4.1).

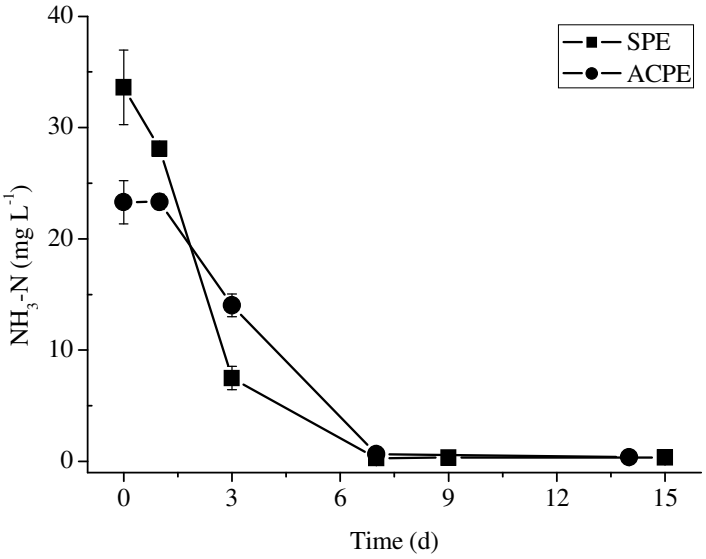

Fig. 4.12 Ammonia removal for *Desmodesmus communis* (SPE) and algal consortium (ACPE) growing in the primary effluent (Tab. 4.1).

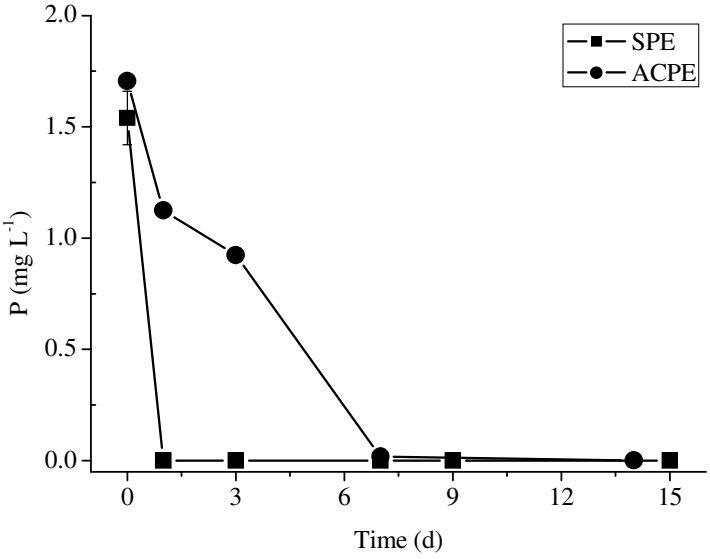

Fig. 4.13 Phosphorus removal for *Desmodesmus communis* (SPE) and algal consortium (ACPE) growing in the primary effluent (Tab. 4.1).

The present study confirmed that nutrients could be almost 100% removed at any N/P ratio, contrary to what Xin et al. (2010b) obtained growing *Scenedesmus* sp. LX1 in a

synthetic medium at different N/P ratios. The nutrient removal data confirmed also the theory expressed by Rhee (1978) according to whom no nutrients were detectable in the surrounding media if the N/P ratio in the water was between the minimum and maximum cellular N/P. A potential limitation of any assimilative nutrient removal processes would be caused by an imbalance of N/P in the wastewater compared to the N/P ratio in the cell tissues. The Stumm empirical formula for microalgae is $C_{106}H_{263}O_{110}N_{16}P$ (N/P ratio of 16:1), however, the average composition of microalgal cells depends on the strain and growth conditions (Xin et al., 2010b). If the classic Redfield atomic ratio of 16:1 N/P was considered unchangeable, P would remain in solution when algae were used to treat wastewater with a N/P ratio lower than 16:1. Rhee (1978) grew *Scenedesmus sp.* at a fixed dilution rate with the N/P ratio in the input medium varying from 5 to 80 (by atoms, as throughout this study). He found that phytoplankton N/P stoichiometry matched the input ratio and that residual nutrients were undetectable. He suggested these levels of nitrogen and phosphorus left in the media were only detectable when the N/P atomic ratio in the feed was lower than the minimum cellular N/P (about 1:4 for *Scenedesmsus* sp.), or higher than the maximum cellular N/P (about 142:1 for *Scenedesmsus* sp.). More recently a mathematical model by Klausmeier et al. (2004) predicts that, at slow growth rates, cellular N/P ratios match media input ratios, from 5 to 80 (by atoms). Also Çelekli et al. (2008) confirmed that *Scenedesmus obliquus* was adaptable to low levels of phosphate and high levels of nitrate.

As the results show, the present study confirmed the validity of Rhee's hypothesis also growing *Desmodesmus communis* in the wastewater and demonstrated that this algal strain is adaptable to low levels of phosphate and high levels of ammonia.

Diversity and community composition of algal consortium

The composition of algal consortium (Fig. 4.14) was assessed in the batch culture for all the length of the experiment and it is represented in the pie charts in Fig. 4.15.

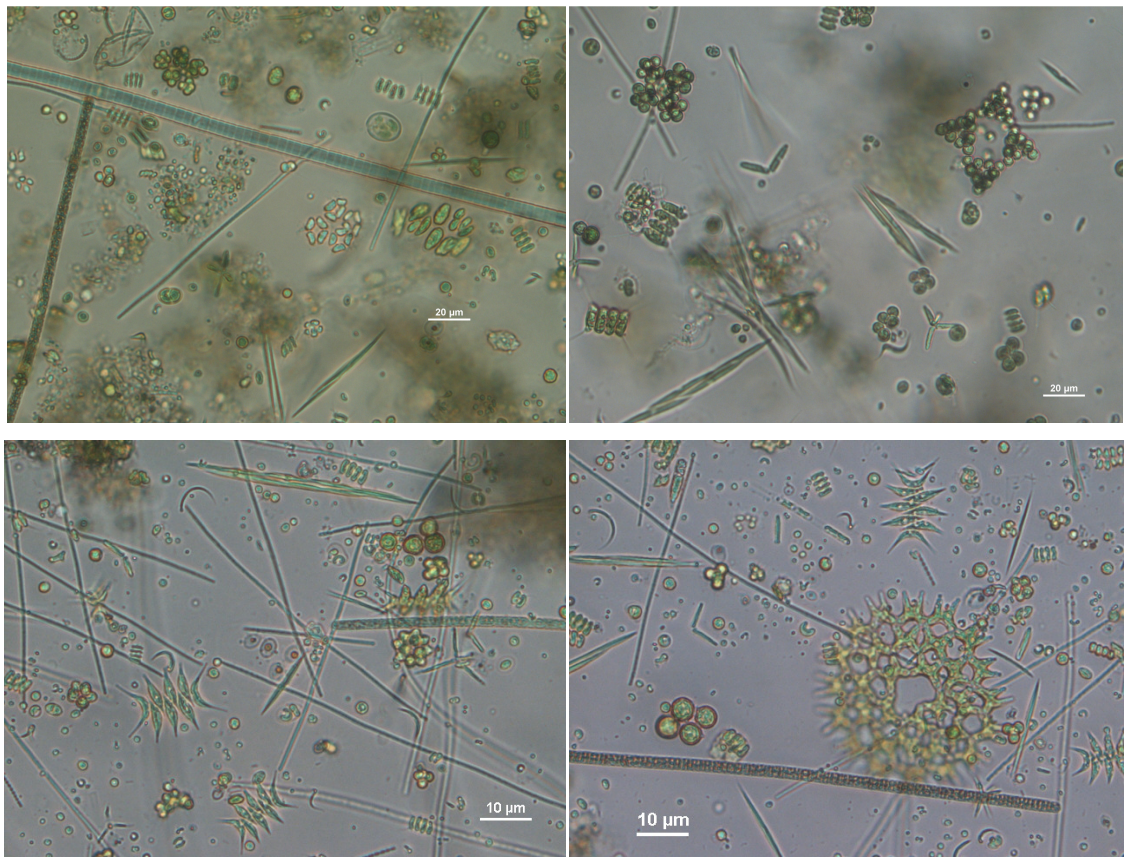

Fig. 4.14 Micrograph, 32X (top pictures) and 20X (bottom pictures), of the natural algal consortium.

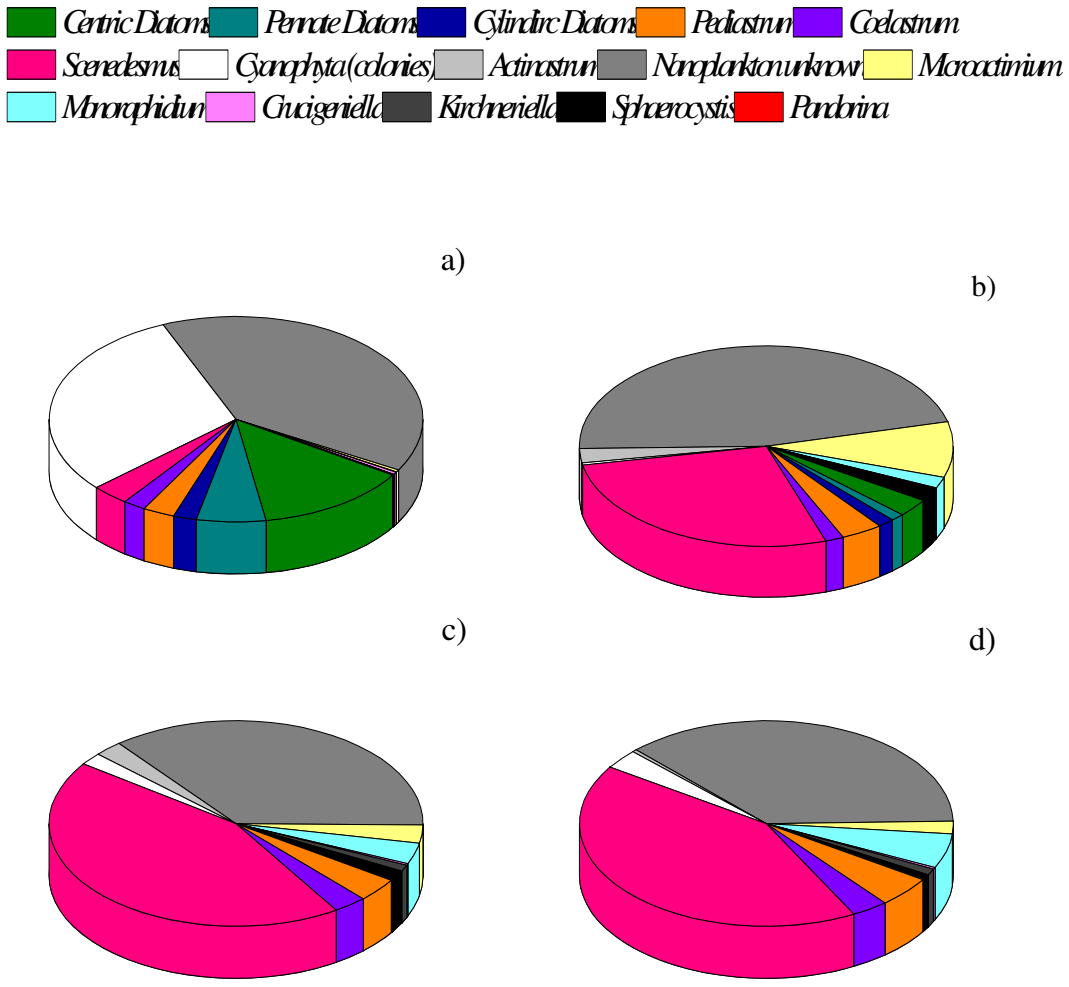

Fig. 4.15 Relative contribution in terms of cell number of the main phytoplankton genera in the algal consortium grown in the primary effluent (ACPE) at Day 1 (a); Day 4 (b); Day 10 (c) and Day 14 (d).

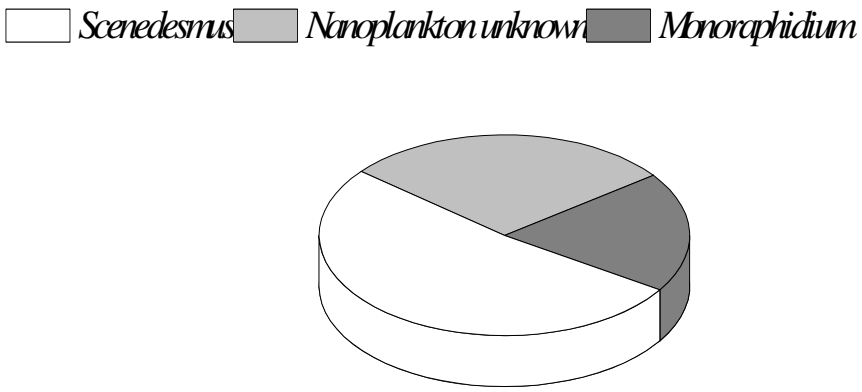

Fig. 4.16 Relative contribution in terms of biovolume of the main phytoplankton genera in the algal consortium grown in the primary effluent (ACPE) at the end of the experiment, Day 14.

As Fig. 4.15 shows, in percentage terms of the number of cells per milliliter, at the beginning of the experiment cyanobacteria, diatoms and nanoplankton not identified were the major groups of algae dominating the culture. After 4 days the community structure changed with a strong reduction of diatoms probably due to the consumption of silica and a high increase of green algae, especially of species belonging to the genus of *Scenedesmus* and/or *Desmodesmus* which dominated all the algal consortium in 10 days. At the end of the experiment *Scenedesmus* and/or *Desmodesmus* and other nanoplankton species not identified were the major groups in the algal community with a percentage of the total number of cells of 42% and 37%, respectively. In terms of biovolume, the percentage distribution showed the genus of *Scenedesmus* and/or *Desmodesmus* to represent the highest cell number constituting 52% of the total volume and the nanoplankton not identified constituted 29%. *Monoraphidium* spp. which didn't reach high numbers of cells counted (5%) constituted 19% of the total volume (Fig. 4.16). Because all the biomass characterization was referred to a biomass dry weight, all the results obtained in the algal consortium study were obviously influenced by the community composition and in particular, as the biovolume results confirmed, strains belonging to the genus *Scenedesmus* and/or *Desmodesmus* were naturally selected as the most abundant microorganisms inside the culture.

PAM fluorometry measurements to assess microalgal growth and nutrient stress

The F_v/F_m ratio obtained from the induction curves, was used for the determination of photosynthetic efficiency and for describing the effects of nutrition status and irradiance on photosynthetic performance of the cultures.

As shown in Fig. 4.17, under nitrogen - limited conditions the efficiency of PSII decreased. Non - limited phytoplankton has F_v/F_m values from 0.6 to 0.7 (Kromkamp and Peene, 1999). Nutrient stress in microalgae is generally detected by a decrease in maximum quantum efficiency F_v/F_m which dropped in this study from almost 0.7 to 0.0. The F_v/F_m ratio trend followed the nutrient availability, indeed we observed that the maximum quantum yield decreased after 3 days in SPE culture when the NH_4-N content was almost completely depleted. The F_v/F_m ratio drop seemed to be less rapid in the SSE culture which was already nutrient - limit adapted. In the SSE condition the ratio decreased from a maximum of 0.6 to a minimum of 0.3; in the SPE condition it decreased from a maximum of 0.66 down to 0.0 (Fig. 4.17a). SPEM and SPEH cultures showed a longer initial phase in which the maximum quantum yield varied around a value of 0.55 - 0.7; on day 9 and 14 the F_v/F_m ratio of SPEM and SPEH respectively, decreased significantly as the NH_4-N was completely depleted (Fig. 4.17b).

The low light exposure, represented by the SPELL curve in Fig. 4.17a, showed a maximum quantum yield of about 0.6 and it started to drop after 10 days. Comparing SPELL and SPE conditions it is possible to observe that under low light irradiance the photosynthetic efficiency is maintained longer probably due to a slower uptake of nutrients, a possible area for future investigation.

Comparing SCC and SCA (Fig. 4.17c) it is possible to observe how the CO_2 supply permitted the photosynthetic efficiency to remain high and constant during a longer period of time, while the C - limitation condition (SCA) seemed to be more influenced by the nutrient consumption. Finally the algal consortium showed a lower F_v/F_m due to an initial acclimatization of the culture and it didn't reach values as high as SPE did: this behavior showed an analogy with the growth rate which is lower in ACPE compared with the SPE one.

The F_v'/F_m' ratio of ACPE and SPE cultures followed the same trend of the F_v/F_m ratio (Fig. 4.18). The SSE F_v'/F_m' ratio trend was clearly influenced by the nutrients starvation

condition of the culture (Fig. 4.18a). As Rodolfi et al. (2008) explained in their work, when nitrogen deprivation is imposed upon a culture exposed to suitable irradiance, photosynthesis continues at a reduce rate, and the flow of fixed carbon is diverted from protein to either lipid or carbohydrate synthesis. SPELL culture condition showed an effective quantum yield higher compared with other conditions which were exposed to high light; its values followed a trend similar to the maximum quantum yield values dropping at day 10 (Fig. 4.18a). In SPEM and SPEH cultures the F_v'/F_m' ratio started to drop earlier when compared with the F_v/F_m ratio that decreased a few days after the nutrient depletion (Fig. 4.18b). From obtained results, it appears that the F_v'/F_m' ratio is the most sensitive parameter for measuring the efficiency of Photosystem II photochemistry correlating with the nutrient removal.

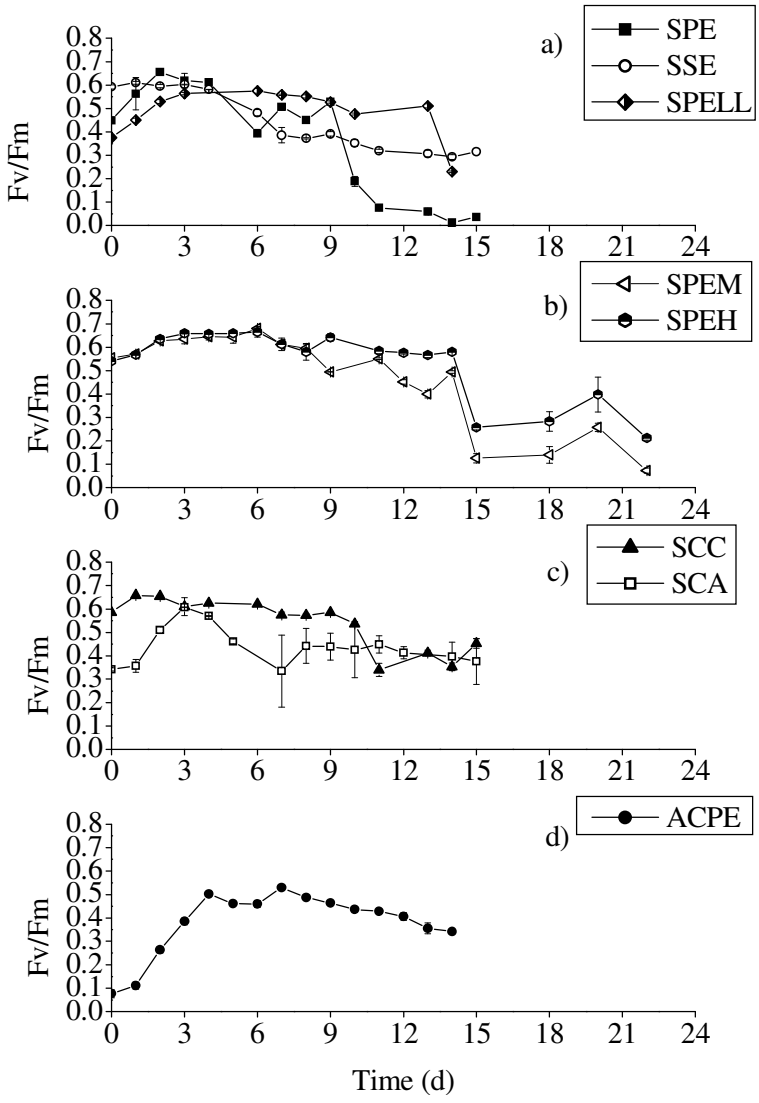

Fig. 4.17 Maximum quantum yield (Fv/Fm) determined from active fluorometry (PAM) as a function of time. (a) represents maximum quantum yields relative at . *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPE); *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 88 µE m^{-2} s^{-1} (SPELL); *Desmodesmus communis* in secondary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SSE). (b) *Desmodesmus communis* in primary effluent wastewater enriched with $(NH_4)_2SO_4$ and insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPEM and SPEH); (c) *Desmodesmus communis* in Chu13 modified medium insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SCC); *Desmodesmus communis* in Chu13 modified medium insufflated with air (SCA). (d) algal consortium in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (ACPE).

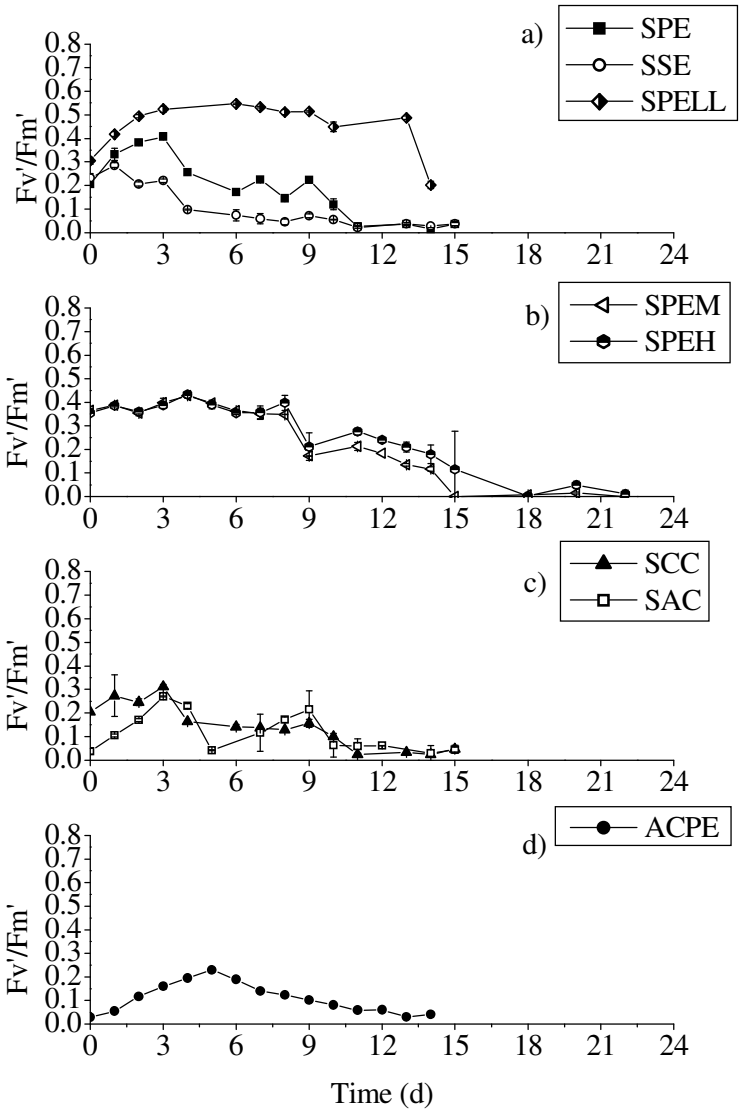

Fig. 4.18 Effective quantum yield (Fv'/Fm') determined from active fluorometry (PAM) as a function of time. (a) represents maximum quantum yields relative at . *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPE); *Desmodesmus communis* in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 88 µE m^{-2} s^{-1} (SPELL); *Desmodesmus communis* in secondary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SSE). (b) *Desmodesmus communis* in primary effluent wastewater enriched with $(NH_4)_2SO_4$ and insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SPEM and SPEH); (c) *Desmodesmus communis* in Chu13 modified medium insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (SCC); *Desmodesmus communis* in Chu13 modified medium insufflated with air (SCA). (d) algal consortium in primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 440 µE m^{-2} s^{-1} (ACPE).

