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Nannochloropsis sp. F&M-M24: Oil production, effect of mixing on productivity and growth in an industrial wastewater

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Abstract

The energy balance of microalgal biodiesel production is rarely considered. Besides, the actual potential of microalgae as triacylglycerol producers is often overestimated. This work was aimed at investigating these critical aspects using the marine eustigmatophyte *Nannochloropsis* sp. F&M-M24, a promising oil producing strain, as model organism and the "Green Wall Panel" as culture system. First, the influence of air-flow rate on volumetric productivity of the microalga was evaluated. At low and medium irradiances, no significant differences in productivity occurred among the three different mixing rates tested, while at high irradiances an increase in the air-flow rate resulted in significantly higher volumetric productivities. These results allow to foresee a strategy of air-flow rate tuning in accordance to radiation, that may lead to substantial energy savings and, consequently, to more favourable energy and economic balances in the cultivation process. Second, the lipid content and fraction distribution of the biomasses produced under nutrient sufficient and nitrogen deprived conditions were analyzed and the neutral lipid fraction was fully characterized. Finally, the alga was grown in bubbled-tubes using a culture medium prepared with an industrial wastewater, to evaluate its ability to use a free of charge source of nutrients, the exploitation of which may improve biomass production economics.

Keywords: microalgae, biodiesel, Green Wall Panel

1. Introduction

Microalgae have been identified as a possible alternative source for biofuel production and, in recent years, the interest in these photosynthetic microorganisms has exploded [1, 2]. The main reasons for such recognition, after years of relative disregard, are that microalgae are considered far more productive than traditional crops used for the production of first generation biofuels (sunflower, sugarcane, rapeseed, oil palm, etc.) and that some strains can accumulate up to 60% of lipids, mainly triacylglycerols (TAG), the fraction suitable for the production of biodiesel after trans-esterification [2, 3]. The enormous expectations placed on microalgae has often led to an overestimate of the true productive potential of these microorganisms [4]. On the other hand, we should recognize that the interest in microalgae as a source of "new" generation biofuels is also supported by facts. Compared with crops used for the production of first generation biodiesel (soybean, sunflower, rapeseed, oil palm), these microorganisms achieve much higher lipid productivities [4]. For example, one hectare of sunflower or rapeseed can attain 700-1000 L of oil per year, while a well managed algal culture, in regions characterized by a high and constant availability of solar radiation along the year and stable climatic conditions, is able to provide over 20 tonnes of lipid per hectare per year [2]. What makes algal biomass interesting and competitive with traditional oil crops is that algal cultures do not compete for fertile soils, do not require pesticides and can be produced in seawater or using agricultural, industrial or domestic wastewaters [4]. Besides, algal cultures consume large amounts of CO_2 (about two kilograms of CO_2 per kg of algal biomass produced) and can be grown using the flue gas of power plant stations [5, 6].

The production of algal biomass, as well as the cultivation of all other energy crops, requires inputs such as electricity, fertilizers, water and raw materials; which means a more or less direct consumption of energy and water and a release of substances with a potential pollution impact (CO_2, NO_x, SO_x, etc.). It is therefore necessary to quantify the energy consumption and the real environmental benefit (sustainability) associated with the production and usage of any biofuel. When economic and energy evaluations of algae biomass production are performed, it clearly emerges that algae cultures are far from industrial sustainability, showing energy balance and production costs still too high for a commercial scale biofuel production [7-10]. In order to make microalgae biomass production a viable solution for the biofuel market, two main targets must be achieved: (i) biomass and oil productivity (t ha^{-1} y^{-1}) have to be maximized and (ii) operational and

capital costs involved in the production process have to be significantly reduced since algal oil price has to compete with that of fossil fuels.

Besides these two main limitations there is a dichotomy, not yet solved, between photobioreactors and open systems as the possible culture solutions to be employed in large-scale microalgae biomass production. Advantages and drawbacks of both systems have been thoroughly examined [11] and the controversy can be simplified as follows. Photobioreactors permit higher concentrations and productivities and lower contamination, but have higher investment costs and are not always easy to be scaled up. On the other hand, open systems, mainly raceway ponds, present low investment and management costs with respect to photobioreactors, allowing biomass production at lower costs, but low productivities, contamination and the high amount of water required, represent the main disadvantages for their application [12]. Currently over 90% of world microalgae biomass production is realized in large raceway ponds [13].