4.2.4 Conclusions

The results of this study showed that the primary (wastewater) effluent from the wastewater reclamation facility of Cesena (Italy) can be used as medium for cultivation of an autochthonous strain of *Desmodesmus communis* leading to the production of an amount of algal biomass (0.138 g L^{-1} d^{-1}) which is even higher than the quantity obtained using a synthetic medium. This algal strain has been proven to be able to grow at high levels of ammonia concentration as those which may be present in the wastewater during the year, reaching values in terms of biomass productivity (0.177 - 0.227 g L^{-1} d^{-1}) which increased with increasing the ammonium level. It also achieved nutrient removal efficiency of almost 100% for NH_3-N and P at any N/P ratio which may characterize the wastewater nutrient composition. The growth rate and the biomass composition of microalgae vary under different medium composition and cultivation conditions; the use of the primary effluent, rich in nitrogen, maximized the biomass production, while the use of the secondary effluent, which represents a nutrient limitation condition, maximized the total fatty acid percentage in the biomass (9.3%) and the C/N ratio (39.3). This study also demonstrated how CO_2 supplementation and high light intensity were used to increase the biomass productivity and the C/N ratio in algal cells. As the photosynthetic process evaluation through the PAM Fluorometer showed, high light condition result in a decrease in the photosynthetic efficiency of *Desmodesmus communis*, in addition the biomass productivity and composition analysis showed that this light condition is worth it.

The algal consortium study showed lower growth rate and biomass productivity (0.078 g L^{-1} d^{-1}) compared with the results obtained for *Desmodesmus communis* monoculture; it also showed a lower nutrient consumption. One of the main limit of algae cultivation technique in open ponds consists in the presence of competitor organisms that can have the upper hand over a monoculture. The community composition study of the algal consortium showed how algal species belonging to the genus of *Scenedesmus* and *Desmodesmus* are good competitors in a mixed population as they reached in 14 days the highest percentage both in terms of cells per liter and on a biovolume basis.

4.3 Induction of lipid synthesis in *Desmodesmus communis* culture grown in urban wastewaters

4.3.1 Introduction

The interest in microalgae for oil production is due to the lipid content of some species, especially of non - polar lipids and hydrocarbons, which are the best substrate to produce biodiesel (Rodolfi et al., 2008). Synthesis and accumulation of large amounts of non - polar lipids and alterations in lipid and fatty acid composition occur in the algal cell when the cultures are placed under stress conditions such as nutrient starvation, high irradiance, unfavorable salinity, (growth – medium) pH and temperature values (Hu et al., 2008; Rodolfi et al., 2008). The physiological role of the triacylglycerols (TAGs) consists in their storage as carbon and energy source, being used especially in algae at the stationary phase or under stress. The *de novo* TAG synthesis pathway serves as an electron sink under photo - oxidative stress. Under light stress, excess electrons that accumulate in the photosynthetic electron transport chain may induce over - production of reactive oxygen species, which may cause inhibition of photosynthesis and damage to membrane lipids, proteins and other macromolecules. The formation of a C18 fatty acid molecule consumes almost 24 NADPH derived from the electron transport chain and increases under high light or other stress conditions (Hu et al., 2008). Green microalgae show a variety of responses to the stress conditions: such as an increase of the TAG synthesis or an increase of energy dissipation as heat. In a previous research (see section 4.2) performed with the microalgae *Desmodesmus communis* in view of its application for wastewater treatment and biomass production, it was shown to contain a total fatty acid (TFA) amount of about 2-4% of dry weight when grown at high nutrient levels (i.e. in a primary effluent); this value increased to nearly 9% in nutrient starvation conditions, as those derived from using a secondary effluent as the culture medium. The aim of this study is to characterize the lipid composition of *Desmodesmus communis* and to observe possible changes, in both cell content and profile, which could be derived from the application of combined stress consisting in nutrient starvation and increased light conditions. The physiological responses of the algae were studied in terms of TFAs production and photosynthetic efficiency.

4.3.2 Overview of experiments

Desmodesmus communis was firstly adapted and grown as a culture batch in the primary effluent wastewater insufflated with air - CO_2 mixture (98/2, v/v) and light intensity of 88 µE m^{-2} s^{-1} for 15 days. The algal culture, after a period of growth at low light intensity was diluted by replacing either 50% or 82% of the culture volume with the secondary effluent ; the batch cultures obtained were insufflated with air - CO_2 mixture (98/2, v/v) exposed to increased light intensity of 140 µE m^{-2} s^{-1} and was followed for another15 days.

Each condition was run in duplicate.

Systems' setting, temperature, light/dark cycle and air - CO_2 flow were set as described in section 4.1.1.

4.3.3 Results and Discussion

Cultures growth rate

Fig. 4.19 illustrates *Desmodesmus communis* growth as optical density over the batch cycle in two step phases described above: in the I Phase *Desmodesmus communis* was grown in the primary effluent while the II Phase consisted in growing the cultures in low nutrients (secondary effluent) and higher light intensity. The initial nutrient concentrations for each Phase are shown in Tab. 4.6.

Condition	NO_3-N (mg/L)	NO_2-N (mg/L)	NH_3-N (mg/L)	P (mg/L)	N/P
I Phase	0.03 ± 0.02	< 0.01	30.12 ± 0.07	2.00 ± 0.28	33:1
II Phase 50%	1.47 ± 0.17	0.04 ± 0.02	< 0.01	nd	nd
II Phase 82%	4.00 ± 0.20	< 0.01	0.20 ± 0.06	nd	nd

Tab. 4.6 Comparison of initial concentration of nutrients in both phases: I Phase with primary effluent; II Phase diluted with secondary effluent at 50% and at 82%.

nd = not detectable

Each culture reached the stationary phase in 3 days: the growth rates didn't show any significant differences ($p > 0.05$, ANOVA) achieving values of 0.37 ± 0.09 d^{-1} for the culture in the I Phase, 0.40 ± 0.03 and 0.46 ± 0.05 d^{-1} for cultures in the II Phase diluted at 50% and 82%, respectively. Despite the strong difference in nutrient concentration between the two phases, during the II Phase algal cells were able to grow to an extent similar to that of the I Phase, probably due to the higher irradiance level. Different results were observed between the two dilution conditions consisting in the more rapid growth of the most diluted culture (II Phase 82%) respect to the 50% diluted culture because of its greater initial concentration of nitrate and greater light exposure.

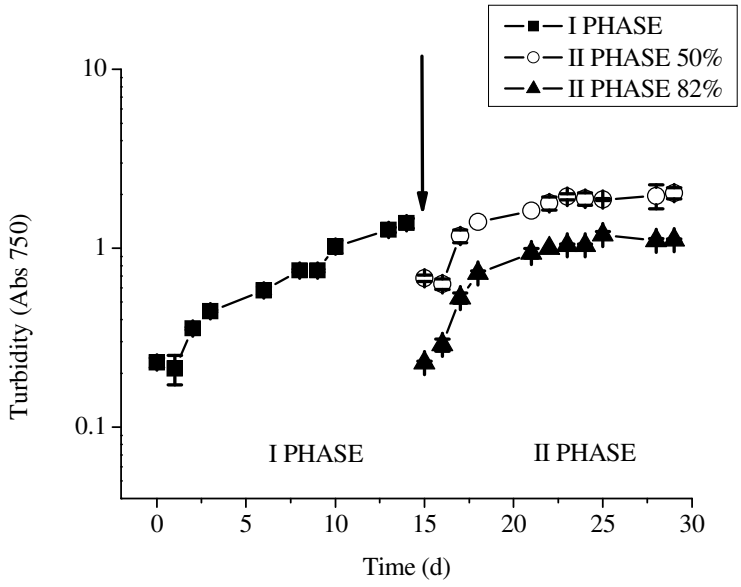

Fig. 4.19 Growth of *Desmodesmus communis* expressed as optical density at 750 nm. In the I Phase *Desmodesmus communis* was grown in the primary effluent under 88 µE m^{-2} s^{-1} irradiance; at the beginning of the II Phase the culture was diluted by replacing either 50% or 82% of the culture volume with the secondary effluent, and at the same time transferred to higher irradiance of 140 µE m^{-2} s^{-1}.

Biomass characterization

The biomass composition was analyzed at the end of each phase and the relative percentage of main cell compounds over the dry biomass is summarized in Fig. 4.20. The biomass productivity achieved 0.018 ± 0.002 g L^{-1} d^{-1} for the culture in the I Phase, 0.081

± 0.001 and 0.049 ± 0.003 g L^{-1} d^{-1} for those in the II Phase diluted at 50% and 82%, respectively. The biomass maximum value was 0.70 ± 0.01 g L^{-1} for the culture in the I Phase, 1.64 ± 0.02 and 0.94 ± 0.04 g L^{-1}, for those in the II Phase diluted at 50% and 82%, respectively.

The initial nutrient limitation condition of the II Phase changed during the experiment in a complete deprivation of nutrients in both conditions. *Desmodesmus communis* culture in N - sufficient media was shown to achieve a N content of 8-10% over dry biomass (see section 4.4). In this study the N limitation at the end of the I Phase was proven by the low N content over dry biomass which achieved 5.1% , and the N deprivation at the end of the II Phase was proven by the N content of only 2.1% and 2.3% achieved in the 50% and 82% culture dilution conditions, respectively. The dilution operation using the secondary effluent affected the cells concentration in the cultures but it didn't influence the N limitation condition reached during the stationary phase of the first (experiment) step, indeed the cells N content was 3% at the beginning of the II Phase for both cultures.

The polysaccharides content increased significantly ($p < 0.05$, ANOVA) between the end of the I Phase (47.3%) and the end of the II Phase for the culture with an initial dilution of 50% (70.1%), while the biomass obtained at the end of the II Phase of the culture with an initial dilution of 82% was characterized by an intermediate polysaccharides value (60.9%) which resulted significantly different only respect to the polysaccharides percentage of the I Phase ($p < 0.05$, ANOVA).

The protein content decreased as the nutrient starvation condition increased. The algal biomass at the end of both the I Phase and the II Phase of the culture with an initial dilution of 50% reached a protein content of 15.8 and 11%, respectively, with no significant differences ($p > 0.05$, ANOVA); the biomass obtained at the end of the II Phase in the culture with an initial dilution of 82% achieved a significant reduced level of protein of 6.6%, respect the other two conditions ($p < 0.05$, ANOVA). This result confirmed that even though the initial concentration of nitrate was higher in the culture diluted at 82% compared with the initial concentration of nitrate of the culture diluted at 50%, the N concentration was still so low in both conditions to induce a similar final N deprivation.

In this study the lipid content representing the total fraction of lipid components such as hydrocarbons, polar and non - polar lipids, was significantly different at the end of each

phases ($p < 0.05$, ANOVA) achieving values in terms of percentage over dry biomass of 11.8% at the end of the I Phase, and 21.3 and 25.8% at the end of the II Phase with an initial dilution of 50% and 82%, respectively. This result confirmed that a severe N-deprivation affected the lipid content in *Desmodesmus communis*, contrary to what was observed by Rodolfi et al. (2008). The decrease in the proteins content and the great increase in the polysaccharides and even more so in the lipids content determined the increase of the C/N value which reached a maximum of 28.1 at end of the II Phase of the culture with an initial dilution of 82% (Tab. 4.7). The most diluted culture thus showed the highest values in the lipid content and C/N ratio, probably due to the higher light intensity inside the culture related to the lower cell concentration.

The polysaccharide and protein percentage obtained in cells grown under the two dilution conditions applied in the II Phase was similar to the percentage of these biomass components obtained in cells grown during the previous study when the secondary effluent was used as the growth medium (SSE condition in section 4.2). However the C/N value obtained in that case was much higher, being nearly 39.3, a result probably due to the high lipid level (not determined) in cells. The main difference between the two experiments consists in the light intensity which was 3 - fold higher in the previous than in the present study. These results reinforce again the hypothesis that the elemental composition of *Desmodesmus communis* biomass is strongly influenced by nutrient starvation and also by light intensity.

Condition	VSS ($g\ L^{-1}$)	Polysaccharides (% w/w)	Proteins (% w/w)	Lipids (% w/w)	Ashes (% w/w)	C/N
I Phase	0.70 ±0.01	47.3 ± 2.0	15.8 ± 1.7	11.7 ± 0.5	9.8 ± 0.5	10.6
II Phase 50%	1.64 ± 0.02	70.1 ± 5.4	11.0 ± 1.2	21.3 ± 0.4	2.1 ± 0.6	24.1
II Phase 82%	0.94 ± 0.04	60.9 ± 0.8	6.6 ± 1.2	25.8 ± 0.8	1.3 ± 1.1	28.1

Tab. 4.7 Biochemical and elemental characterization of *Desmodesmus communis* biomass (express in terms of VSS, volatile suspended solids) obtained in each culture condition in the late stationary phase (day 14).

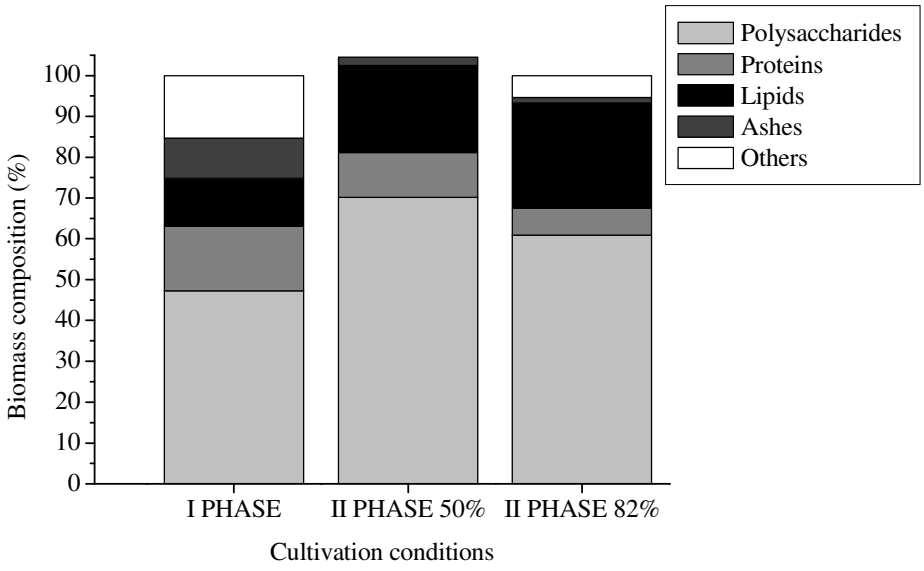

Fig. 4.20 Biochemical composition of *Desmodesmus communis* biomass obtained in each culture condition in the late stationary phase (day 14).

Lipid composition

The algal biomass at the end of both phases in both dilution conditions achieved different values of lipids percentage over dry biomass of 11.7 % at the end of the I Phase, 21.3 and 25.8 % at the end of the II Phase in the cultures with an initial dilution of 50% and 82%, respectively (Tab. 4.8). The lipid content reached by the cells in the I Phase was lower compared with the lipid percentage of other strains studied in similar works (Rodolfi et al., 2008). The lipid accumulation properties of the strain *Scenedesmus* sp. LX1 growing in a secondary effluent has also been studied by Xin et al. (2010a) who reported a lipid percentage of 33%. In the present work the percentage of lipid accumulated in *Desmodesmus communis* biomass doubled proportionally to the nitrogen deficiency level, the emitted irradiance and the initial culture dilution percentage. This result contrasts with the result obtained by Rodolfi et al. (2008) which didn't observe any lipid increase in the selected strain belonging to the genus *Scenedesmus*; on the other hand in *Nannochloropsis* grown in a 110-L panel photobioreactor (Rodolfi et al., 2008), the lipid content increased from 32% to 60% when the culture was transferred from nitrogen sufficient to nitrogen deficient conditions. In accordance to Xin et al. (2010b) *Desmodesmus communis* started to accumulate more lipids when the light had a better penetration through the culture and individual cells were exposed to a large quantity of

light energy (i.e. in the culture diluted at 82%). As the N content in cells attested, the cell culture was already nutrient - limited at the end of the I Phase however this level of nutrient limitation was proven not to be a sufficient stress condition to induce an increment in the cell lipid content, a result which was achieved only during the II Phase characterized by cells achieving a complete nutrient deprivation and exposed to higher light intensity.

With regard to the composition of the total lipid fraction shown in Tab. 4.8 it is possible to observe that some differences were detected between the cells analyzed at the end of the I Phase and those of the II Phase.

The algal cells grown in the I Phase, characterized by low light and high initial concentration of nutrients, accumulated a large amount of polar lipids, almost 84.2% and only 14.2% of non - polar lipids. On the other hand the algal cells grown in the II Phase, characterized by high light and low initial nutrient concentration, increased their content of non - polar lipids which reached a relative percentage of 43-49%, while polar lipids decreased to 49-53% under both dilution conditions. The major polar lipids are usually represented by phospholipids which are indispensable constituents of the cell membrane of all organisms including microalgae (Yamaguchi et al., 1987). A possible explanation for the results observed can be that during the I Phase the algal culture was not stressed by the nutrient limitation and thus invested its energy in cell growth and duplication: this physiological mechanism caused the accumulation of polar lipids in the cell also when the culture reached an advanced state in the stationary phase. During the II Phase the algal cells which derived from a culture in the stationary growth phase were diluted with the secondary effluent and kept under increased light intensity: this operation maintained them in a prolonged nutrient limitation condition which changed very soon into complete nutrient deprivation. In such extreme stress conditions, the cellular organism accumulated energy storage as non - polar lipids which were more abundant in the 82% diluted culture (49.2%).

Lipid	I Phase	II Phase	
		50%	82%
Content	11.7%	21.3%	25.8%
Composition			
Hydrocarbons	0%	0%	0%
Non - polar lipids	14.2%	43.4%	49.2%
Polar lipids	84.2%	53.2%	49.4%

Tab. 4.8 Percentage composition of lipid components in *Desmodesmus communis* biomass obtained in each culture condition in an advanced state of the stationary phase (day 14).

Content and composition of fatty acids

Tab. 4.9 shows the total fatty acid content of the cells, expressed as percentage over dry biomass, as well as the fatty acids profile of the biomass, as percentage in weight over the total fatty acids. Similarly to the total lipid content and relative composition reported before, the total fatty acid analysis also showed an increase in terms of percentage over dry biomass between the end of the I Phase (3.7%) and the end of the II Phase (9.7 and 14.1%), however no significant differences ($p > 0.05$, ANOVA) were observed between the two dilution conditions on the last day of the experiment. The results obtained during this study were in accordance with the previous study (see section 4.2) where it was demonstrated that *Desmodesmus communis* grown in the secondary effluent was able to reach a content of TFAs of 9.3%, which is 3-fold higher than the one obtained using a primary effluent as growth medium. The present results demonstrate that the TFA content increased and its profile changed going from the I Phase to the II Phase. The most abundant saturated fatty acid proved to be palmitic acid (16:0), followed by stearic acid (18:0), as already shown for some strains of the genus *Scenedesmus* in previous works (Martínez et al., 2000; Hodaifa et al., 2008; Zhou et al., 2011). The most abundant of the unsaturated fatty acids were oleic and linoleic acids which together represented more than 50% of the TFA composition. While the proportion between saturated and unsaturated fatty acids wasn't affected by the nutrient conditions and the light intensity, the amount of

C18 fatty acids increased in the II Phase in accordance with the theory expressed by Hu et al. (2008), which explained the tendency of some algae to form C18 fatty acids, using the NADPH derived from the electron transport chain, as a protective mechanism carried out by the algal cell under high light or other stress conditions.

Fatty acids	I Phase	II Phase	
		50%	82%
Content	(3.7 ± 0.5)%	(9.7 ± 0.1)%	(14.1 ± 3.0)%
Composition			
16:2	12.6%	4.2%	4.1%
16:3	4.0%	1.7%	1.7%
16:0	27.9%	24.2%	24.2%
18:2 18:1*	54.3%	65.8%	66.9%
18:0	0.8%	4.0%	3.1%

Tab. 4.9 Summary of the total fatty acid composition (%) representative for each culture conditions in an advanced state of the stationary phase (14 day). (*) Linoleic and Oleic acids were quantified together.

The TFA concentrations were measured at different intervals during the II Phase in order to determine the algal productivity capacity for these compounds. The TFA content over the dry biomass at the beginning of the II Phase was 3.7% and increased to 4.3% and 6.7% in the culture diluted at 50% and 82%, respectively, after only 3 days from the beginning of this phase (day 17 of the whole experiment) (Fig. 4.21). The TFA percentage increased more rapidly in the culture diluted at 82%, reaching its maximum percentage value (13.2 ± 1.3) on day 22 (Fig. 21b) which was 27% higher compared with the maximum percentage value (9.7 ± 0.1) reached at the end of the II Phase in the biomass derived from the culture diluted at 50%. As Fig. 4.21 shows, despite the fact that the percentage of TFAs over dry biomass is higher in the culture diluted at 82%, the TFA productivity is also influenced by the total biomass (VSS) which resulted more

concentrated in the culture diluted at 50%. The TFA productivity obtained was 11.5 ± 1.6 mg L^{-1} d^{-1} and 9.4 ± 2.5 mg L^{-1} d^{-1} in the cultures diluted at 50% and 82%, respectively; this result didn't evidence any differences in productivity between the two cultures ($p > 0.05$, ANOVA).

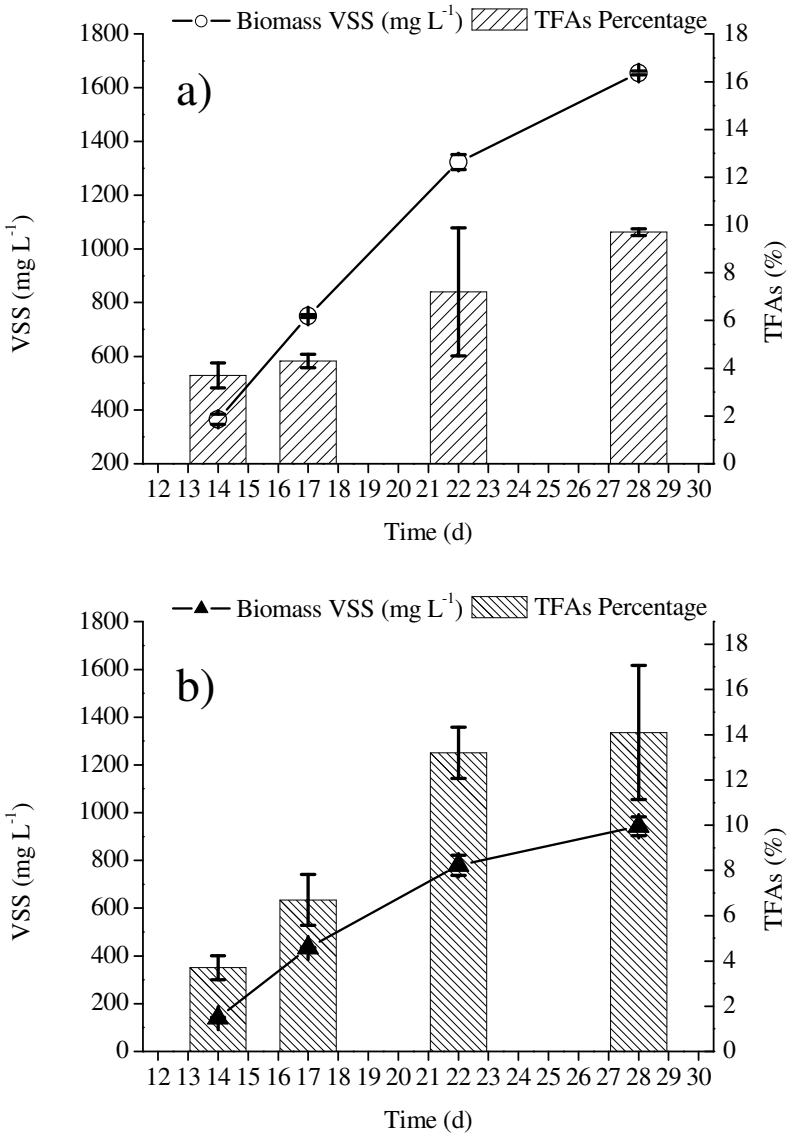

Fig. 4.21 *Desmodesmus communis* growth and total fatty acids (TFAs) content during the II Phase in the cultures diluted at 50% (a) and 82% (b).