In the present work three different aspects of microalgal cultivation, all aimed at reducing the energetic, and also the economic, cost of algal biomass for biodiesel production in bubbled photobioreactors, were analysed. The experiments were carried out using *Nannochloropsis* sp. F&M-M24 as model organism because this alga is one of the most interesting organisms both for biodiesel production and for already established markets (e.g. aquaculture and cosmetics). This strain was largely investigated in the past in outdoor conditions and is known to accumulate lipids under nitrogen starvation [2]. In order to optimize energy consumption without jeopardizing the productivity of the system, the first aspect investigated was the energy consumption for mixing, as this can represent a consistent proportion (up to 100%) of the energy stored into the biomass [14]. The effect of three different air-flow rates (0.05-0.15-0.45 L L^{-1} min^{-1}) on volumetric productivity of *Nannochloropsis* sp. F&M-M24 was evaluated outdoors in vertical first generation "Green Wall Panel" (GWP) photobioreactors [12]. The second aspect investigated was the possibility to reach, in GWP photobioreactors, both high lipid productivities and a good lipid composition. The qualitative analysis of neutral lipid and TAG, the class of lipids necessary for biodiesel production, from outdoor nitrogen-starved cultures of *Nannochloropsis* sp. F&M-M24 was then performed and compared to that of a culture grown under nutrient sufficient conditions. The last aspect investigated was the possibility to use wastewater as a source of nutrients to grow *Nannochloropsis* sp. F&M-M24 to further reduce the operational cost of biomass production. Cultivation experiments were carried out in laboratory bubbled

reactors to investigate the ability of *Nannochloropsis* sp. F&M-M24 to use the nutrients contained in an industrial stream, in comparison with *Scenedesmus*, an alga known for its ability to grow in wastewaters.

2. Materials and Methods

2.1 Experimental plan

The experiments were carried out during 2009 at the experimental station of the Istituto per lo Studio degli Ecosistemi-CNR in Sesto Fiorentino (Florence, Italy) (latitude: 43°50'7"N; longitude: 11°11'46"E) to investigate the effect of mixing on productivity and oil production under nitrogen starvation, and at the Dipartimento di Biotecnologie Agrarie (Florence, Italy) (latitude: 43°47'14"N; longitude: 11°14'59"E) to evaluate the use of wastewater.

The influence of the mixing rate on the productivity of the eustigamtophyte *Nannochloropsis* sp. F&M-M24 cultures was investigated in winter, spring, and autumn using three 20-L GWPs. In late spring, an experiment was carried out with *Nannochloropsis* sp. F&M-M24 in a 590-L GWP to evaluate the increase in lipid content of the biomass under nitrogen starvation. The composition of the neutral lipid fraction of both the control and the nitrogen deprived biomass was also determined.

At the end of September, the ability of two strains of the green alga *Scendesmus* sp. to grow in co-culture using nitrogen from an industrial wastewater was verified in 9-L raceway ponds. Finally, the ability of *Nannochloropsis* sp. F&M-M24 to use nitrogen from the industrial wastewater was compared to that of *Scenedesmus* sp. 3PAV3, under laboratory conditions using 1-L bubbled reactors.

2.2 Cultivation system and culture conditions

2.2.1 Influence of mixing rate on volumetric productivity

Three 20 L "Green Wall Panel" reactors [15] were used to evaluate the influence of the mixing rate on *Nannochloropsis* sp. F&M-M24 productivity. Vertical N-S facing reactors were 1-m high, 0.5-m long and, on average, 4-cm thick. Compressed air was bubbled at the bottom of the reactors through a perforated (Ø 1 mm) plastic pipe. CO_2, used as carbon source, was injected through a gas diffuser. A control unit provided regulation of the culture temperature and pH by automatically activating valves. The cultures were maintained in a pH range of 7 to 7.7 by means of automatic distribution of CO_2. The cultures were heated, to

prevent the temperature from falling below 8 °C, by means of an electric heater for aquaria (50 W) placed inside the reactor. High temperatures at midday, above 25 °C, were prevented by means of water spraying on the reactor surface. The mixing rates adopted were 0.05, 0.15 and 0.45 L^{-1} L^{-1} min^{-1}.

A semi-continuous daily harvesting regime was adopted. Every day in the morning the culture concentration was regulated at the same starting value (0.71 ± 0.02 g L^{-1} on average) in all three GWPs, so that the three cultures received an equal amount of photons per unit volume and biomass at the start of the light period. Every day a variable fraction of the culture volume was withdrawn and replaced with the same volume of fresh medium. *Nannochloropsis* sp. F&M-M24 was cultivated in F medium [16] prepared with artificial seawater (Adriatic Sea Aquarium & Equipment, Rimini, Italy) at 30 g L^{-1} salinity, which was filtered through 80-10-1 μm melt-blown polypropylene cartridges (Everblue, Parma, Italy).

2.2.2 Nitrogen starvation experiment

Nannochloropsis sp. F&M-M24 was cultivated outdoors in a 590-L, 10-m long, 1-m high and 5.5-cm thick (on average) first generation GWP [15]. Cultivation was carried out with a daily dilution of about 40% using nitrogen deprived F medium. At the beginning of the experiment, the residual nitrogen concentration was about 8 mg L^{-1}. Temperature and pH were monitored continuously, pure CO_2 was injected into the culture through a gas diffuser and a water spraying system was installed to avoid overheating.

The whole culture (590 L) was harvested by centrifugation. The biomass (approximately 384 g wet weight) was used for lipid extraction and characterization.

2.2.3 Cultivation in an industrial wastewater

The industrial wastewater, supplied by SARAS S.p.A., consisted of the grey water originating from the TAR Gasification unit of the IGCC (Integrated Gasification Combined Cycle) plant in the Sarroch (Cagliari, Italy) oil refinery. The water, coming out from the quench system of the syngas plant, contains heavy metals (like Ni, V and Fe) and different organic and inorganic substances (like ammonia, iron cyanide and ammonium formate). This water is treated chemically and physically to eliminate heavy metals. At the end of this process, the water is clear and contains sodium, ammonium formate and trace of iron cyanide and is sent to the next treatment unit to remove ammonia and cyanide before the final refinery bio-treatment. The grey

water used in this experiment was only partially purified and its composition was: sulphur 3 mg L^{-1}, cyanide 15.4 mg L^{-1}, formate 1160 mg L^{-1}, chloride 80 mg L^{-1}, ammonium nitrogen 244 (for indoor experiments) or 280 (for outdoor experiments) mg L^{-1}, nitric nitrogen 2.6 mg L^{-1}, phosphate-phosphorus 0.2 mg L^{-1}, iron 0.75 mg L^{-1}, calcium 12.9 mg L^{-1}.

To evaluate toxicity of this industrial grey water, two small-scale (9-L culture volume) raceway ponds were inoculated with two strains, pre-adapted to outdoor conditions, of *Scenedesmus* sp., a robust alga known for its ability to grow in wastewaters. One of the strains, *Scenedesmus* sp. 3PAV3, had been isolated from municipal wastewater and the other, *Scenedesmus* sp. PRA, from a freshwater environment. The strains were morphologically distinguishable in culture. The control culture was set up in BG11 medium [17] with modified nitrogen concentration (25 mg L^{-1}). Grey water was added to the test culture in the amount necessary to furnish 25 mg L^{-1} of nitrogen (ammonium plus nitric nitrogen). All the other nutrients present in the BG11 medium were added to the grey water culture in the same amount as in the control culture. N and P were integrated daily according to volumetric productivity. At the end of the culture period (8 days), grey water was about 17% of the culture volume. Both cultures were then diluted. Grey water was added in an amount equal to 48% of total culture volume. During the experiments, pH was not controlled to avoid formation of cyanidric acid, so that its final value was about 10-11. Carbon was supplied by daily addition (45 mL d^{-1}) of $NaHCO_3$.

For laboratory cultures in 1-L bubbled reactors, *Nannocholorpsis* sp. F&M-M24 and *Scenedesmsus* sp. 3PAV3 were inoculated from cultures grown in bubbled tubes in F or BG11 medium. For both strains a control culture in F or BG11 medium containing 100 mg L^{-1} of nitrogen was prepared. The industrial grey water was used to set up three cultures for each strain: one with 10%, one with 25% and one with 50% grey water over the total culture volume, corresponding to 25, 62 and 123 mg L^{-1} of nitrogen, respectively. Each grey water culture was added with the same nutrients as the control culture, except nitrogen. *Nannochloropsis* sp. F&M-M24 was cultivated at a salinity of 30 g L^{-1}. The tubes were incubated at 25 °C under continuous illumination of 200 µmol photons m^{-2} s^{-1} provided by metal halide lamps. Mixing, pH control, and carbon supply were obtained through bubbling with an air:CO_2 mixture (98:2, v/v). The outlet gases were vented to the ouside. The state of the cells was monitored daily by microscopic observations. The experiment was repeated twice.