Induction curves

PAM fluorescence induction kinetics of *Desmodesmus communis* cultures, measured during both exponential and stationary growth phase for each condition, exhibited patterns related to the stress conditions (Fig. 4.22). During the exponential growth of *Desmodesmus communis* in the I Phase, the maximum fluorescence levels (Fm) reached the highest value of 900 A.U. and the maximum fluorescence yield (Fm') induced by the saturating pulse of light on the light - adapted culture, didn't show any decreases compared to the Fm value (Fig. 4.22a). These results are indicative of a good performance of the photosynthetic process under low light as it maintained a high efficiency also during the light exposure. The fluorescence level at the steady state of electron transport (F) remained constant during the whole measurement and it showed the same value and trend also at the end of the I Phase (Fig. 4.22b). During the stationary phase of the I Phase the kinetic curve of *Desmodesmus communis* showed a decrease in the Fm value of 35% compared to the Fm observed during the exponential phase, due to the nutrient limitation, however no differences were observed between Fm and Fm', thus the nutrient limitation at low light didn't affect the flux of electrons in the photosynthetic electron transport chain. The F0' value, which represents the minimal fluorescence in a light-adapted state, was 22% higher in the stationary phase compared with the one obtained in the exponential phase, proving that no damage in the photosynthetic apparatus had occurred during this growth phase. The induction curves measured during the II Phase are shown in Fig. 4.22, for both the culture diluted at 50% (Fig. 4.22c,d) and at 82% (Fig. 4.22e,f). As soon as the II Phase began, an increment in the maximum level of fluorescence, Fm, was observed for both the dilution conditions, probably due to the addition of nutrients and to the increase of the irradiance level (140 $\mu E\ m^{-2}\ s^{-1}$ of the II Phase against the 88 $\mu E\ m^{-2}\ s^{-1}$ of the I Phase) (Fig. 4.22c,e). In this condition the Fm value was even 20% higher than the Fm obtained during the exponential growth in the I Phase; this result confirms that the light intensity has a higher effect on the maximum photosynthetic efficiency than the nutrient availability which was greater in the I Phase. In addition, the maximum fluorescence yield of the light - adapted sample, Fm', decreased compared to the Fm value by 45% and 52% in cultures diluted at 50% and 82%, respectively, during the II Phase, phenomenon that was not observed during the I Phase at any growth stage. These results may prove that the effective efficiency of the photosynthetic process in light - adapted cultures decreased under high irradiance

conditions. The F0' values didn't show any differences compared to the value obtained during the exponential growth of the I Phase. At the end of the II Phase the induction curves clearly showed that the cultures were highly stressed. As shown in Fig. 4.22d,f, the maximum fluorescence level, Fm, decreased by 46% and 53%, with respect to the level measured in the exponential growth of the II Phase, in the cultures diluted at 50% and 82%, respectively. This drop in the Fm value was higher than the one observed during the I Phase (35%) between the exponential and the stationary phase; this result may prove the extreme stress condition achieved during the II Phase. Moreover the maximum fluorescence yield of the light - adapted sample, Fm', decreased compared to the Fm value, by 46% and 41% in the cultures diluted at 50% and 82%, respectively, indicating the almost complete absence of flux of electrons in the photosynthetic electron transport chain. The minimal fluorescence in a light-adapted state, F0', also decreased by 20% compared to the value obtained in both cultures at the beginning of the II Phase, proving that now the photosynthetic apparatus was damaged.

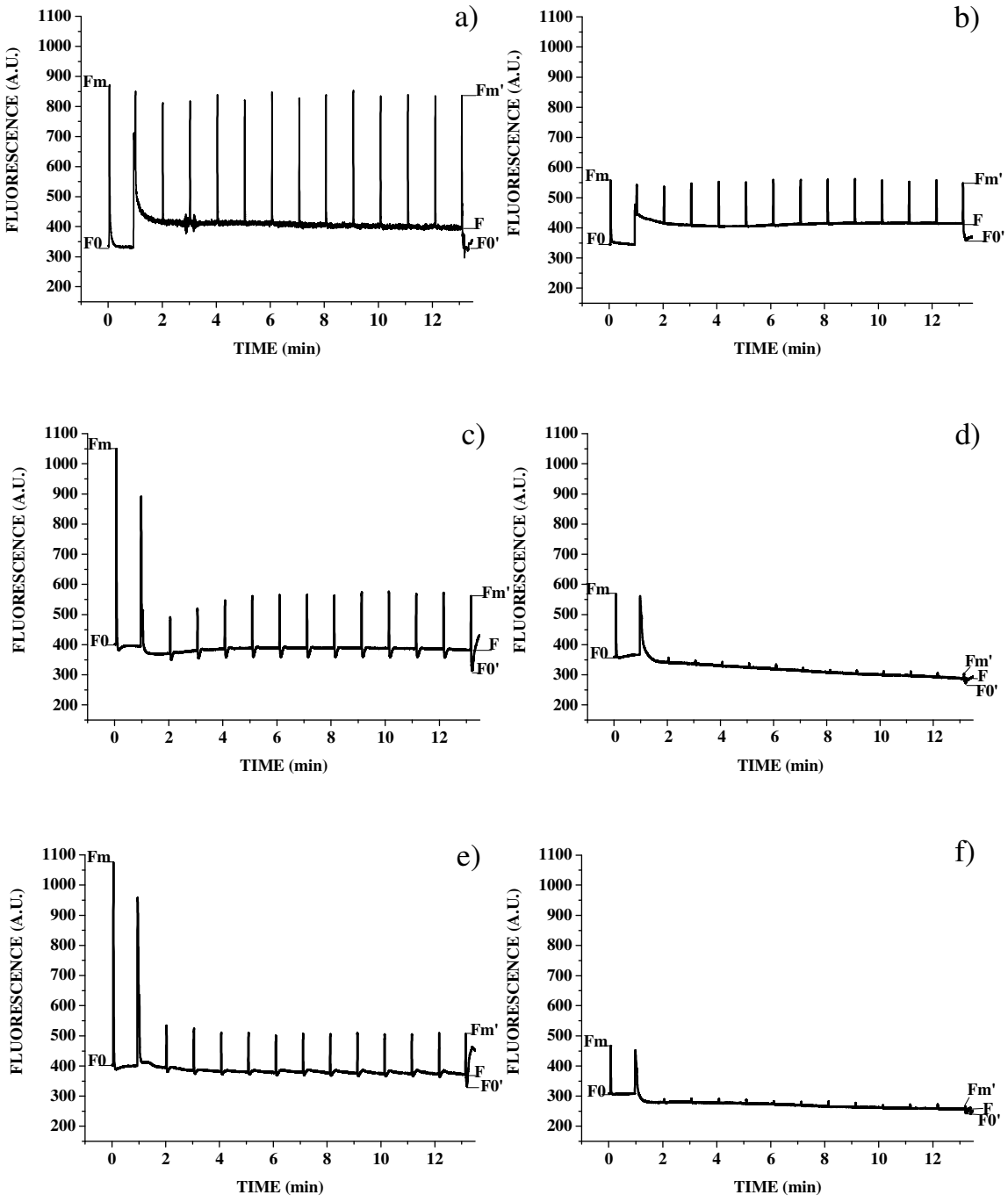

Fig. 4.22 PAM fluorescence induction kinetics of *Desmodesmus communis* culture measured: a) in the exponential phase of the I Phase; b) in the stationary phase of the I Phase; c) in the exponential phase of the II Phase of the culture diluted at 50%; d) in the stationary phase of the II Phase of the culture diluted at 50%; e) in the exponential phase of the II Phase of the culture diluted at 82%; f) in the stationary phase of the II Phase of the culture diluted at 82%.

The maximum quantum yield of PSII (Φ_{PSII}, Fv/Fm) maintained high values during the whole I Phase, decreasing rapidly in the advanced state of the stationary phase while it dropped in a few days for both cultures in the II Phase (Fig. 4.23). During this phase the Fv/Fm ratio showed higher values in the culture less diluted, which was probably less inhibited by the light. The effective quantum yield (Φ_{PSII}', Fv'/Fm') in the I Phase maintained high values which were not so much lower compared to the Φ_{PSII}, while in the II Phase this parameter collapsed after only 3 days, due to the complete deprivation of nutrients (Fig. 4.24). Low light and high nutrient concentrations kept the photosynthetic apparatus in the optimum condition and in these culture conditions the photosynthetic efficiency was high from the end of the I Phase, on the other hand high irradiance levels increased the stress effect of nutrient starvation in the II Phase.

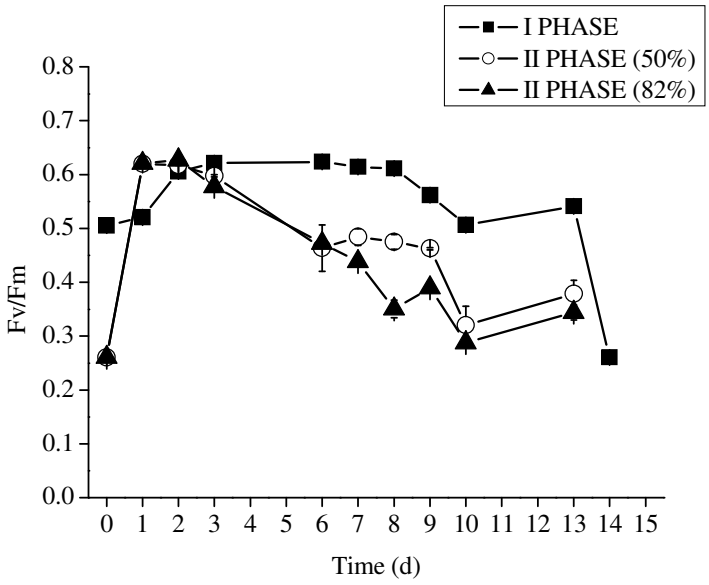

Fig. 4.23 Maximum quantum yield (Fv/Fm) determined from active fluorometry (PAM) as a function of time in *Desmodesmus communis* cultures in both phases.

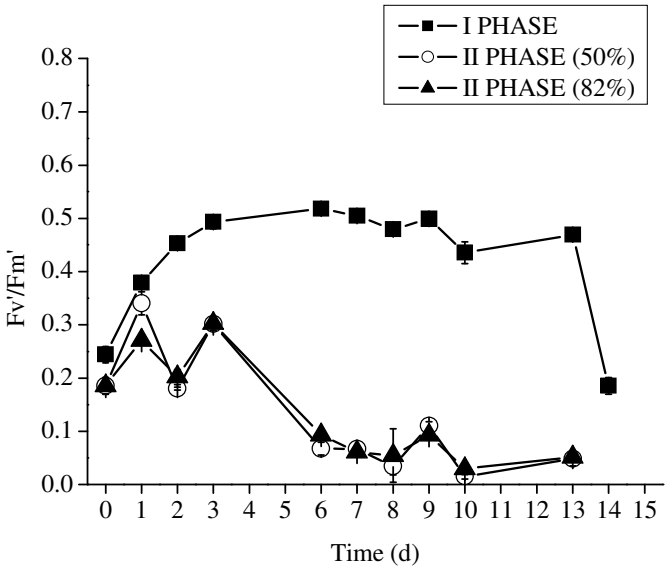

Fig. 4.24 Effective quantum yield (Fv'/Fm') determined from active fluorometry (PAM) as a function of time in *Desmodesmus communis* cultures in both phases.

Under nutrient deprivation and increased light conditions the efficiency of PSII decreased rapidly during the II Phase and as the Fv/Fm ratio dropped the TFA content over dry biomass increased (Fig. 4.25). An inverse correlation was measured between the decline in the photosynthetic efficiency and the ability of *Desmodesmus communis* to synthesize the fatty acids, as also observed by White et al. (2011) using the microalgae *Chlorella* sp. As represented in Fig. 4.25b, the most diluted culture showed the lower Fv/Fm ratio and the highest content in TFAs; this result probably means that not only the nutrient deficiency but also the light intensity has an effect on both physiological process.

In microalgae, exposure to high light intensity triggers the operation of energy dissipating processes that include the dissipation of energy in terms of heat, known as the non photochemical quenching (NPQ) process. The NPQ measurements maintained low values closed to zero during the whole I Phase, meaning no presence of photosynthetic stress, unlike what was observed during the II Phase where these values increased (Fig. 4.26a,b). One day after the dilution of the cultures and the increase in the light intensity the NPQ parameter increased from almost zero of the I Phase to almost 0.7 in the culture diluted at

50% and to 1.1 in the culture diluted at 82%; this increment was probably due to the photo - stress of the cells.

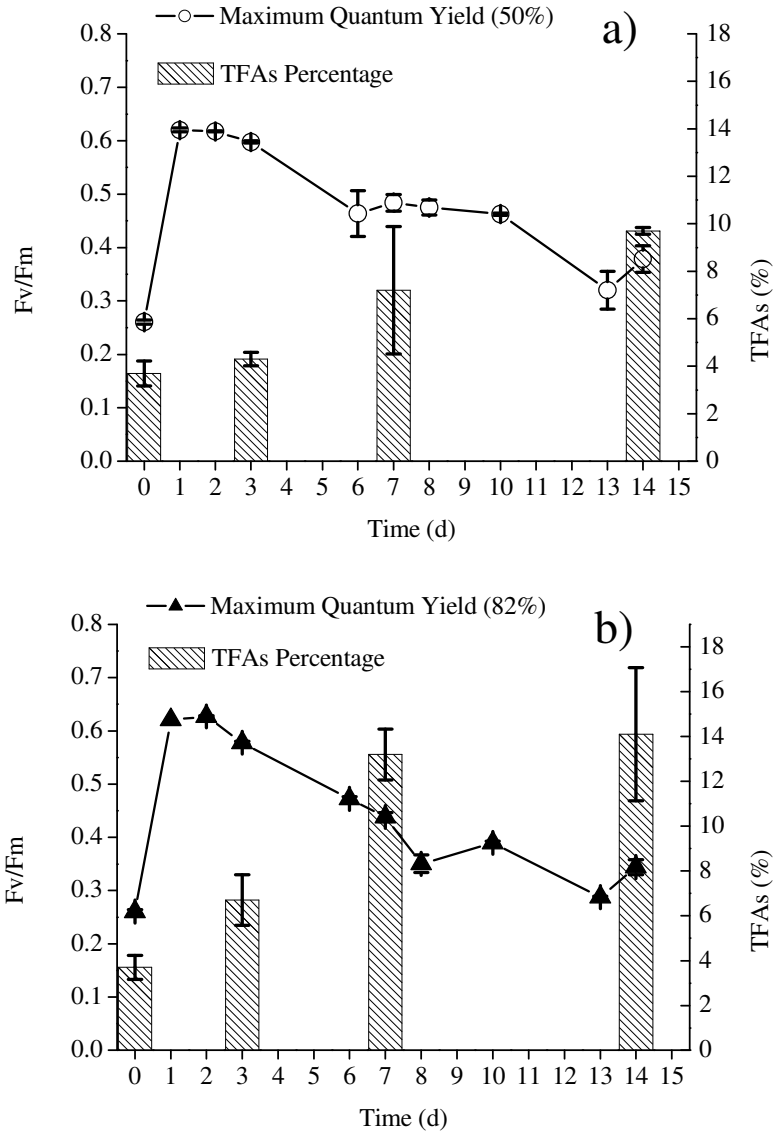

Fig. 4.25 Maximum quantum yield (Fv/Fm) recorded during the II Phase in *Desmodesmus communis* culture at both dilution conditions, 50% (a) and 82% (b), and the corresponding cellular total fatty acids (TFAs) yields as percentage over the total dry biomass.

As soon as the NPQ values dropped after one week from the beginning of Phase II, the TFA content over dry biomass increased (Fig. 4.26a,b). In accordance with White et al. (2011), who observed similar responses of the same physiological parameters derived

from PAM without representing the NPQ trend over the batch cycle, the high values of the NPQ during the II Phase, values which have been determined in this study during the whole growth curve, may firstly be justified as a photo - adaptation process that involves the functioning of the xanthophyll cycle. On the other hand observing the trend of TFA content and NPQ in Fig. 4.26a,b, the drop of NPQ values after one week of nutrient deficiency condition and high light exposure may be due to the prevalence, over the heat dissipation process, of the physiological process that synthesized fatty acid molecules by the utilization of the electrons that were accumulated in the photosynthetic electron transport chain.

Correlating all the PAM information *Desmodesmus communis* increased its lipid content, especially the TFA fraction, when the algal culture was under high stress condition moreover, as the kinetic curves shown, when the electron flux in the photosynthetic electron transport chain was almost nonexistent.

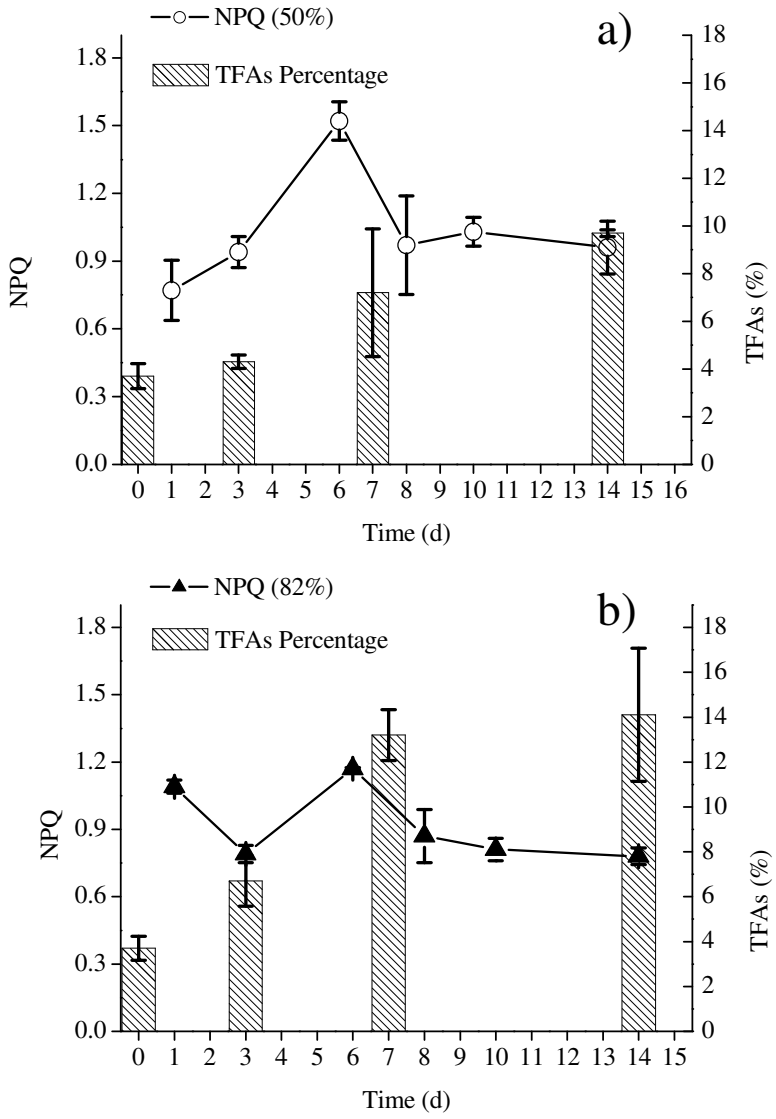

Fig. 4.26 Non photochemical quenching (NPQ) recorded during the II Phase in *Desmodesmus communis* culture at both dilution conditions, 50% (a) and 82% (b), and the corresponding cellular total fatty acids (TFAs) yields as percentage over the total dry biomass.

Rapid light curves

The physiological stress, induced by the nutrient deficiency and the light intensity, was also recorded using parameters such as relative electron transport rate (rETR) and its maximum ($rETR_{max}$) as a function of time, derived from the rapid light curves analysis using a PAM Fluorometer. rETR, which is an approximation of the rate of electrons pumped through the photosynthetic chain (Saroussi and Beer, 2007) as a function of PAR irradiance is shown in Fig. 4.27. During the exponential growth phase *Desmodesmus*

communis cultures in each condition showed a similar trend and values of rETR as a function of PAR, except for the early photo - inhibition status which was reached at the same PAR value of almost 600 µmol m^{-2} s^{-1}, while the culture diluted at 50% of the II Phase maintained the light - saturated condition until 1500 µmol m^{-2} s^{-1}. The slope of the curve in the light - limiting region (α), which is proportional to the efficiency of light capture, was similar at the beginning of the each phase, but decreased rapidly during the II Phase while it was 2-3 times higher during the whole I Phase (Fig. 4.28). As the fit curves shows, (Fig. 4.27b, d, e) during the stationary phase the culture in the I Phase showed a higher slope of the curve, as also confirmed by the α graph, and reached higher values of rETR compared with the fit curves obtained for the cultures in the II Phase. The $rETR_{max}$ trend, represented in Fig. 4.29, was similar to the α one; thus the culture in the I Phase maintained a good capacity in the electron transport chain while in the II Phase the photosynthesis was strongly limited by a low $rETR_{max}$. This result confirms the previous hypothesis made on the induction curves, that there was an accumulation of electrons in the photosynthetic electron transport chain due to the high stress conditions of the cultures in the II Phase which may justify the increase of non - polar lipid, especially of TFAs such as C18.

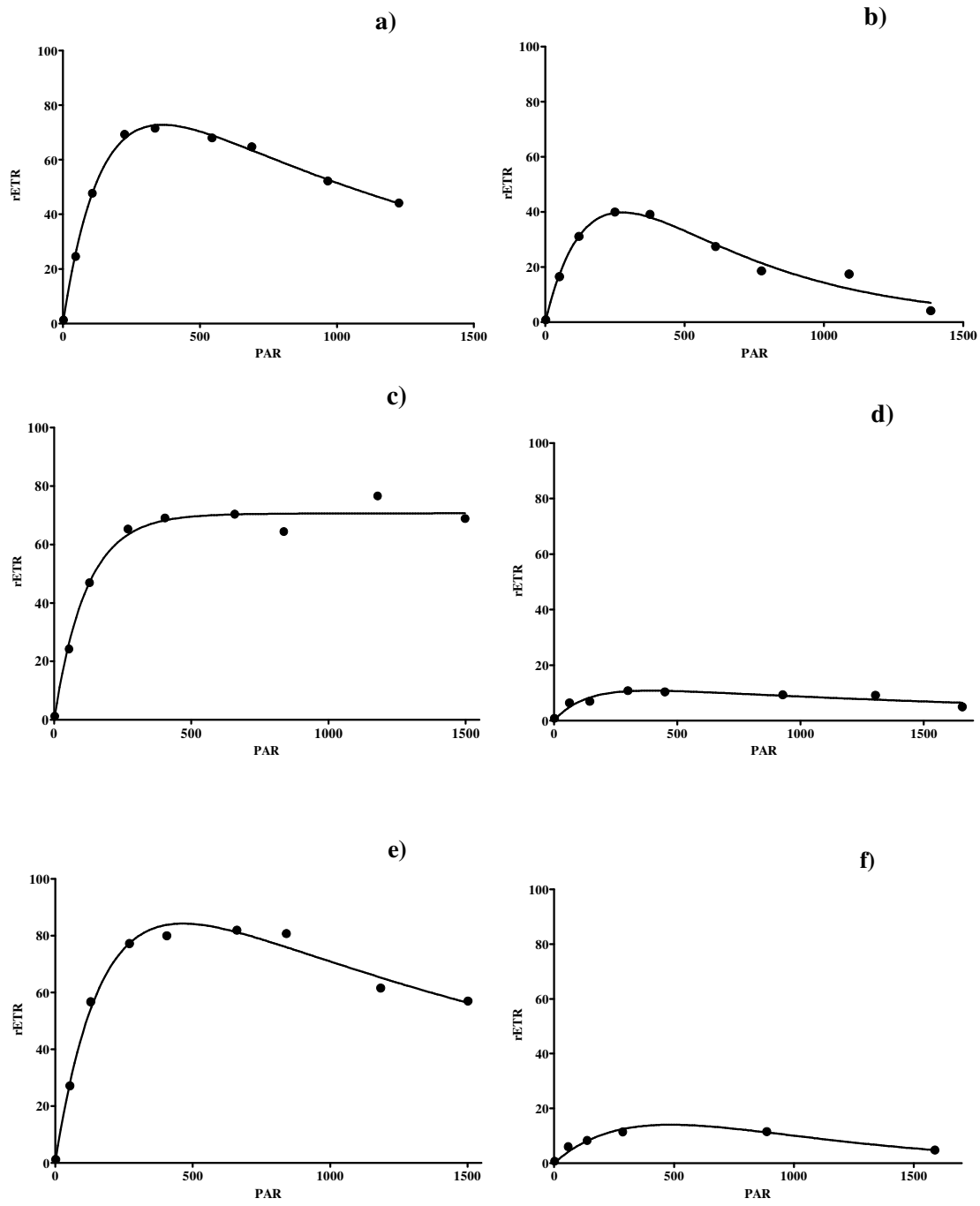

Fig. 4.27 Rapid light curves of *Desmodesmus communis* culture measured: measured: a) in the exponential phase of the I Phase; b) in the stationary phase of the I Phase; c) in the exponential phase of the II Phase of the culture diluted at 50%; d) in the stationary phase of the II Phase of the culture diluted at 50%; e) in the exponential phase of the II Phase of the culture diluted at 82%; f) in the stationary phase of the II Phase of the culture diluted at 82%.