2.3 Analytical determinations

Culture growth and productivity were determined by measuring the dry biomass in culture aliquots according to Chini Zittelli *et al.* [18]. Each sample of outdoor cultures was collected at the end of the dark period. For the outdoor cultures in wastewater, dry weight was determined only at the start and at the end of the experiment, while the growth was followed daily by measuring the optical density at 750 nm (Varian Cary 50 spectrophotometer). For 1-L culture experiments the growth was followed daily by dry weight determination.

Lipid content of samples collected during growth was determined according to Marsh and Weinstein [19]. The neutral lipid fraction was separated by using an organic solvent extraction method, which was developed *ad hoc*, and characterized via gas chromatography. The algal mass was extracted in pure methanol using sonication (35 °C for 30 minutes) and vortexing (30 seconds) for 3 times. Then water was added to increase polarity and petroleum ether was added to extract the methanolic phase. This sample was in turn treated with vortex, sonicated and then centrifuged to separate the petroleum ether phase. The procedure was repeated twice. n-hexane was then added and the vortex-sonicator-centrifuge procedure was applied again. All the extracts were mixed together and activated carbon was added to eliminate pigments. After centrifugation the solvent was evaporated under nitrogen stream and the oil weight was determined. The oil was analyzed via gas chromatograph equipped with a fid detector and a capillary Zebron™ Inferno™ ZB 5HT column (Phenomenex Inc., USA).

Before the set up of tube cultures, nutrient analysis of wastewater was carried out following Solórzano [20] for ammonium nitrogen, Crumpton *et al.* [21] and Ferree & Shannon [22] for nitric nitrogen and by using Merck Spectroquant 1.14848.0001 kit for orthophosphate phosphorus.

Daily global solar radiation on the horizontal surface was obtained from LaMMA Agrometeorological Station (CNR-IBIMET, Sesto Fiorentino, Italy). Solar radiation impinging on the reactor surface was calculated from horizontal radiation data as described by Kreith and Kreider [23].

The velocity profile was determined by a frame-by-frame analysis of the diffusion rate of a dye injected inside the reactor according to Giannelli *et al.* [24] and Hu and Richmond [25].

In the mixing experiments, volumetric productivities at different mixing rates, for each level of irradiance considered, were analysed by one-way ANOVA. Difference between treatments (different air-flow rates) were compared by Tukey's multiple comparison test, with a significance level of $P<0.05$. Differences among productivities obtained during the growth with industrial grey water in bubbled tubes for each strain were also compared with one-way ANOVA and Tukey's multiple comparison test with a level of significance of $P<5\%$.

3. Results and discussion

3.1 Influence of mixing rate on volumetric productivity of Nannochloropsis *sp. F&M-M24*

The influence of mixing in algae cultivation has been studied extensively because of its relevance in determining yield and stability [26]. Typically studies show that higher turbulence enhances productivity [25, 27, 28], although data demonstrating no effect have also been reported [29].

Table 1 reports the average volumetric productivity obtained at the three different air-flow rates tested for different ranges of solar radiation impinging on the GWPs. As expected, a rise in solar radiation led to an overall increase in *Nannochloropsis* sp. F&M-M24 productivity. A less expected result was that the effect of turbulence on productivity depended on the level of solar radiation. During the experimental period, the daily global solar radiation impinging on the reactors varied in the range 2.1 - 23.8 MJ m^{-2} [reactor surface] d^{-1}. At low and medium irradiance levels (2-8 and 8-16 MJ m^{-2} [reactor surface] d^{-1}) volumetric productivities did not show a defined relationship with the mixing rate, while for radiation levels above 16 MJ m^{-2} [reactor surface] d^{-1} the culture subjected to the highest level of turbulence (0.45 L L^{-1} min^{-1}) reached the highest volumetric productivity (Tab. 1). At 0.45 L $L^{-1}min^{-1}$, the productivity increased approximately of 10% and 45% with respect to the cultures bubbled with 0.15 and 0.05 L L^{-1} min^{-1}, respectively. The highest volumetric productivity (0.205±0.085 g L^{-1} d^{-1}) attained corresponded to an areal productivity of 8.2 g m^{-2} [reactor surface] d^{-1}.

The data obtained in this study confirm the positive effect of mixing rate on productivity of algal cultures at high solar irradiances [25, 28]. This effect is significant only at relatively high culture concentrations, while for low concentrations an increase in mixing rate does not lead to positive effects on productivity [28]. The effect of mixing on photosynthesis is mainly related to the creation of a more favourable light regime

inside the reactors. Increasing the air-flow results in increased liquid velocities and light/dark cycle (L/D) frequency and therefore in higher photosynthetic efficiency [24, 27, 28, 30]. In a 20-L GWP, the velocity of the liquid phase increased with increasing air-flow rate (6.2, 9.3, 22.3, 30.2 and 35.6 cm s^{-1} with air-flow rates of 0.05, 0.15, 0.30, 0.45 and 0.60 L L^{-1} min^{-1}, respectively). This contributed to the creation of more favourable L/D cycle frequency that resulted, in the sunniest days, in a greater volumetric productivity for the GWP with the highest level of turbulence (0.45 L L^{-1} min^{-1}). As a general principle the higher the intensity of the light source, the higher becomes the optimal population density and the greater will be the effect that mixing has on productivity [25, 28]. It is however difficult to establish with certainty whether the increase in productivity with higher turbulence measured at high levels of radiation was due to an improved light regime (shorter L/D) or to a combination of factors. High air-flow rates in fact, also reduce dissolved O_2 thanks to an increased outgassing. This prevents the build-up of high levels of oxygen in the culture that could reduce the overall solar conversion efficiency by increasing losses of biomass due to photorespiration [4] and may damage cell components (photooxidation). In our experiments, dissolved oxygen concentrations in a typical sunny day reached the maximum level of 270-290% of air saturation in 0.05 and 0.15 L L^{-1} min^{-1} bubbled GWPs, with respect to 130% in the 0.45 L L^{-1} min^{-1} bubbled reactor (Fig. 1). High oxygen levels, in combination with high irradiance, may have further contributed to reduce volumetric productivity in reactors at lower levels of mixing. Experiments carried out with *Arthrospira platensis* in Flat Alveolar Panels showed that the higher productivities obtained with higher air-flow rates derived from an improved light regime (increasing productivity with increasing air-flow rates at constant low-level pO_2) as well as from increased oxygen degassing (increasing productivity with decreasing pO_2 at constant high-level air-flow rate) [31].

The energy consumption for mixing in photobioreactors is not negligible. In GWP of 4.5-cm thickness, an air-flow rate of 0.45 L $L^{-1}min^{-1}$ and an average volumetric productivity of 0.3 g L^{-1} d^{-1} the energy consumption equals the energy stored into the biomass, reducing to zero the energy gain. It is therefore clear how pneumatically induced mixing represents one of the major expenditures in the management of GWP reactors [14]. A tuning of the mixing rate, according to hour by hour changes of irradiance will lead to higher algal productivity or at least to the same productivity with significant energy savings. In particular, at low light intensities (for example early in the morning or late in the evening for N-S oriented vertical GWP or during the central hours for an E-W facing GWP, or in cloudy weather) turbulence can be kept low. On the

contrary, when irradiance is high and able to sustain high photosynthetic rates and productivities, mixing intensity should be increased accordingly. Mixing may be reduced to the lowest air-flow rate during the night.

3.2 Effect of nitrogen starvation on Nannochloropsis *sp. F&M-M24 and lipid characterization of the biomass*

Cultivation of *Nannochloropsis* sp. F&M-M24 was carried out outdoors in a 590-L, 10-m long, first generation GWP [15]. A daily dilution of about 40% using nitrogen deprived F medium was applied.

Biomass concentration progressively decreased from 1.32 g L^{-1} on the second day of culture to 0.38 g L^{-1} on the last day (6^{th}) of the experiment. The decrease very likely was due to lack of nitrogen for cell division processes.

The lipid content increased from 28% of the dry biomass at the start of the experiment, to 46% at day 3, 50% at day 4, 53% at day 5 and 64% at the end of the experiment (day 6). Similar results in terms of lipid content of nitrogen starved cultures were obtained with the same strain, though lipid content higher than 60% was reached in a shorter time [2], also thanks to a more stable sunny weather. An average lipid productivity of 0.11 g L^{-1} d^{-1} was obtained, which is about half the value reported by Rodolfi et al. [2], due to the longer time needed to start lipid accumulation.

In Figure 2 the composition of the neutral lipid fraction of *Nannochloropsis* sp. F&M-M24 biomass obtained at the end of the nitrogen starvation experiment is compared to that of a biomass obtained in nitrogen sufficiency. The TAG content of the neutral lipid fraction, which under nutrient sufficiency was below 5%, under nitrogen starvation increased to about 95%. This confirms previous findings [32] that this strain accumulates mainly neutral lipids as energy reserve during nitrogen starvation. Although the biomass obtained under nitrogen sufficiency usually has a lower lipid content than starved biomass ([2], [32]) and a lower neutral lipid level [32], it showed a composition of some interest for biodiesel production, as the main components were free saturated and monounsaturated fatty acids or their derivatives and phytol. Free fatty acids can be used as a feedstock for biodiesel production using an acidic catalytic system [32], and phytol was found to be suitable for gasoline production [33].