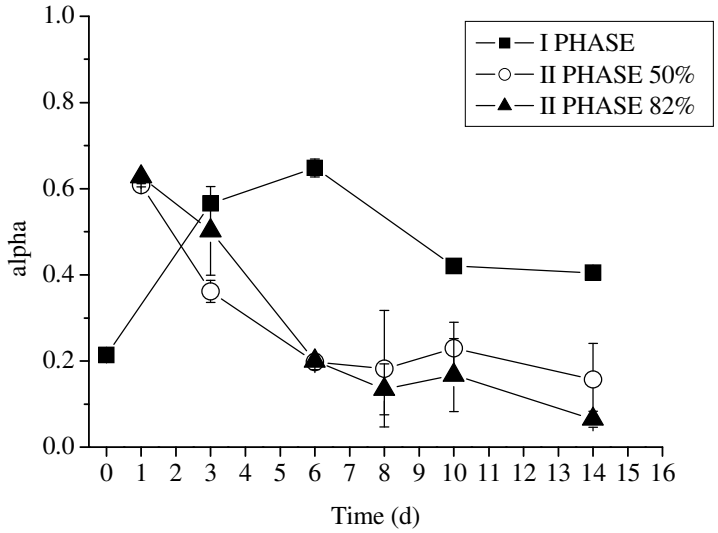

Fig. 4.28 Variation of fluorescence parameter values α as a function of time in *Desmodesmus communis* cultures in both phases.

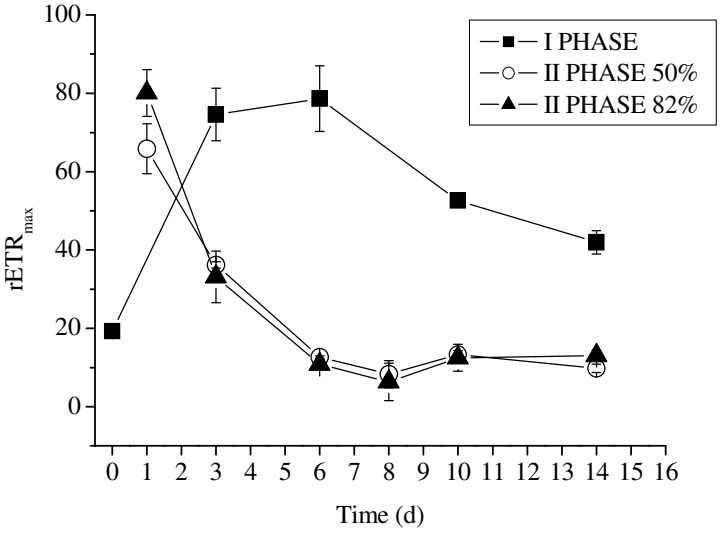

Fig. 4.29 Variation of fluorescence parameter values $rETR_{max}$ as a function of time in *Desmodesmus communis* cultures in both phases.

4.3.4 Conclusions

Desmodesmus communis which is one of the photosynthetic microorganisms frequently used in laboratory studies because it can easily be isolated and cultivated has never been selected for optimal lipid production. Many algal species are able to increase the lipid content when cells are subjected to stress conditions, such as photo - oxidative stress or nutrient starvation. This work demonstrated that the autochthonous algal strain *Desmodesmus communis* under complete N deprivation was able to double the lipid content and moreover increasing the percentage of the non - polar lipid content. The TFA content reached a final percentage over dry biomass of 13.2%, three times higher than the typical TFA content observed in *Desmodesmus communis* in nutrient sufficient conditions. The lipid increase is usually associated with growth cessation, in the present study it occurred under N deprivation coupled with light increment which seemed to stimulate the growth rate. The algal biomass increased during the stress phase as well as it did during the nutrient sufficient phase at low light. In another work (see section 4.2) it was demonstrated how this algal strain was able to grow at light intensity of 440 $\mu E\ m^{-2}\ s^{-1}$, which is higher than the light irradiance of the II Phase, reaching high growth rate values. On the other hand, both the induction curves and the light curves showed that *Desmodesmus communis* photosynthetic apparatus worked better at low light intensity (88 $\mu E\ m^{-2}\ s^{-1}$) without any heat dissipation even during the stationary phase. As the light intensity increased the photosynthetic process became less efficient, inducing from the beginning heat dissipation but also restarting the cellular growth. As the nutrients were completely depleted the photosynthetic process reduced considerably the maximum quantum yield, no electron flux were evident in the photosynthetic electron transport chain and the TFA content started to increase significantly. *Desmodesmus communis* showed rapid growth , great biomass productivity, easy adaptation at different media and culture conditions, good competitive performance with other algal species and resistance to zooplankton grazing; moreover in the present work *Desmodesmus communis* showed positive response in terms of non - polar lipids and TFA increment under stress condition. This last result confirm its potential application in the renewable energy field.

4.4 Algae growth and nitrogen removal in small scale semi - continuous *Desmodesmus communis* culture in view of urban wastewater treatment

4.4.1 Introduction

Industrialized countries produce a great volume of urban and industrial wastewater. These effluents should not be drained into superficial water bodies before treatment to reduce contaminants to environmentally safe levels. Microalgae can play an important role in the wastewater treatment absorbing excess nutrients; releasing oxygen through the photosynthetic process which is used by the bacterial community involved in the organic matter oxidation; producing a biomass which can be transformed in renewable energy form; removing heavy metals and xenobiotic substances.
As observed in the previous studies, the autochthonous green microalgae *Desmodesmus communis* has shown extraordinary vitality in urban wastewater, registering growth rates similar to those reported for a completely synthetic medium. This freshwater algae tolerates a wide range of N/P ratio and high ammonia levels (see section 4.2).

In the present work, the nitrogen removal efficiency of *Desmodesmus communis* from the primary effluent of the wastewater reclamation facility (WRF) of Cesena (Italy) has been studied in a laboratory scale under different hydraulic retention times (HRTs): 1.5-, 3- and 5-days which are much shorter than those typically used in waste stabilization ponds (Oswald, 1995).
The aim of this study was to determine the influence of the time of permanence of the primary effluent collected from the WRF in the culture system on the removal of nitrogen and on the biomass productivity. In addition, to forecast the future application of the biomass generated during the wastewater treatment, elemental and biochemical analyses were performed, especially on the resulting compounds of commercial interest: proteins, polysaccharides, total fatty acids (TFAs) and C/N ratio.

4.4.2 Overview of experiments

Three sets of conditions with different HRTs were run in parallel in semi - continuous monoculture of *Desmodesmus communis*

All three experiments started as a batch culture condition, without any nutrient supply until the beginning of the stationary phase. As soon as the cultures stably reached the stationary phase they were switched under the semi - continuous operation for 7 days to study the effects of HRTs on algae growth and nutrient removal.

The culture volume in each bottle was 900 mL at the end of the batch phase. Wastewater was introduced into the bottles daily at the end of the dark period and the total volume was maintained constant until the end of the experiment. Three hydraulic loading rates were tested, 600, 300 and 180 mL of primary effluent, in order to achieve 5-, 3- and 1.5-day HRTs, respectively. Each HRT condition was run in duplicate. From the beginning of the batch phase condition the culture media were bubbled with a mixture of air and 2% CO_2, under light intensity of 440 μE m^{-2} s^{-1}. As previous studies have demonstrated CO_2 supplementation and high light intensity represent the best conditions to increase the biomass productivity (see section 4.2).

The systems' setting, temperature, light/dark cycle, and air - CO_2 flow were set as described in section 4.1.1.

4.4.3 Results and Discussion

Growth under semi - continuous culture conditions

The batch culture of *Desmodesmus communis* growing in the primary effluent of the WFR of Cesena reached the stationary phase in 7 days with a biomass concentration of 0.923 ± 0.030 g L^{-1} (VSS). At this stage the cultures were operated in a semi - continuous mode for 7 days as showed in Fig. 4.30.

The semi - continuous cultures nearly reached the steady - state biomass concentration after 4 days of daily feed operation (day 11) with VSS values of 0.20 ± 0.01, 0.44 ± 0.03 and 0.74 ± 0.02 g L^{-1} at 1.5-, 3- and 5-day of HRT conditions, respectively. Despite the

different treatment the biomass productivity achieved a value of 0.14 ± 0.01 g L^{-1} d^{-1} in all conditions.

The VSS results conformed to a previous work of Woertz et al. (2009) which studied HRTs of 2-, 3 and 4-days in a mixed population culture on municipal wastewater for nutrient removal and lipid production.

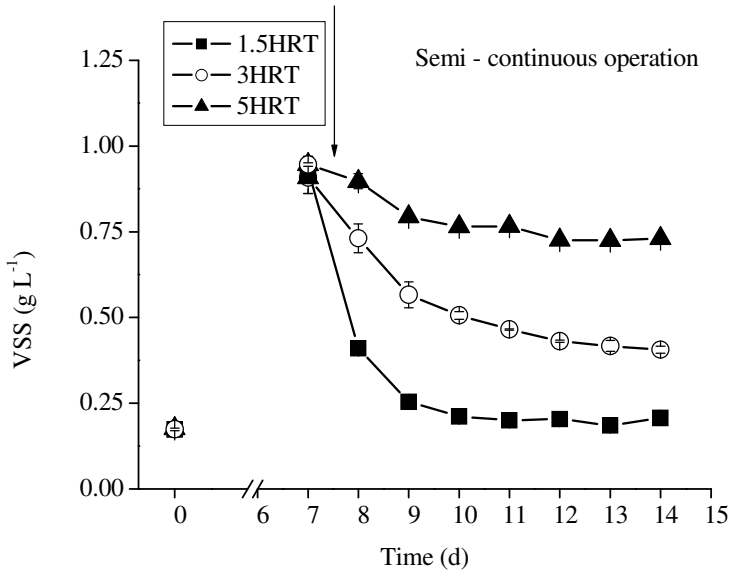

Fig. 4.30 Biomass concentrations at the beginning and the end of the batch phase (days 0 and 7) followed by the biomass concentrations during semi - continuous flow treatment of municipal wastewater. The arrow indicates the beginning of dilutions.

Ideally, sunlight is the main limiting "nutrient" parameter in algae production, with all other nutrients provided in abundance (C) or just at sufficiency (N, P). The typical depth of high rate ponds (0.3 m) dilutes sunlight to an extent that only 0.2 - 0.3 g L^{-1} of algal volatile suspended solids (VSS) can be sustained (Sheehan et al., 1998). These algal concentrations are high compared to waste stabilization ponds growing green (chlorophyte) algae, however a 0.2 - 0.3 g L^{-1} value is low compared to the biomass concentrations in activated sludge. This low biomass concentration contributes to the need for an extended hydraulic residence time in high rate ponds (3-5 days) compared to hours needed for activated sludge. However in a laboratory scale experiment using 1 L

bottles the light showed to be less limiting and cultures in 3- and 5-day HRT conditions reached a biomass concentration 2 to 3-fold higher than 0.2 - 0.3 g L^{-1}. No light limitation, due to the high cell density in the culture, seems to have occurred as all three cultures achieved the same final biomass productivity.

The effects of different HRT conditions on the biochemical composition of the algal biomass

Fig. 4.31 represents the biochemical composition of *Desmodesmus communis* biomass expressed as a percentage over dry biomass (VSS) and measured in the three different HRTs (5-, 3- and 1.5-days) conditions at the end of the semi - continuous phase. As shown before, the culture of *Desmodesmus communis* treating the primary effluent at different HRTs achieved a common value in terms of biomass productivity of 0.14 g L^{-1} d^{-1}, nevertheless the biochemical composition of the algal biomass obtained for each culture condition changed in terms of percentage of polysaccharides, proteins, TFA and chlorophyll *a* content.

Specifically, the amount of proteins inside the cells increased as the residence time of the untreated wastewater in the system decreased; thus the percentage of protein over dry biomass obtained from the culture at 1.5-day of HRT (26.9%) is significantly higher than the percentage obtained for the culture at 5-day of HRT (14.0%) ($p < 0.05$, ANOVA).

The polysaccharides percentage over dry biomass increased significantly from a minimum value in the culture characterized by the lowest HRT to a maximum value in the culture characterized by the highest HRT ($p < 0.05$, ANOVA), achieving 16.9, 26.6 and 57.2 % in the cultures with 1.5-, 3- and 5-day HRTs respectively.

The total fatty acid content didn't show any change among the conditions, the values measured as percentage over dry biomass were 2, 3.5 and 3% in the cultures with 1.5-, 3- and 5-day HRTs, respectively, and were similar to the TFA values obtained in the previous study using *Desmodesmus communis* grown in a batch culture with the primary effluent as culture medium (see section 4.2).

The chlorophyll *a* percentage showed significant differences between cultures with 3 and 5-day of HRTs and between cultures with 1.5- and 3-day of HRT ($p < 0.05$, ANOVA). No differences were evidenced between cultures with 1.5- and 5-day of HRT which

achieved values of 8.9 and 7.2 % respectively, while the culture at 3-day of HRT reached the maximum value of 11.2%.

The polysaccharides percentage over dry biomass measured daily during the semi - continuous operation treatment (Fig. 4.32) didn't show any change in the culture at 5-days of HRT which maintained a range of 56-57%. On the other hand the polysaccharides percentage showed a substantial decreasing trend in the culture at 1.5-day HRT dropping from an initial value of 56.3% to 38.8% and then to 24.8% in 24h and 72h, respectively; it reached a stable value of almost 17% at day 4. In the biomass obtained from the 3-day HRT culture condition the polysaccharides percentage slowly decreased until a value of 25-26% at the end of the experiment.

The protein percentage over dry biomass (Fig. 4.33) showed an inverse trend compared with the polysaccharides one; this percentage increased rapidly in the culture treated with 1.5-day of HRT in only 48h, reaching a value of almost 30% which remained constant until the end of the experiment. 3-day HRT operation conditions induced again a slow change in the protein percentage content which resulted in a gradual increase from 8.6 to 23.6%. On the other hand the biomass obtained at 5-day HRT reached a maximum value of protein percentage of 12/13% in 72h which remained stable until the end of the experiment. These changes in the proteins and polysaccharides percentage are clearly dependent on the nutrient abundance inside the culture, the lower is the HRT applied the higher the nutrient supply is into the culture. High concentrations of nitrogen resulted in a production of a biomass rich in protein and lacking in polysaccharides as obtained in the 1.5-day of HRT condition.

The variation in the nutrient intake didn't show any effects on the TFA percentage (Fig. 4.34) unlike what observed for the polysaccharides and proteins content.

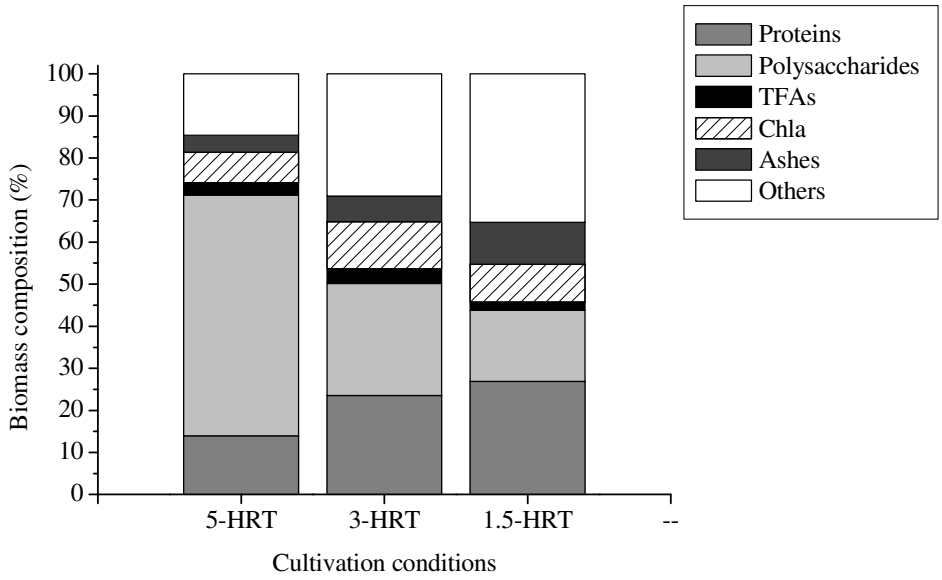

Fig. 4.31 Biochemical composition in terms of percentage over dry algal biomass obtained at the end of the semi - continuous treatment at 5-, 3- and 1.5-day of HRT.

The high production of chlorophyll *a* is usually related to an adaptation to the self - shading effect which could be present in a more concentrated culture as in the 5-day HRT condition. The high chlorophyll *a* content is also related to the growing energy requirement (light) for higher production of biochemical compounds like proteins (Ruiz-Marin et al., 2010). However, in this study the chlorophyll *a* like the protein content kept a lower level in the 5-day HRT condition probably as a consequence of the nitrogen limitation which did not occur in the 3-day HRT condition which showed the highest content of chlorophyll *a* (Fig. 4.35).

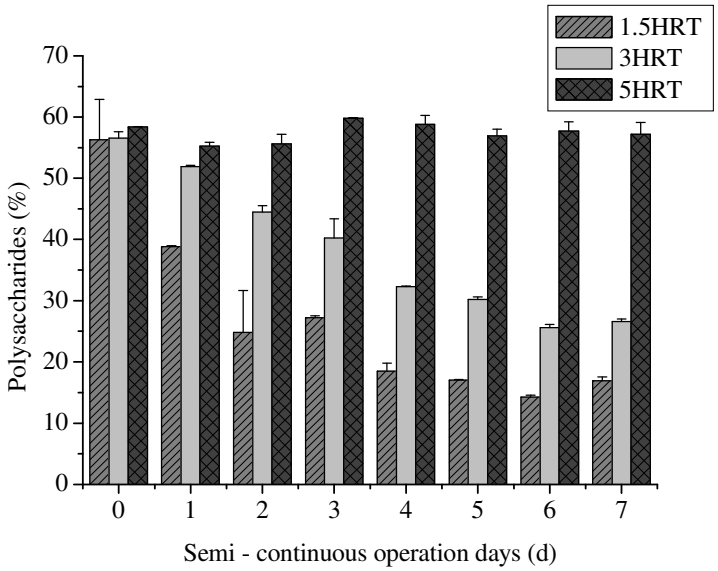

Fig. 4.32 Polysaccharides percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

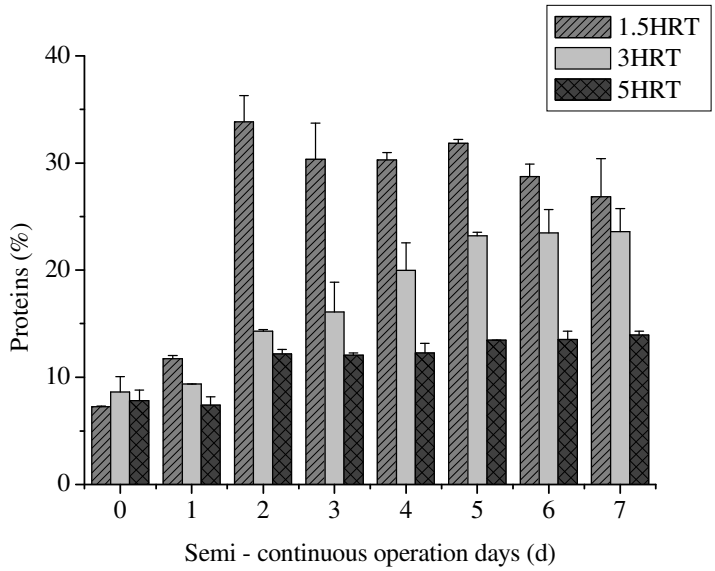

Fig. 4.33 Proteins percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

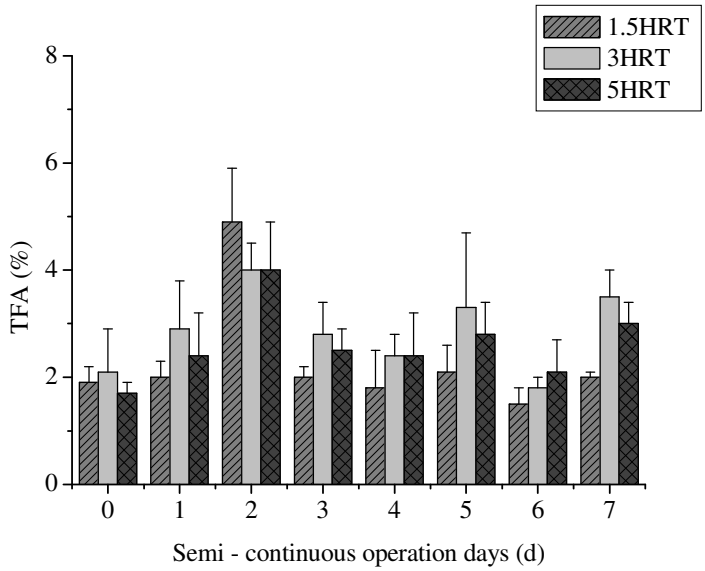

Fig. 4.34 Total fatty acids (TFAs) percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

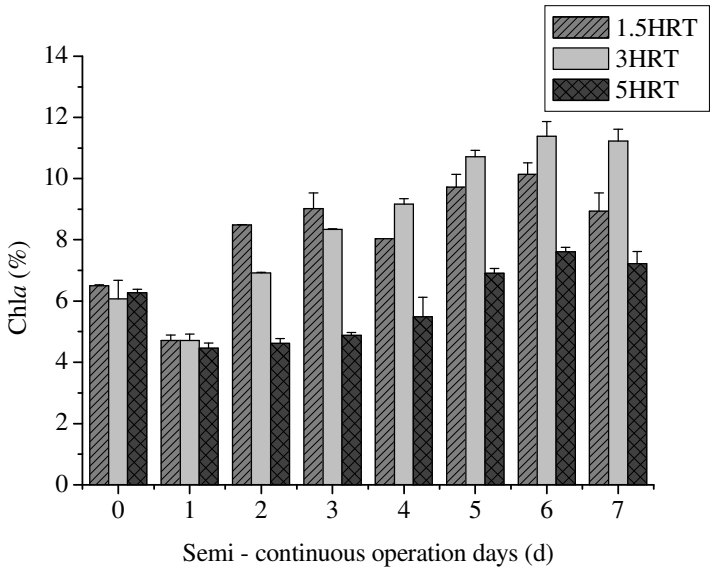

Fig. 4.35 Chlorophyll *a* (Chl*a*) percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

Fig. 4.36 shows the absolute concentrations of each compound, in terms of mg per liter, that is possible to extract from the algal culture at different HRT conditions. As the semi - continuous treatment reached a stable trend in the biomass production it was possible to observe a proteins productivity of 19.5, 33.0 and 37.6 mg L^{-1} d^{-1} at 5-, 3- and 1.5-day HRT, respectively; a polysaccharides productivity of 80.1, 37.2 and 23.7 mg L^{-1} d^{-1} at 5-, 3- and 1.5-day HRT, respectively; a TFAs productivity of 4.2, 4.9 and 2.8 mg L^{-1} d^{-1} at 5-, 3- and 1.5-day HRT, respectively, as resumed in Tab. 4.10.

Condition	**Biomass (mg L^{-1} d^{-1})**	**Polysaccharides (mg L^{-1} d^{-1})**	**Proteins (mg L^{-1} d^{-1})**	**TFA (mg L^{-1} d^{-1})**
5 HRT	140.0	80.1	19.5	4.2
3 HRT	140.0	37.2	33.0	4.9
1.5 HRT	140.0	23.7	37.6	2.8

Tab. 4.10 Productivity of biomass and resulting compounds of commercial interest such as polysaccharides, proteins and total fatty acids (TFAs) of *Desmodesmus communis* cultures during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

The biochemical composition analyses were supported by the elemental analysis of carbon (C) and nitrogen (N) in the biomass. The C percentage over dry biomass didn't show any difference between the different HRT culture conditions, probably due to the constant CO_2 supply which avoids the carbon limitation condition and also due to a good performance of the cells to fix the inorganic carbon at all HRTs (Fig. 4.37). On the other hand, the different HRT conditions allowed to obtain a biomass with a N per cent content of 8-10%, 6-8% and 4-5% for 1.5-, 3- and 5-day HRTs, respectively. The N percentage over dry biomass showed a similar trend to the protein one (Fig. 4.38). This result was also strengthened by the correlation between the protein and the nitrogen percentage (Fig. 4.39) which may allow the elemental analysis instead of the proteins' determination to be used in future studies.

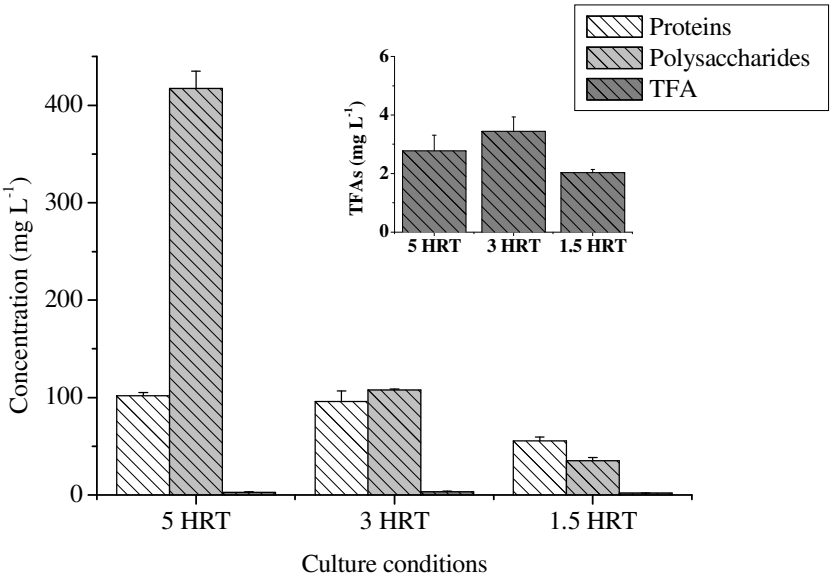

Fig. 4.36 Concentrations of each compound of commercial interest such as polysaccharides, proteins and total fatty acids (TFAs) resulting from the *Desmodesmus communis* cultures during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

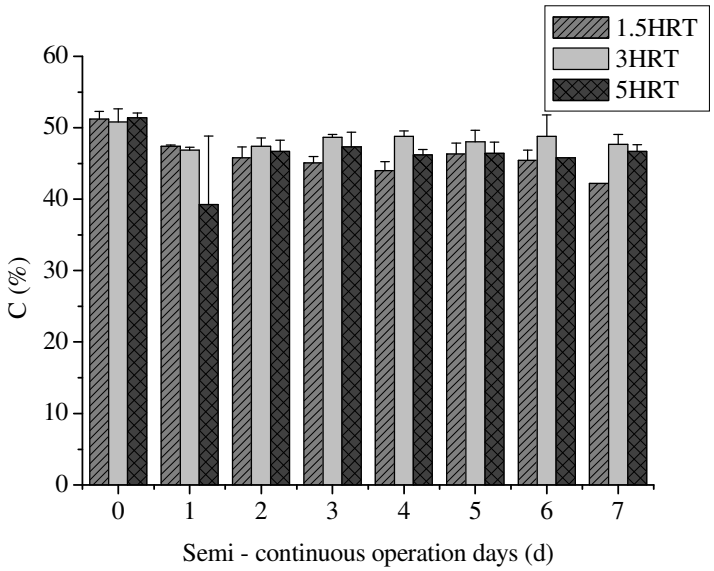

Fig. 4.37 Carbon percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

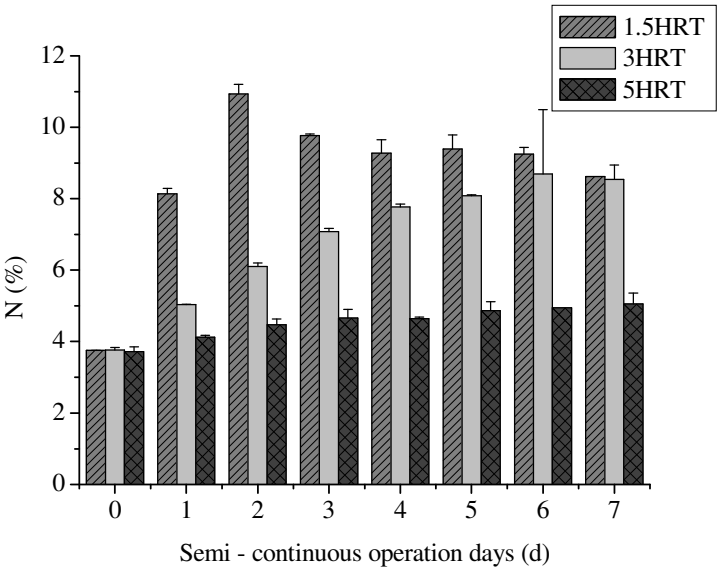

Fig. 4.38 Nitrogen percentage over dry biomass during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

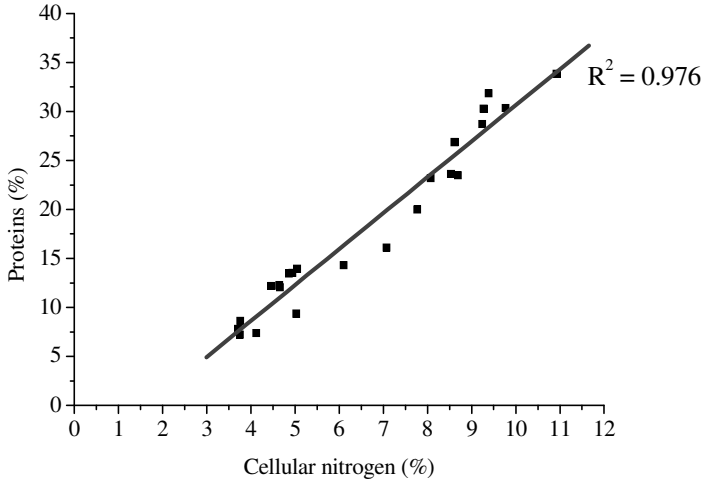

Fig. 4.39 Linear correlation between cellular nitrogen and protein content express in terms of percentage over dry biomass obtained during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

Some further information obtained from the elemental analysis is the C/N ratio values which are shown in Fig. 4.40. During the semi - continuous operation phase the C content didn't change while the N content increased, especially in the lowest HRT condition (1.5 days), resulting in a decrease of the C/N ratio from 15.9 to 10.8, 5.7 and 6.5 in 5-, 3- and 1.5-day of HRT condition, respectively. The C/N ratio obtained in the 1.5-day of HRT condition is much lower compared with the ratio obtained in the previous study regarding *Desmodesmus communis* grown in a batch culture using the same wastewater effluent and similar culture condition (see section 4.2), moreover this ratio seems also to be underestimated compared with the typical C/N value of 10 obtained for an algal biomass (Sialve et al., 2009).

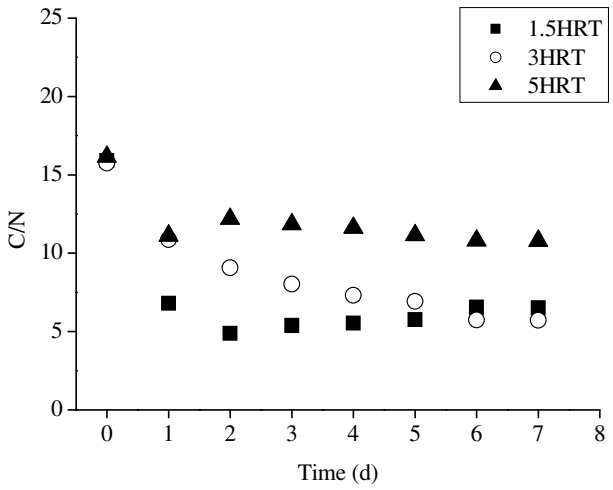

Fig. 4.40 Cellular carbon and nitrogen ratio (by atoms) trend during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

The effects of N/P ratio on nutrient removal

The wastewater characteristics of the influent were daily monitored before the dilution and the average values are reported in Tab. 4.11.

Wastewater characteristic	
NO_3-N (mg/L)	0.08 ± 0.03
NO_2-N (mg/L)	0.02 ± 0.01
NH_3-N (mg/L)	32.39 ± 1.05
P (mg/L)	2.39 ± 0.67
N/P	30:1
TSS (mg/L)	123 ± 22
VSS (mg/L)	68 ± 12

Tab. 4.11 Initial wastewater characteristic.

As shown in Fig. 4.41, over 99% ammonia and phosphate removal was achieved for treatments with both 3- and 5-day HRT (Tab. 4.12). The treatment with 1.5-day HRT achieved 99% phosphate removal but it was not so efficient in the ammonia reduction; the effluent going out from this system reached a concentration of 17.05 ± 0.92 mg L^{-1} NH_3-N which is higher than the maximum level of 14 mg L^{-1} according to the Italian law (DLgs 152/2006).

	NH_3-N (mg L^{-1})			P (mg L^{-1})		
	Influent	**Effluent**	**% Removal**	**Influent**	**Effluent**	**% Removal**
5 HRT	32.39 ± 1.05	0.27 ± 0.13	> 99%	2.39 ± 0.67	< 0.01	> 99%
3 HRT	32.39 ± 1.05	0.35 ± 0.02	99%	2.39 ± 0.67	< 0.01	> 99%
1.5 HRT	32.39 ± 1.05	17.05 ± 0.92	47%	2.39 ± 0.67	< 0.01	> 99%

Tab. 4.12 Nutrient removal during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

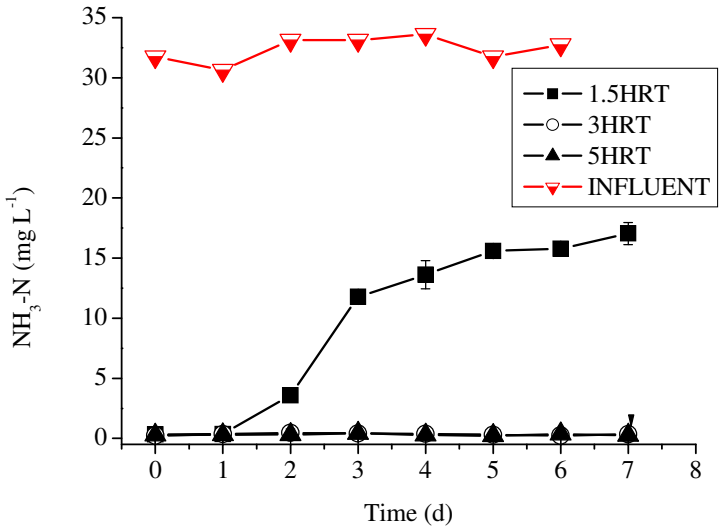

Fig. 4.41 Ammonia removal during the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

To determine the fate of the removed ammonia, a nitrogen balance was calculated each day since the culture reached the steady - state biomass concentration after 4 days of daily feed operation. The results were similar on all days and Fig. 4.42 shows the balance calculated with the data obtained on the last day of treatment.

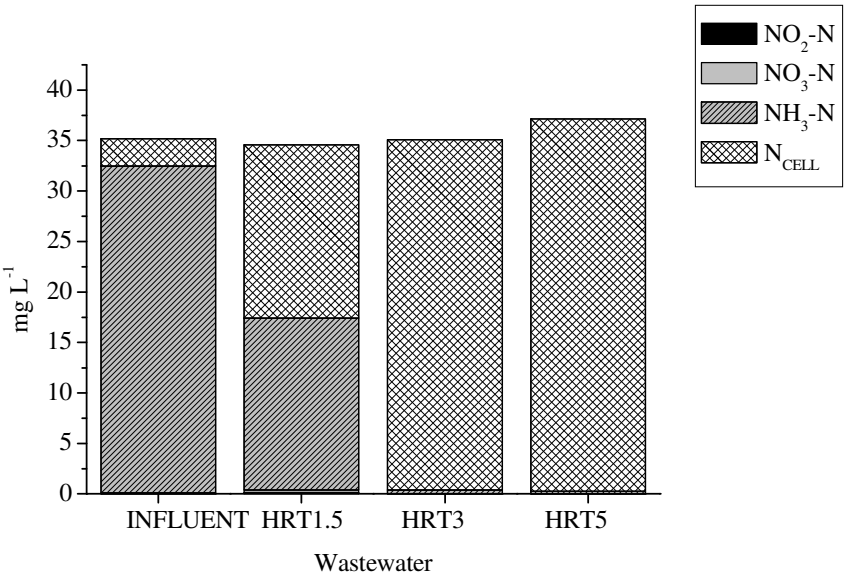

Fig. 4.42 Nitrogen balance for *Desmodesmus communis* cultures in municipal wastewater on day 7 of the semi - continuous flow treatment at 1.5-, 3- and 5-day of HRT.

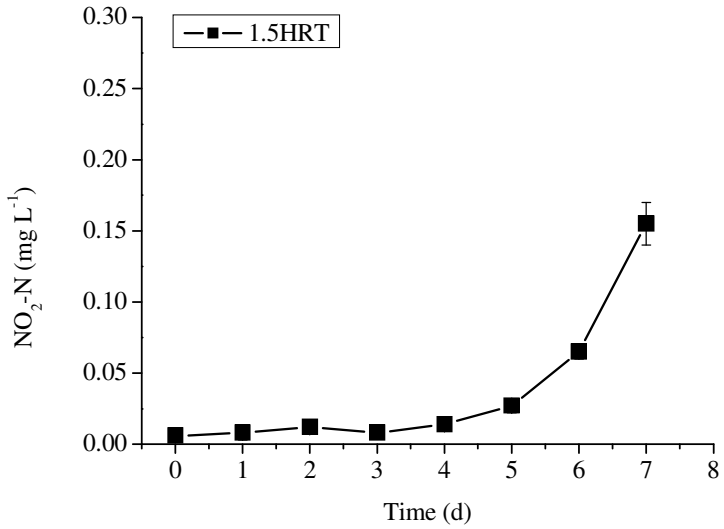

Fig. 4.43 Nitrogen as nitrite concentration trend in the 1.5.day HRT cultures during the semi - continuous flow treatment.

Ammonia was the main form of nitrogen in the influent wastewater, and after algal growth, cellular nitrogen was predominant in both 3- and 5-day HRTs effluents. In the 1.5-day of HRT condition the ammonia observed in the effluent confirmed the inefficiency of this short retention time. The increase of nitrite in 1.5-day HRT effluent indicated incomplete nitrification of ammonia for this condition (Fig. 4.43). Similar ammonium and phosphorus removal efficiency was observed by Woertz et al. (2009) at high HRTs. In that work they reached a higher efficiency in the ammonium removal process of 98% with 2-day HRT comparing with the removal percentage of 47% achieved in this study with 1.5-day HRT. On the other hand the removal of phosphate was > 99% at1.5-day HRT which was a better result compared with the removal of 93% obtained by Woertz et al. (2009).

Rapid light curves

The effect of the semi - continuous operation process on *Desmodesmus communis* culture was also studied in terms of photosynthetic responses using a PAM Fluorometer. Rapid light curves were measured every day during the semi - continuous operations, except on the last day, and the curve fit, which was very good ($r > 0.96$) in all cases, is shown in Fig. 4.44 and it is representative for the second last day of the treatment. Fig. 4.44 shows plots of the relative electron transport rate (rETR) which is an approximation of the rate of electrons pumped through the photosynthetic chain (Saroussi and Beer, 2007) as a function of PAR irradiance for 1.5-, 3- and 5-day of HRT culture conditions respectively. 3- and 5-days HRT culture showed elevated rETR at elevated irradiance, while 1.5-day HRT culture had a rETR of about 55-60% of the 3- and 5-day HRT rETRs. All the cultures reached a plateau in rETR at the same PAR irradiance of ca. 500 $\mu mol\ m^{-2}\ s^{-1}$. The slope of the light - limiting regions (α), which is proportional to the efficiency of light capture, was similar for 3- and 5-day HRT and lower for the 1.5-day HRT.

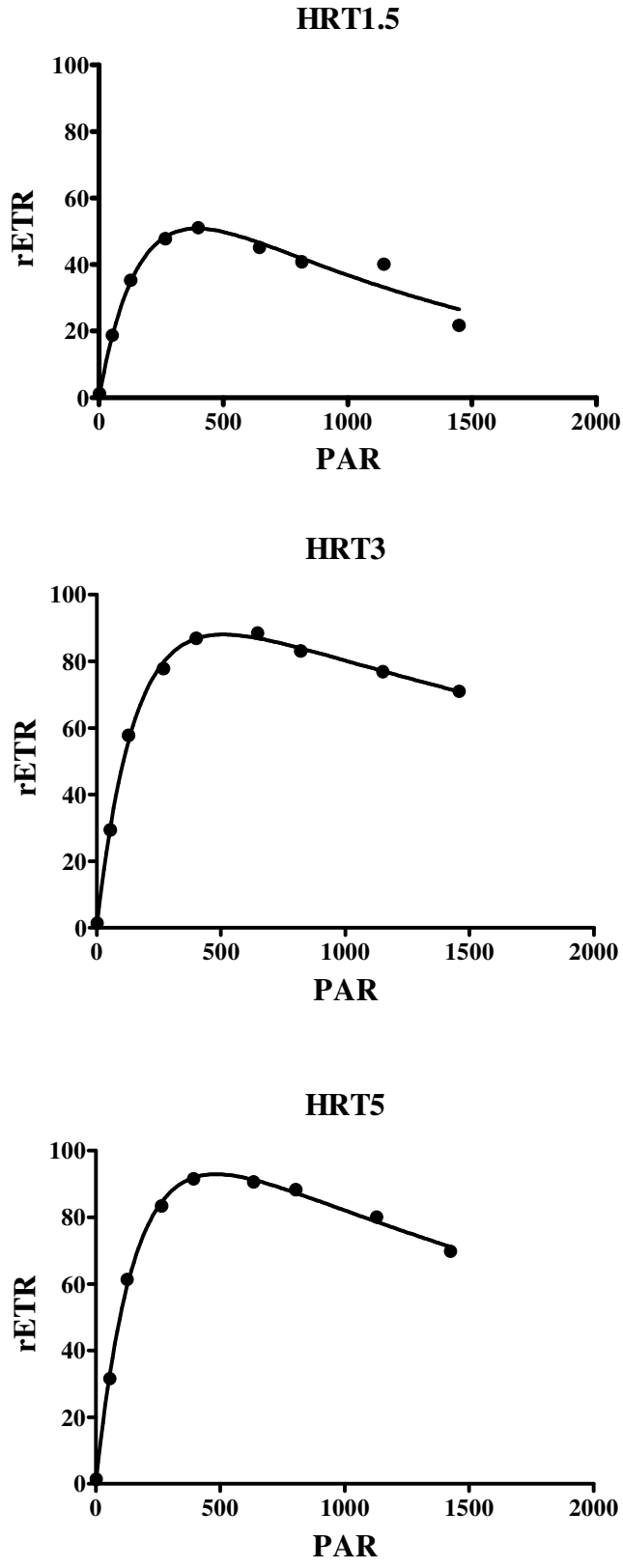

Fig. 4.44 Rapid light curves , on day 6, of *Desmodesmus communis* cultures using in the primary effluent treatment with 1.5-, 3- and 5-day of HRT.

According to Saroussi and Beer (2007), the maximum quantum yield was derived from the initial Y - value of the rapid light curve instead of deriving it from the initial slopes since the data are plotted against available irradiance instead of absorbed light (Fig. 4.45). No significant differences in the maximum quantum yield appeared between HRT treatments and it seems that no changes occurred during the semi - continuous dilution process from the beginning until the end of the experiment ($p < 0.05$, ANOVA). Comparing the trend of this parameter obtained in semi - continuous cultures with the one obtained in a batch cultures (see section 4.2) it appeared that in the semi - continuous culture conditions that maximum quantum yield maintained high levels probably due to non - stress nutrient conditions which didn't affect the photosynthetic apparatus.

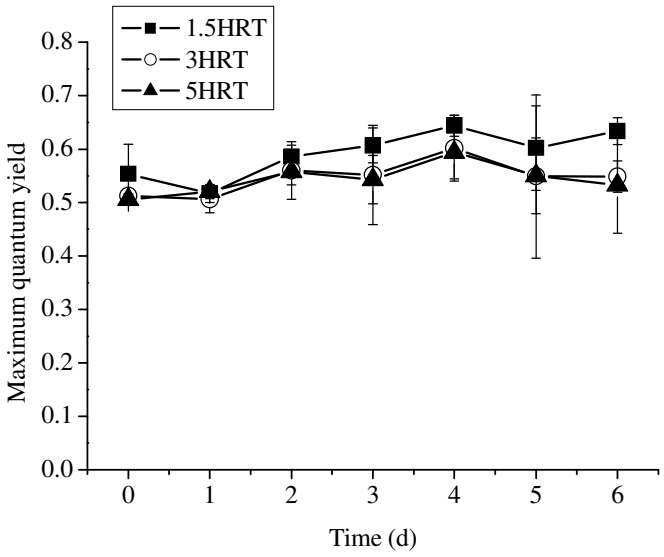

Fig. 4.45 Variation of the maximum quantum yield (derived from the rapid light curves) of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

To compare rapid light curves obtained for each HRT condition, they need to be described by several characteristic parameters such as initial slope (α), the photoinhibition parameter (β), the light - saturation parameter (E_k) and the maximum relative electron transport rate ($rETR_{max}$) (Sakshaug et al., 1997). Even if it was not possible to estimate an absorbing factor for the cultures, their optical densities were maintained similar by dilutions to permit the comparison of the parameters above.

The α values, represented in Fig. 4.46, didn't show any significant differences for the three conditions at the beginning of the semi - continuous operations ($p > 0.05$, ANOVA). The initial slope of the 1.5-day HRT condition didn't increase during the whole experiment while 3- and 5-day HRT α values showed a significant increment with respect to the 1.5-day HRT α values on the last days ($p < 0.05$, ANOVA). A possible hypothesis about the low values of α maintained in the 1.5-day HRT condition can be the high dilution rate; thus the culture was exposed to high intensity light which induced a photo - protection mechanism. An increase in the amount of photoprotective pigments (i.e. the xanthophyll cycle), that dissipate the absorbed energy as heat instead of transferring it to the photosynthetic reaction centers, results usually in a decreasing of α but also of the maximum quantum yield which didn't decrease at any treatment conditions of this study, as shown before. A similar trend was observed comparing the $rETR_{max}$ values (Fig. 4.47): no significant differences were observed between the three culture conditions at the beginning of the semi - continuous operations ($p > 0.05$, ANOVA), while the highest value were obtained in 3- and 5-day HRT cultures on the last days determining a significant difference with respect to the 1.5-day HRT condition ($p < 0.05$, ANOVA). No significant differences were shown between β parameters at each condition ($p > 0.05$, ANOVA) (Fig. 4.48). The light - saturation parameter E_k didn't exhibit any differences between treatments even if the E_k values for each HRT increased slightly as the semi - continuous operation started ($p > 0.05$, ANOVA) (Fig. 4.49).

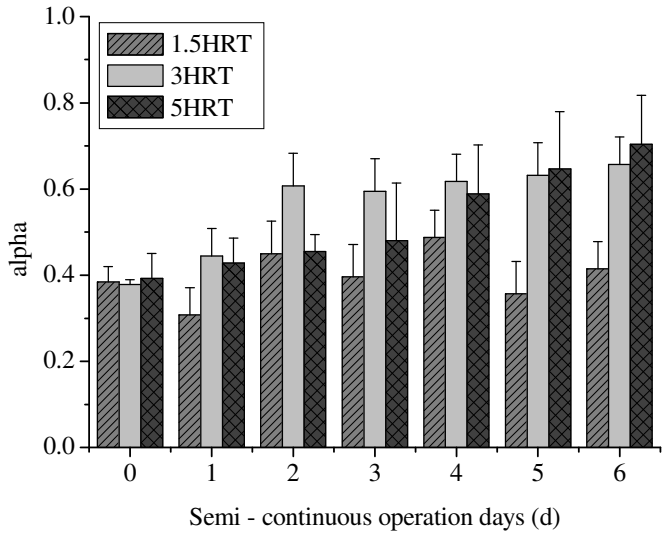

Fig. 4.46 Variation of fluorescence parameter values α of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

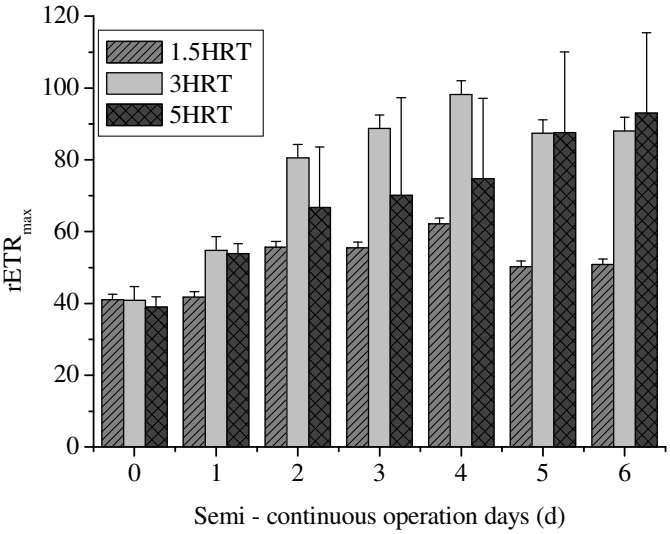

Fig. 4.47 Variation of fluorescence parameter values $rETR_{max}$ of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

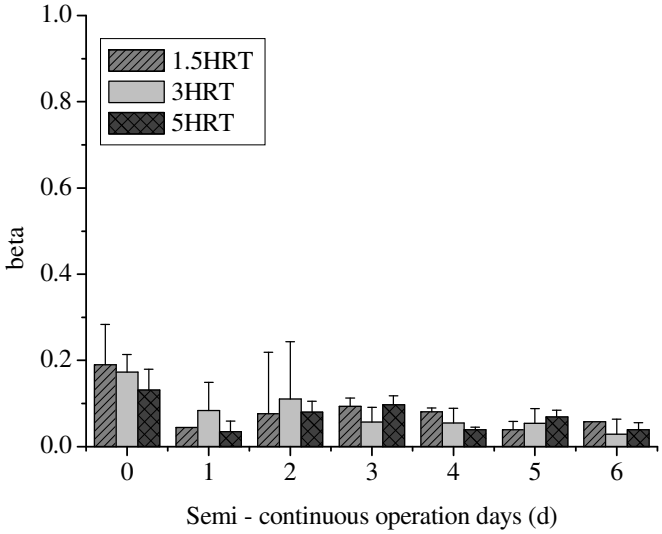

Fig. 4.48 Variation of fluorescence parameter values β of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

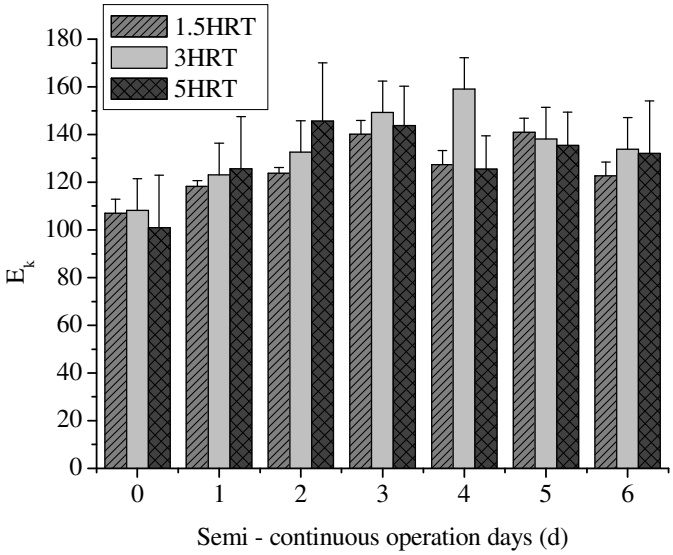

Fig. 4.49 Variation of fluorescence parameter values E_k of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

Induction curves

The induction curves were monitored every day from the beginning of the semi - continuous operation; since the curves trend didn't show any differences day by day, they were represented in Fig. 4.50 which shows fluorescence induction kinetics of *Desmodesmus communis* culture under 5-, 3- and 1.5-day HRT conditions, observed on the second last day of the experiment. The fluorescence responses of *Desmodesmus communis* under 3- and 5-day HRT conditions didn't show any relevant differences. The maximum levels of fluorescence (Fm) reached the highest value in the 3-day HRT culture and was lower by 8% and 18% in the 5- and 1.5-day HRT culture respectively. In the 1.5-day HRT culture, this lower Fm value compared to the other HRT cultures' Fm may indicate a partial inhibition of the PSII electron transport activity. The maximal fluorescence yield (Fm'), which was induced by the saturating pulse of light on the light - adapted culture, decreased compared to the Fm value by 13% in both 3- and 5-day HRT and by 15% in 1.5-day HRT condition.

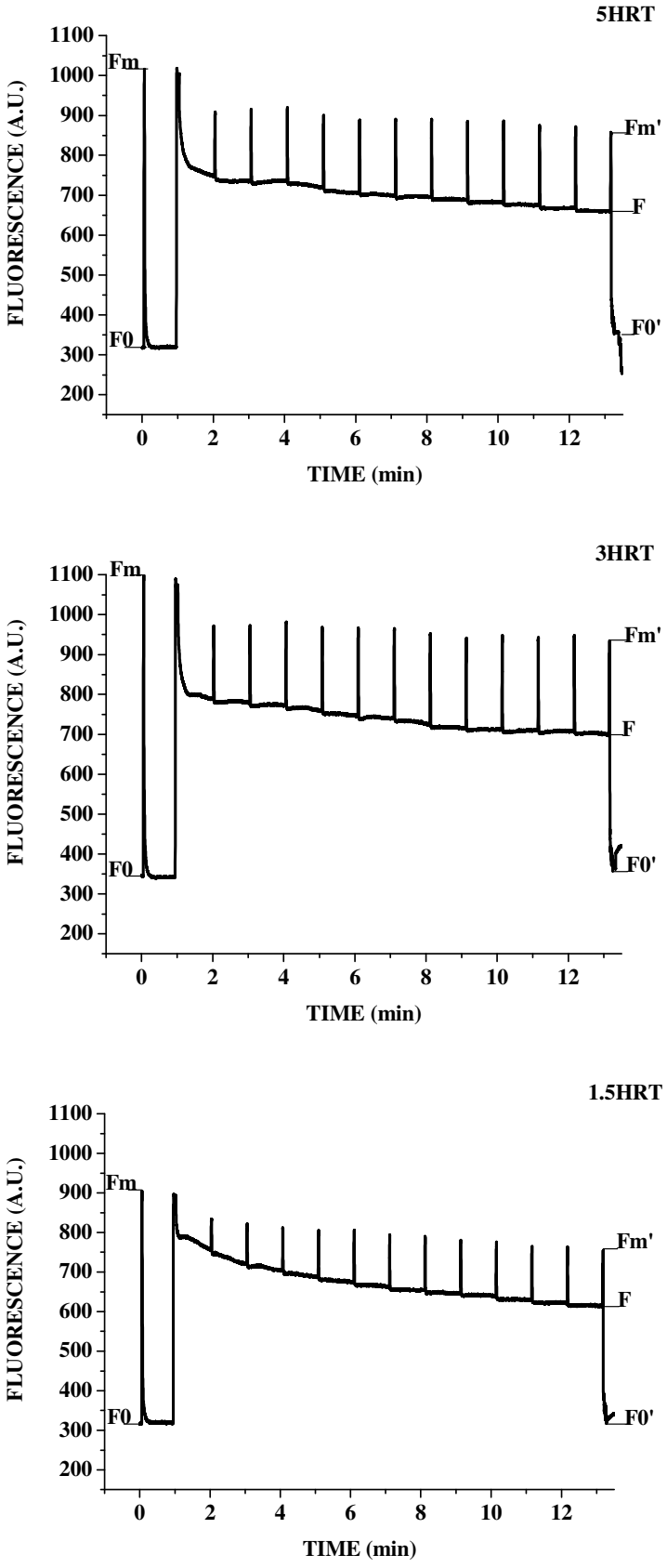

Fig. 4.50 PAM fluorescence induction kinetics of *Desmodesmus communis* on day 6 of the semi - continuous flow treatment of the primary effluent at 5-, 3- and 1.5-day of HRT.

Observing the value of the fluorescence yield (F0'), obtained when all PSII reaction centers are in a open state for a light adapted sample, the 1.5-day HRT culture showed a value which was lower by 10% compared with the one obtained for the 3-day HRT culture; while the F0' of the 5-day HRT culture differed from the one obtained for the 3-day HRT culture by only 3%. Data from the induction curves confirmed what was observed with the α and $rETR_{max}$ obtained in the rapid light curves and enforced the hypothesis about the reduced efficiency of the photosynthetic process in the 1.5-day HRT culture.

The maximum quantum yield of PSII (Φ_{PSII} or Fv/Fm) and effective quantum yield of PSII (Φ'_{PSII} or Fv'/Fm') are reported in Fig. 4.51, 4.52. The maximum quantum yield of PSII obtained through the induction curves showed some discrepancies with the one derived from the initial Y - value of the rapid light curve. No differences in the maximum quantum yield appeared between HRT treatments using the rapid light curve analysis, considering that on the last day the data were not detected, on the other hand as Fig. 4.51 shows, the maximum quantum yield at the end of the experiment is lower in the 1.5-day HRT culture confirming the previous hypothesis of a inhibition of the photosynthetic process probably due to the high light exposition, which was a consequence of a high rate dilution of the culture. A low efficiency in the photosynthetic process of the culture treated with 1.5-day of HRT was clearly observed through the effective quantum yield (Fig. 4.52) which showed lower values from the third day of the semi - continuous operation.

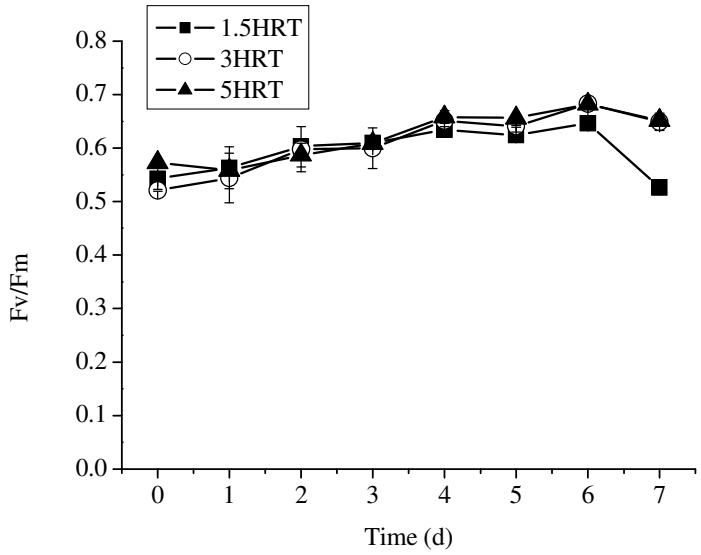

Fig. 4.51 Variation of the maximum quantum yield (derived from the induction curves) of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

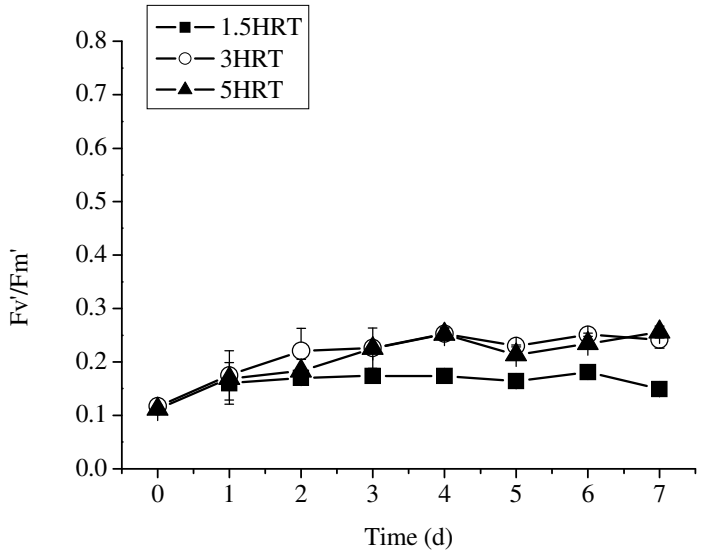

Fig. 4.52 Variation of the effective quantum yield of *Desmodesmus communis* during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

Photochemical (qP) and non - photochemical (NPQ) quenching parameters describe the relative influence of the energy dissipation pathways (Ralph and Gademann, 2005). The qP shows the proportion of light excitation energy "trapped" by open PSII reaction centers used for electron transport (Juneau et al., 2002). On the other hand, NPQ value represents the amount of light energy dissipation as heat, which is the energy not involved in the photochemical reactions.

As Fig. 4.53 shows, the qP values increased from the beginning of the semi - continuous operation, due mainly to the continuous nutrient supply and due also to the photoacclimation process; this parameter reached a stationary state at day 3. At this state, qP values resulted higher by 30% in the 3- and 5-day HRT cultures compared with the values characterizing the 1.5-day HRT culture. Again, in the low HRT condition the culture showed through the qP measurement a light saturation condition due to the high light exposure.

When the photochemical energy - consuming process is partially inhibited, dissipation of energy through nonphotochemical processes appears to be appropriate and should increase (Schreiber et al., 1986). Indeed, as Fig. 4.54 shows, the NPQ values slightly increased at the beginning of the experiment because as the dilution procedures began the incident light increased causing a response from the algal cells. No relevant differences appeared between all conditions ($p > 0.05$, ANOVA), except for day 5 in which 1.5-day HRT culture showed the highest NPQ value confirming the previous hypothesis about the photoinhibition in the culture treated at low HRT. The sudden decrease of NPQ values observed on the last day of the experiment can be consequence of an instrumental problem instead of a consequence of the culture adaptation, especially considering that all the other parameters (Φ_{PSII}, Φ_{PSII}', and qP) maintained a difference between 1.5-day HRT culture and the other cultures.

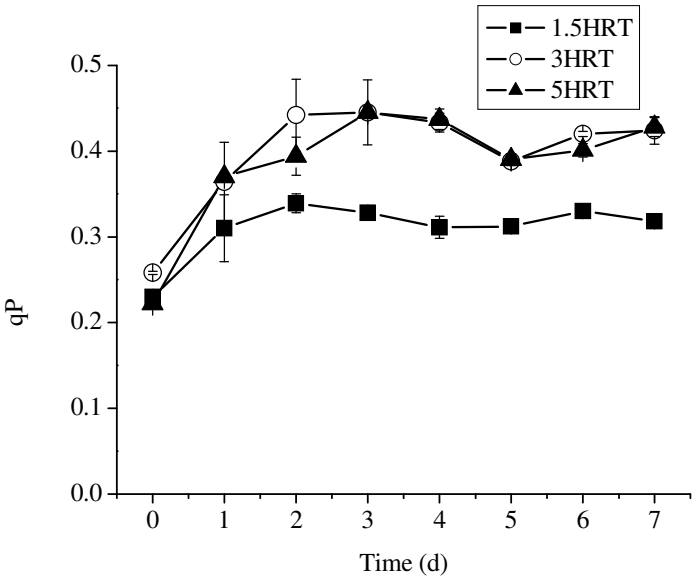

Fig. 4.53 Variation of *Desmodesmus communis* fluorescence parameter values qP during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

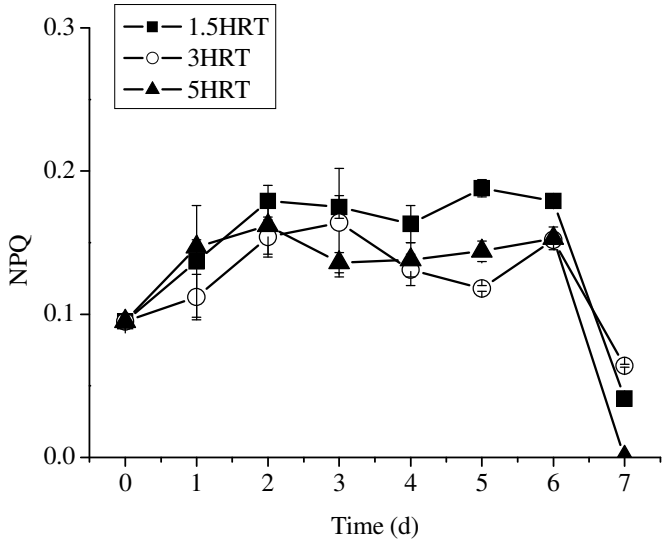

Fig. 4.54 Variation of *Desmodesmus communis* fluorescence parameter values NPQ during the semi - continuous flow treatment of the primary effluent at 1.5-, 3- and 5-day of HRT.

4.4.4 Conclusions

In this research it was demonstrated that application of the algae *Desmodesmus communis* for a wastewater treatment process has the dual role of combining nutrient removal and algal biomass production for potential use as a biofuel feedstock in a semi - continuous process. This work aimed to reproduce on a laboratory scale what can be realized in a open pond system working as a continuous treatment process with different HRTs. Algal biomass productivity achieved the same value of 0.14 g L^{-1} d^{-1} in 1.5-, 3- and 5-day HRTs, on the other hand the biomass composition was influenced by the different HRT conditions as well as the nutrient removal efficiency. 1.5-day HRT may represent a good operative condition to treat a large amount of wastewater per unit of time but it has been shown to be inefficient in terms of ammonia removal (47%) which was nearly complete for both 3- and 5-day HRTs. This low performance of the algal strain *Desmodesmus communis* at 1.5-day HRT may be correlated to the low photosynthetic efficiency shown through both rapid light curves and induction curves analysis which demonstrate that the culture is partially photo - inhibited due to the high light exposition which is a consequence of high rate dilution. This study also contributed data on both biochemical compounds' content and productivity as well as on elemental characterization of the algal biomass, a rarely addressed topic. Algal biomass composition was strongly influenced by the HRT conditions in terms of polysaccharides and proteins content which reached the maximum value of 57% with a productivity of 80 mg L^{-1} d^{-1} at 5-day HRT and 27% with a productivity of 37 mg L^{-1} d^{-1} at 1.5-day HRT, respectively. The maximum C/N ratio of 16 was achieved in the 5-day HRT culture. In addition, the elemental analysis demonstrated that the N content of the biomass was dependent on the ammonia supply at different HRTs. At 1.5-day HRT the culture was considered completely N - sufficient as the cells didn't deplete all the nitrogen present in the effluent and the algal biomass reached an optimal N content of 8-10%. At 3-day HRT the ammonia removal was almost complete but the culture was again not considered N-limited as the N content was still about 8%, on the other hand at 5-day HRT the culture became N-limited with N content of 5%. Anyway the 5-day HRT N limitation didn't affect the fatty acids production which didn't show any significant differences at any conditions ($p > 0.05$, ANOVA) but it reached a productivity of 4.2 and 4.9 mg L^{-1} d^{-1} at 5- and 3-day HRT conditions, respectively, and only 2.8 mg L^{-1} d^{-1} at 1.5-day HRT condition. Considering the future application for biofuel production of the algal biomass obtained from the wastewater

treatment process, the 5-day HRT condition seems to be the most promising condition to apply. The low content of total fatty acids and the high C/N ratio suggest an algal utilization in the anaerobic digestion or combustion process as a more convenient application of *Desmodesmus communis* biomass compared with its utilization for biodiesel production.

5. Baseline study on the wastewater treatment performance and algal biomass productivity of an open pond pilot plant

This section summarizes the technical skills in operating an open pond pilot plant developed during a training period at the Civil and Environmental Engineering Department of California Polytechnic State University.

The High Rate Ponds (HRPs) were used to study how an open pond system works and to evaluate the effects of natural variables and explore the effects of nighttime aeration on water treatment performance and on algal biomass productivity. The HRPs pilot plant was constructed in 2007 at the City of San Luis Obispo Wastewater Reclamation Facility (WRF) in coastal central California (Fig. 5.1) by the Civil and Environmental Engineering Department of the California Polytechnic State University. A process flow diagram of the pilot plant is shown in Fig. 5.2. The four HRPs were named Northwest (NW), Northeast (NE), Southwest (SW), and Southeast (SE) based on their geographical position.

Operations, maintenance, water quality analysis and data analysis are described in this section as well as the treatment performance and experimental observation for the baseline study when the HRPs were operated with and without a nocturnal aeration system during the winter and the spring of 2010.

Fig. 5.1 Aerial view of the City of San Luis Obispo WRF showing the location of the pilot plant and the primary clarifier, which provided the wastewater feed to the pilot plant. Source: Google Earth.

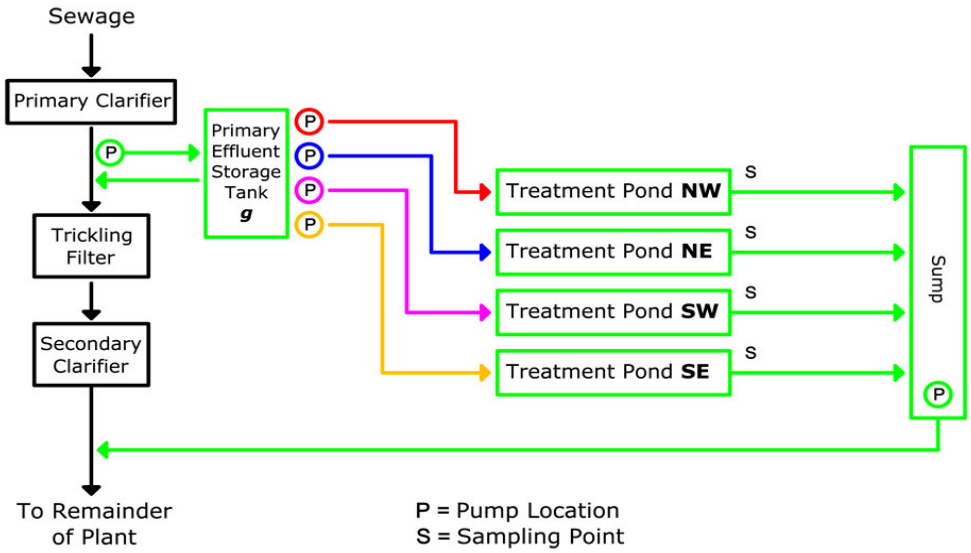

Fig. 5.2 Schematic of the pilot plant and its connection to the SLO Wastewater Reclamation Facility.

5.1 Pilot plant layout

This paragraph summarizes the technical specifics of the pilot plant extracted from a master's thesis written by Frost (2008).

The four HRPs were placed in a secondary containment made of lumber and covered with 45 - mil PVC as a precaution to protect nearby storm drains (Fig. 5.3). Each high rate "pond" was constructed from a rectangular fiberglass tank (3 m x 1.5 m x 0.76 m deep) with a central baffle (King Starboard® marine plastic) and a custom paddle wheel (Starboard® blades bolted to an aerator paddle from Aquatic Ecosystem, Inc.) (Fig. 5.4). Each paddle wheel was fixed to a stainless steel shaft and powered by a 1/8-hp motor (Bodine, Inc.) with a speed controller (Minarik Corp.). The paddle wheels provided gentle mixing to prevent algae sedimentation and promote the exposure of the cells to the sunlight to optimize the biomass productivity. The paddle wheel motor and controller were mounted to a plywood platform bolted to the lip of the fiberglass tank. Effluent outflow was controlled by a 4 cm PVC stand - pipe that was installed in each tank to maintain a constant water level. The standpipe directed effluent flows through a PVC tank adapter connected to another PVC pipe. This pipe from all tanks directed the effluent flow to a sump in one corner of the containment area. A sump pump, with a float switch, directed water from the sump back to the main flow of the WRF.

The pilot plant received primary effluent directly from one of the WRF primary clarifiers. A 1/4-hp submersible pump placed in the weir of the primary clarifier, the water flowed to a constant - head feed tank (76 L) located at the center of the pilot plant facility (Fig. 5.5). The primary effluent in the feed tank continuously flowed through PVC manifold to four peristaltic pumps that individually fed the HRPs. The influent flow rates provided a residence time of 4 days. The pond water depth was 68 cm, and the volume was 2200 L for each pond.

Fig. 5.3 HRPs tank showing placed in a secondary containment, showing paddle wheels.

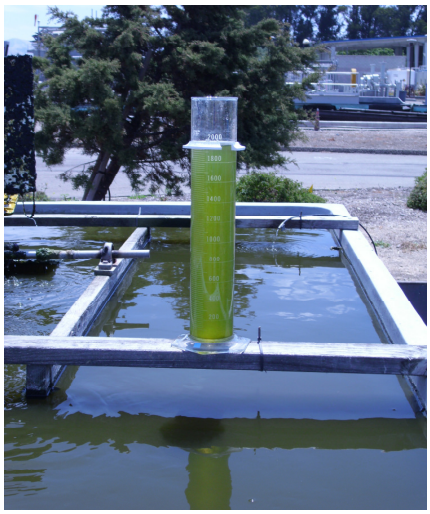

Fig. 5.4 Top view of a high - rate pond tank, showing the central baffle and a culture sample.

Fig. 5.5 Influent feed tank**.**

5.2 Routing monitoring and maintenance

Pilot plant operations required the monitoring of two operator - controlled variables such as flow rate and wheel rotational speed. These variables were monitored daily and adjusted when necessary to maintain constant operation conditions. The influent flow rate to each pond was checked measuring the inflow volume of water pumped into the system in a 250-mL graduated cylinder over the course of 30 seconds in order to keep a constant inflow rate. The rotational paddle wheel speed was adjusted to maintain a flow velocity of 23-25 cm/s in order to prevent over - mixing.

Influent and effluent tubing and piping were inspected daily for leakage and clogging and repaired or cleaned when necessary. Filamentous algal grew rapidly on the upper few centimeters of the submerged tank wall and the paddle wheel and they needed to be removed brushing them every few days, always more than three days before the sampling day to prevent any influence on the TSS measurements.

During the whole study it was necessary to clean, lubricate and less frequently, replace pumps and motors.

Interruption of the weekly sampling and analysis occurred during storm events, which were frequent during the winter months. Flow rates and paddle wheel speeds were reset after every power outage.

5.3 Experimental procedures

The aim of this work was to test the operational efficiency of two ponds in terms of wastewater treatment performance and biomass productivity as a response to two different aeration conditions and the seasonal variability.

From January until July 2010 two of the HRPs, NW and SW, were operated with 4-day HRT corresponding to an inflow rate of 380 mL/min/pond. The SW pond was aerated during the night to maintain a high level of dissolved oxygen also when the photosynthetic process stopped during the dark period, while NW pond served as a control pond without any air supply.

The ponds have been previously inoculated with a community of different algal species derived from samples collected from several wastewater treatment pond systems in California. The prominent algae genera were *Actinastrum*, *Ankistrodesmus*, *Scenedesmus*,

Chlorella, *Closterium*, *Chlorococcum*, *Coelastrum*, *Spirogyra*, *Micractinium*, *Golenkinia*, *Gleocystis* and *Westella*.

Tab. 5.1 includes some of the field activity and laboratory analysis that were carried out during the whole experiment to collect data necessary to evaluate the treatment performance in terms of BOD removal, ammonia removal, biomass production, and biomass settleability. Temperature, pH and dissolved oxygen of the HRPs were measured daily; flow rate, paddle wheel and weather data were also collected daily, while water quality analysis was carried out once or at maximum twice per week. Algae identification and culture observation was performed less frequently using an optical microscope.

Parameter	Location	Meas. Frequency
TSS/VSS	INF/HRPs	Twice per week
BOD_5	INF/HRPs	Weekly
Ammonia	INF/HRPs	Twice per week
Soluble reactive phosphorus; nitrate; nitrite	INF/HRPs	Weekly
Dissolved oxygen	HRPs	Daily
pH	HRPs	Daily
Temperature	HRPs	Daily
Laboratory settling tests	HRPs	Weekly
Algae identification	HRPs	3-4 times per month

Tab. 5.1 Measurement performed in the field and in the laboratory to characterize the pond operation. The analysis were performed in the influent (INF) and/or in the effluent (HRPs) water. TSS and VSS represent the total suspended solids and volatile suspended solids, respectively. BOD is soluble biochemical oxygen demand.

5.4 Methods

5.4.1. Measurement of biomass production

The total suspended solids (TSS) and the volatile suspended solids (VSS) were measured according to the APHA Methods 2540 B and E, respectively. The filters used for solids tests were Fisher Scientific G4 glass fiber filters with a nominal pore of 1.2 µm. The filtrate was used for nutrient determination.

Biomass productivity ($g/m^2/d$) of the HRPs was calculated following the equation:

$P = VSS_{effl} * Q * A$

where VSS_{effl} was the biomass concentration ($g\ L^{-1}$) determined in the effluent overlooking the VSS determined in the influent, Q was the inflow rate of the influent ($L\ d^{-1}$) and A was the upper - water layer area of the ponds ($4.42\ m^{-2}$).

5.4.2 Determination of ammonium

Ammonium was determined in the HRP influent and effluents using the Ammonia - Selective Electrode Method (APHA 4500-NH_3 D). After bringing samples to room temperature, Orion Ammonia pH - adjusting ISA (# 1310-73-2) was used to raise the pH of the standard and samples over 11 with NaOH solution (10 N). Calibration curves were developed using 0.1 mg L^{-1}, 1 mg L^{-1}, 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 1000 mg L^{-1} and 2500 mg L^{-1} stock solution. The ammonia probe was rinsed between readings using de - ionized water.

5.4.3 Determination of nitrate and nitrite

Nitrate and nitrite were both determined by ion chromatography (Dionex DX 120) with an AG9-HC Ion Pac Guard Column, AS9-HC 4-mm IonPac IC column, DS4-1 Detection Stabilizer, and an AS40 Automated Sampler. The analysis were carried out on the HRP influent and effluent samples filtrated through 0.22 µm Whatman filters.

5.4.4 Determination of reactive phosphorus

Dissolved reactive phosphorus was determined in the HRP influent and effluent filtrates using the Vanadate - molybdate method (APHA 4500-P C); no pH adjustment nor color removal were necessary.

5.4.5 Biochemical oxygen demand

Total biochemical oxygen demands were determined in accordance with the APHA method 5210 B.

5.4.6 Settling tests

The settleability of the biomass was determined using 1-L Imhoff cones, in accordance with the standard device for determining settleable solids in wastewater laboratories (APHA Method 2540 A). Supernatant samples were collected after 2 hours and 24 hours of settling time. The initial supernatant samples were obtained from the TSS/VSS measurements. For the settling test, the sample bottle was gently inverted several times to homogenize the solids suspended and then the sample was poured into the settling vessel. At 2 and 24 hours later, 100 ml of supernatant was pipetted from 1 - cm below the water surface without mixing or shacking the cone. These measurements are useful to evaluate the capacity of the biomass to be collected by gravity.

In the field some settling tests were performed occasionally using 100 - mL glass beakers. The approximate settling rate, the color of the culture, and other visible annotations, such as the presence of zooplancton were recorded.

5.4.7 Algae observations and identifications

Algae identification was performed by observation through the optical microscope. Algae were identified to the genera level using information in Standard Methods and other identification materials. Some photomicrographs of the culture placed onto a standard glass microscope slide with a cover slip were taken at 100x, 400x, and 1000x total

magnifications using an Olympus CX41 optical microscope with phase contrast coupled with an Infinity 2 digital camera and Infinity Analyze software.

When identifying algal cells, any matter that appeared to originate from wastewater, bacterial component or zooplankton, mainly rotifers and ostracods, were noted and they were present as a minor component.

5.4.8 Temperature and pH

Temperature and pH were measured in the field using a portable Oakton Acorn® Ion 6 meter. The pH 4, 7 and 10 buffers were checked weekly. Measurements were made directly in the HRPs.

5.4.9 Dissolved oxygen

Dissolved oxygen (DO) was measured in the field using a portable YSI 58 DO meter. The YSI meter was calibrated according to the manual's instructions prior to each use. Measurements were taken by rinsing the DO electrode with de - ionized water and placing the electrode directly in to the HRPs and gently rotating the electrode in the water.

5.4.10 Weather data

Weather data, such as daily solar irradiation, air temperature, precipitation and other climatic information were obtained from the California Irrigation Management Information System through the Department of Water Resources Office of Water Use Efficiency online database. The data was obtained from San Luis Obispo Station No. 52 located at the California Polytechnic State University campus (35°18'22"N, 120°39'37"W), which was approximately 5 Km from the pilot plant.

5.4.11 Quality assurance and quality control

Quality assurance and quality control (QA/QC) procedures were carried out for the laboratory analysis and for each batch of samples, more than 90% of matrix spike recoveries were between 85% and 115% and more than 99% of split results were between 90% and 110%

5.5 Results and discussion

Weather data

Variability in environmental conditions is one of the main barriers to the application of an open pond system. Sunlight intensity and temperature variations, which affect biomass productivity, are a challenging problem in the use of open ponds in many areas.

As Fig. 5.6 shows, no significant differences occurred in the average air temperature during the whole study, despite the seasonal change. During the winter the air temperature ranged from 6.9°C to 17.8°C, with an average value of 12°C; during the spring the air temperature ranged from 8.4°C to 20.2°C, with an average value of 13.7°C. As a consequence of such a low air temperature variability, the average water temperature reached the maximum value of 21°C and the minimum value of 9°C, and the average temperature over the course of the whole experiment ranged from 17°C to 12°C.

The light intensity also affects the photosynthetic efficiency, the productivity of cell biomass and the activity of cellular metabolism. Solar irradiation increased significantly during the study and changed from an average of 122.5 W m^{-2} in the winter to an average value of 279 W m^{-2} in the spring due to longer days and the higher sun angle (Fig. 5.7).

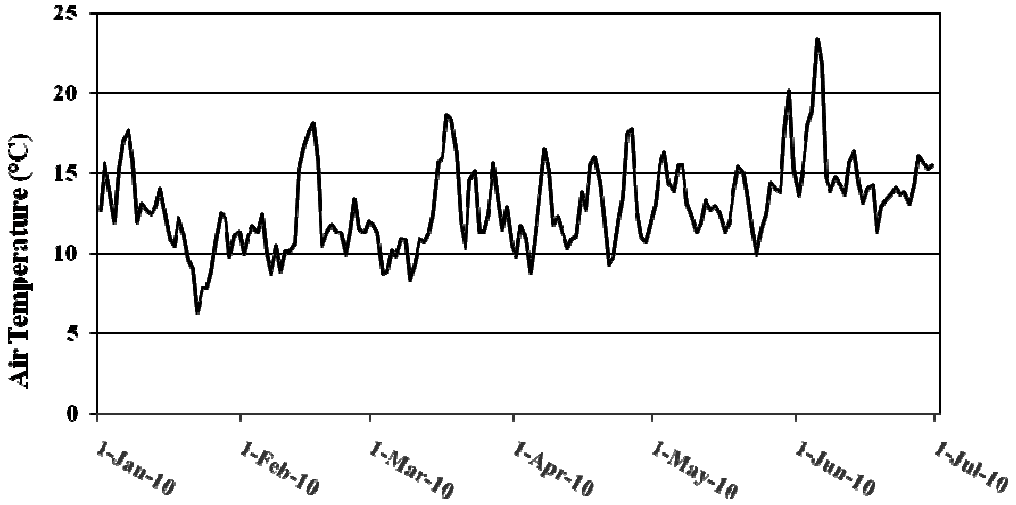

Fig. 5.6 Average air temperature obtained from the California Irrigation Management Information System through the Department of Water Resources Office of Water Use Efficiency online database.

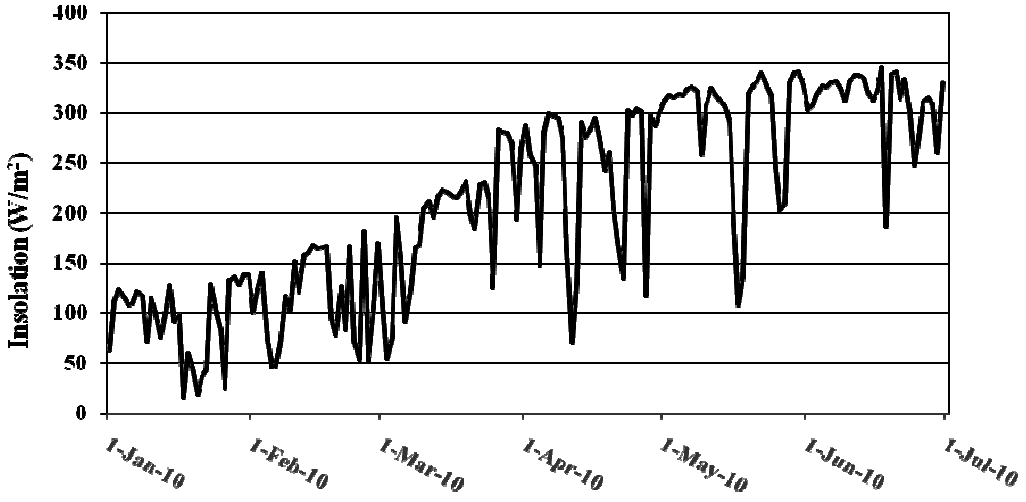

Fig. 5.7 Insolation data obtained from the California Irrigation Management Information System through the Department of Water Resources Office of Water Use Efficiency online database.

Influent wastewater characteistic

The primary wastewater characteristics were monitored weekly and the average, maximum and minimum values are reported in Tab. 5.2 for both seasons. The organic matter, represented as BOD_5 concentration, and the ammonium were the most abbundant compounds in the influent. These parametes were affected by the seasonal change; indeed the BOD_5 and the ammonium concentrations increased from an average of 52.7 mg L^{-1} and 28.2 mg L^{-1}, respectively, in the winter to an average value of 103.9 mg L^{-1} and 40.8 mg L^{-1}in the spring. The soluble reactive phosphorous started to be monitored from April and its average during the last months of the experiment was 5.5 mg L^{-1}, ranged from a maximum of 7.4 mg L^{-1} to minimum of 3.8 mg L^{-1}.

Wastewater characteristics	**WINTER**			**SPRING**		
	Average	**Max**	**Min**	**Average**	**Max**	**Min**
TSS (mg L^{-1})	52	117	28	68	104	31
VSS (mg L^{-1})	42	74	28	62	91	31
NH_3-N (mg L^{-1})	28.2	40.6	22.6	40.8	55.9	22.8
NO_3-N (mg L^{-1})	1.5	2.5	0.06	0.3	1.6	< 0.001
NO_2-N (mg L^{-1})	0.5	0.8	0.3	0.8	1.6	< 0.001
BOD_5 (mg L^{-1})	52.7	66.3	38.0	103.9	188.0	68.0

Tab. 5.2 Influent wastewater characteristics during winter and spring.

24h variations in chemical - physical parameters

Diurnal variations in physical - chemical parameters such as dissolved oxygen (DO), temperature and pH were measured during an occasional overnight experiment in June.

As Fig. 5.8 shows, the concentration of DO had a different trend during the 24 hours in the two pond conditions. The aerated pond showed a decrease in the DO values as the sunlight started to be less intense from the early afternoon and the oxygen concentration dropped to zero around 9 pm rising again at sunrise. DO level achieved a maximum value of 11 mg L^{-1} at midday. On the other hand the aerated pond, which started to receive air from 8 pm until 8 am, kept a high level of dissolved oxygen that ranged from 5.3 mg L^{-1} during the dark period to 10.8 mg L^{-1} at midday. It appears clearly how the algae provided the dissolved oxygen concentration in the water through the photosynthetic process during daytime and also how high the oxygen depletion was at night probably due to the parallel oxygen consumption by algae and bacteria.

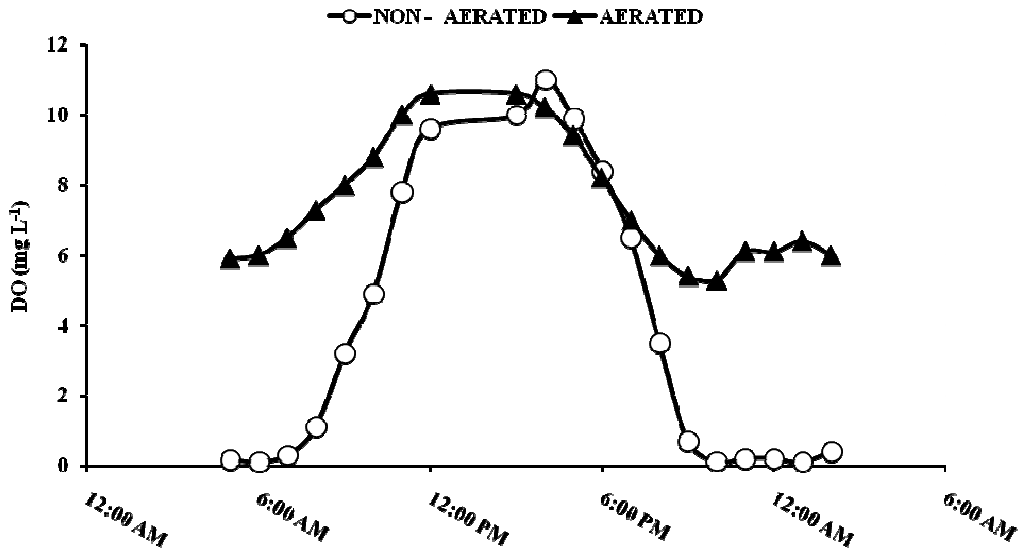

Fig. 5.8 Dissolved oxygen (DO) concentration variability during an overnight experiment in June 2010.

Culture pH is one of the most important factors in algae cultivation; algae vary greatly in their pH optima but several studies confirm that the optimal medium pH at which to add CO_2 is over 8.5. At high pH values, minerals (especially calcium salts, carbonates and

phosphates) precipitate leading to nutrient deficiencies. The pH values in both HRPs ranged between 7.2 and 8.4, which means no carbon limitation on algal growth (Fig. 5.9). Fig. 5.10 shows the water temperature diel variation which ranged from a maximum of 21.1°C to a minimun of 14.6°C.

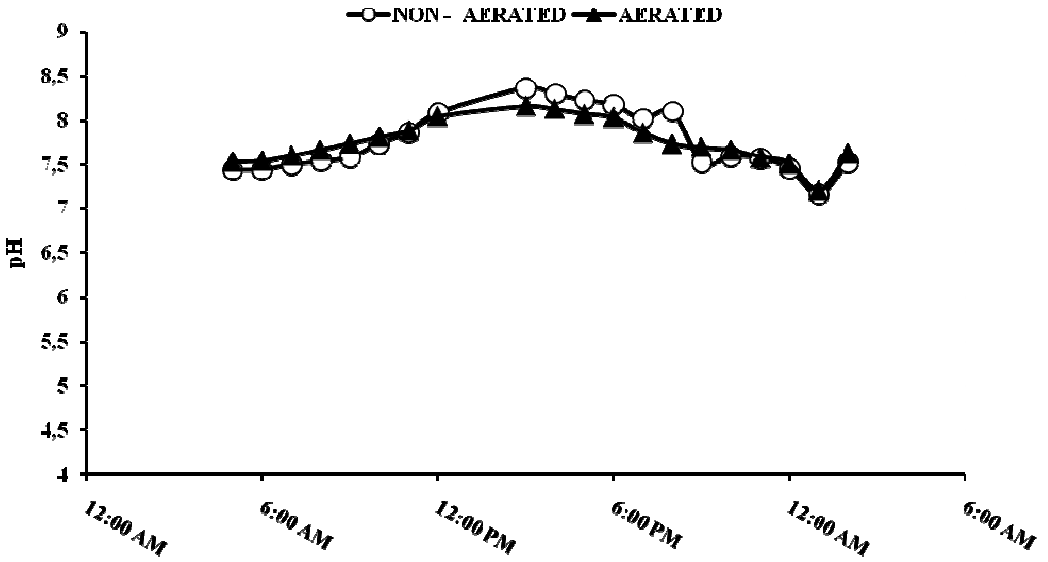

Fig. 5.9 pH variability during an overnight experiment in June.

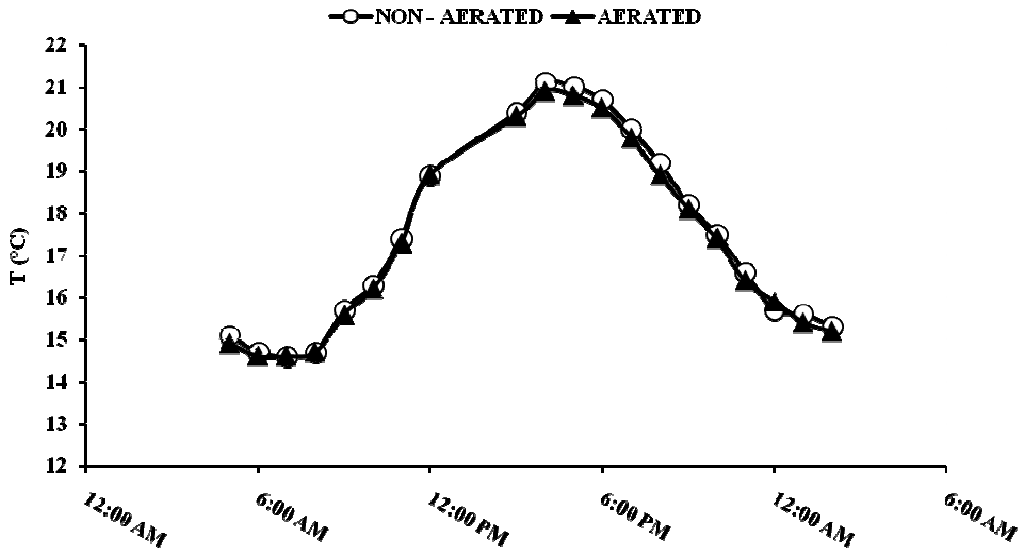

Fig. 5.10 Water temperature variability during an overnight experiment in June.

Biomass productivity

The effluent VSS concentrations for both HRPs varied during the whole study, increasing rapidly at the beginning of spring (Fig. 5.12). The HRP effluent VSS was a mixture of algae and bacteria, as shown in Fig. 5.11, and its trend estimated both the heterotrophic (bacteria) and autotrophic (algae) growth. The VSS average changed from 82 mg L^{-1} in the winter to 249 mg L^{-1} in the spring for the non - aerated pond; while the increase in VSS average concentration was lower in the aerated pond and it ranged from 83 mg L^{-1} in the winter to 146 mg L^{-1} in the spring.

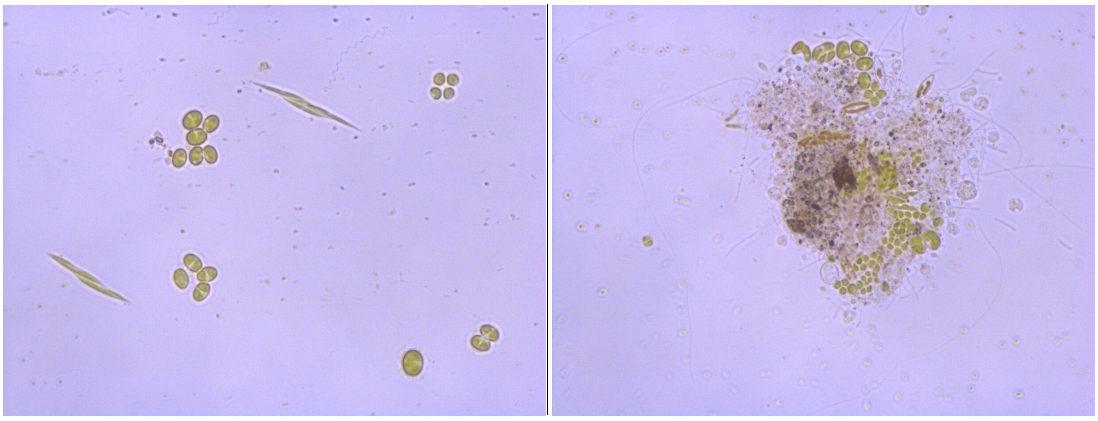

Fig. 5.11 Micrograph of water samples in the HRPs. The samples contained many green algae species (left) and flocs of algae-bacteria (right).

The biomass productivity was calculated in both seasons (Fig. 5.13); during the winter both ponds produced approximately 10 g/m^2/d, during the spring months the biomass productivity increased reaching a value of 31 g/m^2/d and 18 g/m^2/d in the non - aerated pond and in the aereted pond, respectively.

VSS and biomass productivity results showed their depedence on light irradiation, thus the biomass increased by 67.7% and 44.4% in the non - aeretaed and aerated ponds, respectively, during the spring.

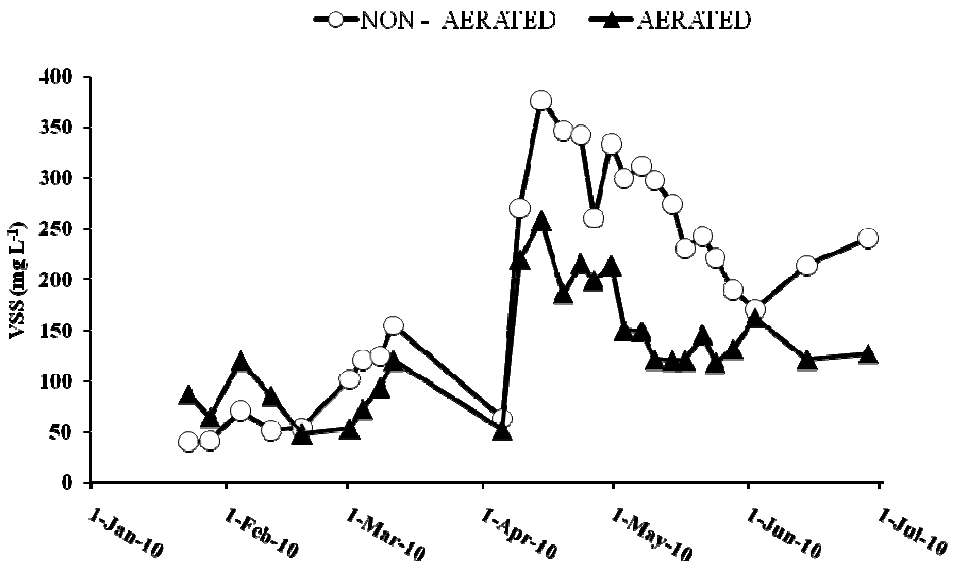

Fig. 5.12 Volatile suspended solids concentrations in the HRPs effluents during the whole experiement.

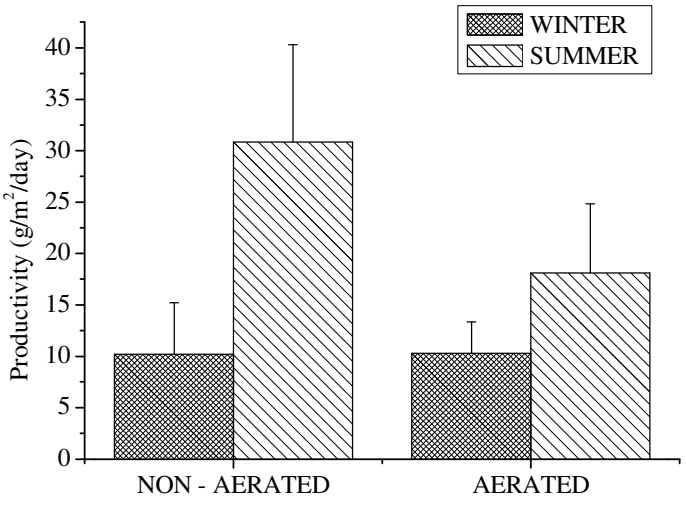

Fig. 5.13 Seasonal average of biomass productivity determined in both non - aerated and aerated HRPs.

Wastewater treatment

Ammonium was the main form of nitrogen in the influent wastewater (Tab. 5.2); the ammonia nitrogen removal was quite good during the spring, achieving 95.3% and 88.3% in the non - aerated and aerated pond, respectively (Fig. 5.14). During the winter months the treatment efficiency was characterized by only 61.7% and 73% of ammonium removal in the non - aerated and aerated pond, respectively. Increased ammonium removal during the spring months was probably due to the high concentration of the algae and bacteria in the ponds. Ammonia volatilization was considered insignificant because the pH never reached high values. Accumulation of nitrate was observed in the aerated pond which showed an average concentration of 16.5 mg L^{-1} both in the winter and in the spring. In the non - aerated pond the nitrate concentration was about 14.8 mg L^{-1} in the spring but no accumulation was observed during the winter. The nitrite concentration was lower than 2 mg L^{-1} in both pond conditions. The nitrate accumulation may be caused by the nitrification process during the day and by the absence of the denitrification process, which causes the reduction of nitrate as the electron acceptor to nitrite and ultimately to nitrogen gas. The denitrification process occurs under anoxic conditions which were never achieved in the aerated pond justifying the nitrate accumulation in this pond. On the other hand, the nitrate accumulation during the spring months in the non - aerated pond was totally unexpected and needed a more detailed nitrogen balance to understand the transformation of nitrogen species in the pond systems.

The BOD_5 removal efficiencies for both HRPs are summarized in Fig. 5.15. In the non - aerated pond the BOD_5 concentration ranged during both seasons from 23.3 mg L^{-1} to 10.1 mg L^{-1} while the average removal efficiency increased from 71.3% during the winter to 87.1% in the spring months. In the aerated pond the BOD_5 concentration ranged from 15.8 mg L^{-1} to 7.7 mg L^{-1} during the winter and from 38.1 mg L^{-1} to 10.8 mg L^{-1} during the spring but the removal efficiency didn't show any significant increment between the seasonal change and varied form 78.9-82%. The greater BOD_5 removal efficiency in the non - aerated pond may have been due to the greater concentration of microorganisms (as VSS) in this pond (Fig. 5.12).

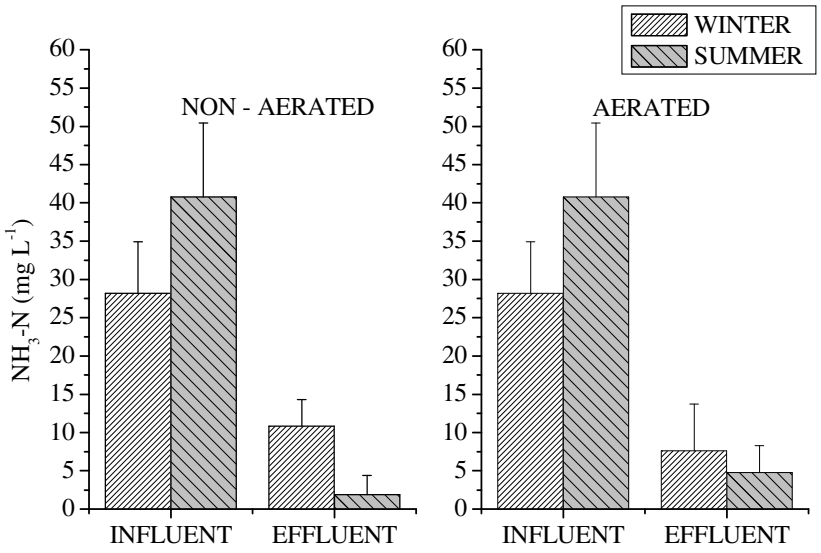

Fig. 5.14 Seasonal average of total ammonia nitrogen determined in the influent and in both non - aerated and aerated HRP effluents.

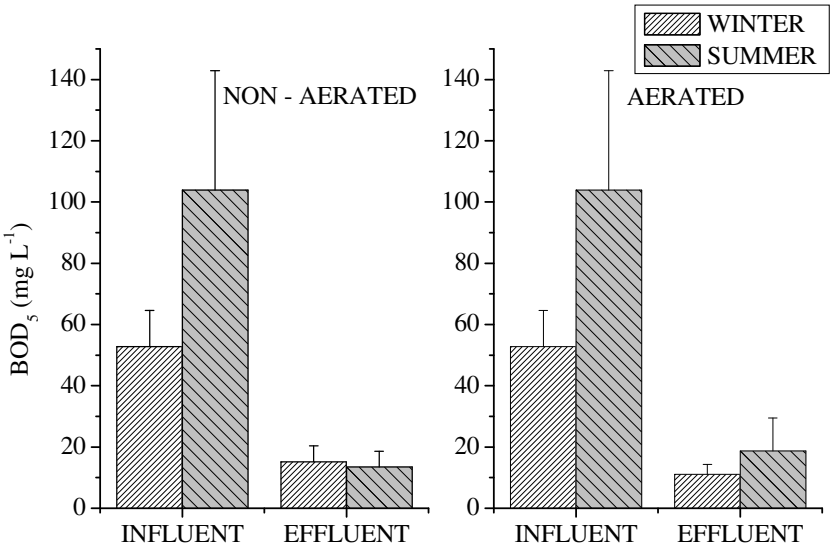

Fig. 5.15 Seasonal average of biochemical oxygen demand (BOD) determined in both HRPs Seasonal average of biochemical oxygen demand (BOD) determined in the influent and in both non - aerated and aerated HRP effluents.

Settleable biomass test

The settleability of the biomass was tested weekly using 1-L Imhoff cones to evaluate the sedimentation efficiency of the pond biomass (Fig. 5.16).

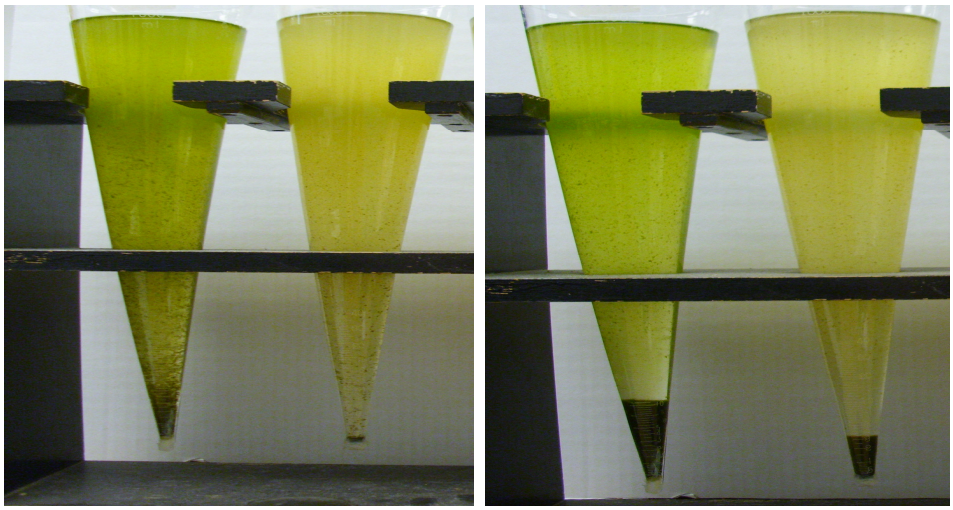

Fig. 5.16 Biomass settling in 1-L Imhoff cones after 2hours (left) adn after 24 hours (right).

Supernatant TSS was determined after 2h and 24h of settling time (Fig. 5.17). The percentage of sedimentation in only 2 hours reached the highest value of 75.7% then 87.9% in 24 hours in the aerated pond during the winter, while the non - aerated pond showed only 51.7% of biomass sedimentation and 74.2% in 24 hours. During the spring months the biomass sedimentation increased in the non - aerated pond reaching the 71.6% after 2 hours and 85% after 24 hours, probably due to a high VSS concentration in the pond. On the other hand the percentage of sedimentation decreased in the aerated pond with values which ranged from 52.7% in 2 hours and 81% in 24 hours. No good correlations were observed between the supernatant TSS and the influent BOD and TSS, what's more no correlations were noticed between the sedimentation capacity and the TSS measured in the pond (data not shown).

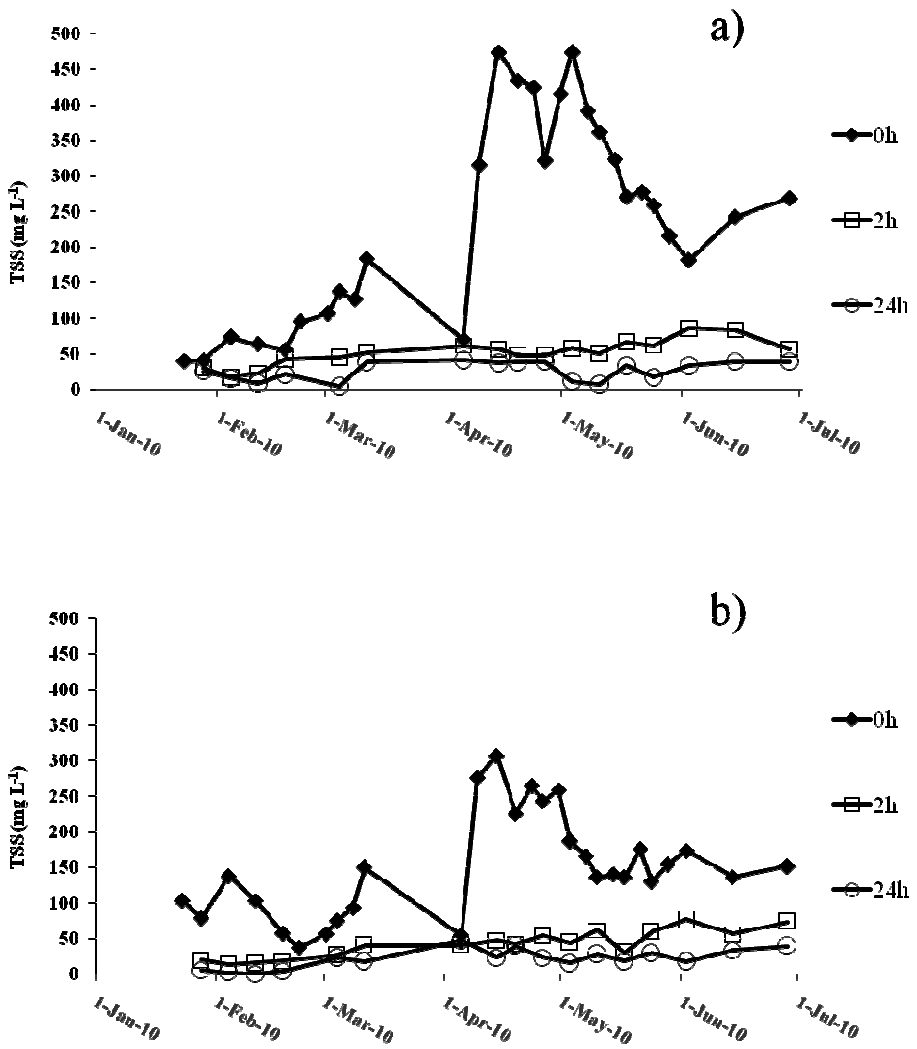

Fig. 5.17 0 hour, 2 hours and 24 hours supernatant TSS concentration in the non - aerated (a) and nighttime aerated (b) ponds.

5.6 Conclusion

The preliminary screening experiment designed to explore the effects of nighttime aeration and seasonal changes on algae production and wastewater treatment performance belonged to a major research project directed by Dr. T. Lundquist at the California Polytechnic State University. The main purpose of this baseline study was to acquire technical competences in the field and in the laboratory operating outdoor algae culturing systems for the purposes of biofuel production and wastewater treatment. The data collected during this study demonstrated how the seasonal variation affected the biomass productivity and the treatment performance. Sunlight appeared as one of the main limiting factors in algae production, when carbon, nitrogen and phosphorus are sufficient. Under high levels of irradiance the biomass productivity achieved a maximum of 31 $g/m^2/d$ which is comparable with other typical biomass productivity such as 35.15 $g/m^2/d$ and 37.68 $g/m^2/d$ reported for monoculture of *P. carterae* and *D.salina*, respectively, in open pond system (Moheimani and Borowitzka, 2006). Also the lowest value of biomass productivity of 10 $g/m^2/d$ achieved during the winter months were in accordance with the long - term productivity in large commercial raceways. Also the wastewater treatment performance increased during the spring due to the higher concentrations of microorganism such as algae and bacteria. On the other hand the nighttime aeration didn't seem to affect the productivity, which was similar in both ponds during the winter and reached lower level during the spring in the aerated pond. Many discrepancies were observed comparing the treatment performance results in both conditions and further information derived from the grazing monitorning and the quantitative and qualitative evaluation of the bactera community are probably necessary to better understand such a complex biological system.

6. General conclusions

This study highlights the potential utilization of the newly isolated microalgae *Desmodesmus communis* in an alga - based wastewater treatment for biofuel production.

This algal strain was isolated during the winter months in a lake close to Cesena proving its natural resistance at low temperature and adaptation at low sunlight irradiance. During the present study *Desmodesmus communis* showed a great capacity to compete with other microorganisms, like other microalgae, bacteria and zooplankton that were naturally present in the wastewater utilized. Especially during the algal consortium study the species belonging to the genus *Desmodesmus* and/or *Scenedesmus* appeared to be the most abundant in just a few days, despite the fact that they didn't represent the majority group at the beginning of the experiment.

The isolated strain of *Desmodesmus communis* showed great vitality in the primary effluent collected from a local wastewater reclamation facility in Cesena (Italy), reaching high values in terms of biomass productivity of 0.177 - 0.227 g L^{-1} d^{-1} in batch cultures with also high levels of ammonia. High ammonia concentration, CO_2 addition (2%) and high light intensity positively affects the biomass production, on the other hand nutrient deprivation, which is achieved growing the algae in a secondary effluent, is shown to be an efficient factor to increase lipid accumulation. Both biomethane and biodiesel production need a biomass rich in the lipid fraction, especially in the total fatty acid (TFA) content, lipid compounds which are the substrate for biodiesel synthesis. For this reason, this work was focused particularly on the study of the TFA accumulation in the biomass and the environmental conditions that affect this process. In a batch culture system, *Desmodesmus communis* grown in a secondary effluent achieves a biomass productivity of 0.023 g L^{-1} d^{-1}, 9.3% over the dry biomass of TFAs and a C/N ratio value of 39.9, on the other hand the same strain grown in a primary effluent achieves a biomass productivity which ranges from 0.138 to 0.227 g L^{-1} d^{-1}, 2.1 - 4.9% over the dry biomass of TFAs and a C/N ratio value of 7.6 - 16.3.

Desmodesmus communis reaches an optima nitrogen content over the dry biomass of 8-10%, in N sufficient culture conditions; while it shows the effect of N limitation culture conditions when its N content decreases until 4-5%. In these conditions the algal cells

don't accumulate any lipids as cellular energy storage, but the total lipid fraction and the TFA content increase only when the N content over the dry biomass decreases below 3%, thus when the culture is completely N deprived. It has been demonstrated that *Desmodesmus communis* grows more rapidly under high light conditions but on the other hand its photosynthetic apparatus doesn't work at its maximum level at such high light intensity (i.e 140 - 440 μE m^{-2} s^{-1}). Nitrogen deprivation and high light intensity were shown to induce a great accumulation of electrons in the electron transport chain of the photosynthetic system, reducing the photosynthetic efficiency and on the other hand affecting the increase of the non -polar lipid fraction and especially the TFA content, which achieved 13% over the dry biomass in N deprivation conditions. High light intensity also stimulates the growth rate when the culture is subjected to nutrient stress conditions. The best choice that the results of the present study suggest is a two - phase strategy: in the first step of the process the biomass productivity is optimized using a primary effluent; in the second step this biomass obtained from the first one is stressed by nutrient deprivation and high light exposure, using a secondary effluent to dilute the culture and prevent the complete nutrient deficiency and culture death. As the maximum lipid content achieved in *Desmodesmus communis* was only 25.8% with a TFA amount of 13% over the dry biomass, the anaerobic digestion of the whole biomass appears to be a good strategy to investigate in further studies for the energetic recovery of cell biomass.

As one of the main purposes of this work was to investigate *Desmodesmus communis* biomass productivity and composition in order to develop an alga - based pilot plant for the wastewater treatment and biomass production, a semi - continuous experiment has been carried out to evaluate the nutrient removal efficiency of this algal strain at different hydraulic residence times (HRTs). In the batch culture it was proved that the nutrient removal was complete at any N/P ratio, also in P limited condition which are common in this wastewater. Algal biomass productivity in semi - continuous operation achieves the same value of 0.14 g L^{-1} d^{-1} in 1.5-, 3- and 5-day HRTs but its composition is strongly affected by the different HRTs. The longest residence time provides complete nutrient removal, as well as the 3-day HRT condition, and a biomass with the highest value of polysaccharides of 57% and the highest C/N ratio value of 16. The shortest residence time achieves only 47% of the ammonia removal and also produces a biomass rich in protein and with a high N content. The TFA content doesn't show any differences at the three

HRT conditions probably because even at 5-day HRT the N content in the cells is not below 4%, thus the culture is not N deprived.

The present work which aimed to study the growth rate, the biomass characterization and productivity coupled with the nutrient removal capacity of the newly isolated microalgae *Desmodesmus communis* has been concomitant with other research projects which aimed to investigate the same algal strain biomass application in the energy recovery field, especially using the pyrolisis process for bio - oil production. Moreover, *Desmodesmus communis* characterization conducted in the laboratory during the whole thesis will be used as a preliminary study for the operation of an open pond pilot plant realized in 2010 within the Ca.Re.Te. (CArbon REduction TEcnologies) project funded by Regione Emilia Romagna. Two open ponds, with 3 m^3 of volume and 60 cm depth, have already been constructed at Romagna Compost S.R.L., in Cesena. With the data collected during the present work and the training experience acquired at the California Polytechnic University, *Desmodesmus communis* will be utilized as a monoculture in the wastewater treatment and biomass production for biomethane synthesis through anaerobic digestion, in the open pond system with the initial aim of studying the biomass productivity and wastewater treatment performance as a response to seasonal and environmental condition variability.

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