Also the fatty acid composition of TAG changed drastically during the starvation process (Fig. 3). In particular, the TAG profile of starved algal oil was characterized by very high amounts of palmitic, palmitoleic and oleic acids and by low levels of linoleic acid, C20:2 and C20:1 compared with the profile of algal oil obtained from biomass grown in nutrient sufficiency (except palmitoleic acid, present in similar percentages). These changes in nutrient starved *Nannochloropsis* have been widely reported in the literature [2; 34; 35]. On the basis of all the modifications described above it is possible to point out that through nitrogen starvation the quality of the algal oil can be modified, thus making it suitable to comply with biodiesel standards [32].

3.3 Cultivation in an industrial wastewater

Preliminary experiments were performed with two *Scenedesmus* strains using industrial grey water originating from the TAR Gasification unit of IGCC (Integrated Gasification Combined Cycle) of an oil refinery plant. The aim was to assess grey water toxicity and suitability to sustain algal growth using this algal genus known for its ability to grow in polluted waters.

In outdoor laboratory scale raceway ponds, the productivities reached were very low but similar in the control and the grey water cultures of two co-cultivated strains of *Scenedesmus* sp. In the first experiment, the culture grown in a medium containing 17% grey water reached a productivity of 3.7 g m^{-2} d^{-1} while the control attained 3.4 g m^{-2} d^{-1}, with an average global solar radiation of 4.0 ± 0.5 MJ m^{-2} d^{-1}. In the second experiment, the productivities were of 2.0 and 2.1 g m^{-2} d^{-1} in 48%-grey water and control cultures, respectively, with an average global solar radiation of 3.3 ± 0.7 MJ m^{-2} d^{-1}. The low productivities were partly due to the low solar radiation available and supra-optimal pH values.

Microscope observations performed on the cultures grown at the lower concentration of grey water showed mostly healthy algal cells morphologically similar to those of the control culture. *Scenedesmus* sp. 3PAV3 resulted always dominant over *Scenedesmus* sp. PRA. The latter showed suffering cells when cultured in 48% grey water, whereas *Scenedesmus* 3PAV3 was still healthy and similar to the control.

Given the lack of lethal toxicity of grey water towards *Scenedesmus*, the water was used in experiments with *Nannochloropsis* sp. F&M-M24 in bubbled tubes. The experiments showed that this strain was able to grow in grey water at concentrations up to 50% of the culture medium. At this wastewater concentration, the

growth started with a slight delay compared to the cultures at lower concentrations and to the control (Fig. 4). Productivities (Fig. 5) were not significantly different (P>5%) among cultures grown in grey water and in the control medium. The productivities obtained with 10% wastewater were, however, lower than those of the control (-33%) and of the cultures grown in 25 and 50% wastewater (-33 and -18%, respectively), likely because of the low nitrogen concentration in the medium. *Scenedesmus* sp. 3PAV3 showed as well a delayed onset of the growth, that was also significantly hampered, when cultured at a 50% wastewater concentration (Fig. 4). The productivities in the control culture of *Scenedesmus* sp. 3PAV3 were similar to those of *Nannochloropsis* sp. F&M-M24 (Fig. 5). In grey water, productivities of *Scenedesmus* sp. 3PAV3 were lower than in the control and progressively decreased as wastewater concentration increased. A significant difference emerged between the productivity obtained in the control culture and that obtained in the 50% wastewater culture (P<1%).

The ability of *Nannochloropsis* sp. F&M-M24 to grow in a medium containing formate and cyanide is worth emphasizing. Formate metabolism has been studied mainly in higher plants. This molecule can also be a product of algae metabolism [36]. It can be used as basis for biosynthesis of other molecules and as a source of carbon dioxide in the absence of bicarbonate [37-39]; it possesses radical scavenging activity and can protect the cell against photoinhibition [40]. On the other hand, formate can inhibit carbonic anhydrase [41]. No data on toxicity towards algae are reported in the literature. Cyanide is a well known poison for algal cells, as it blocks photosynthesis, respiration (except, in some algae, the so-called cyanide-resistant respiration) and inhibits nitrate absorption. Österlind [42, 43] found a highly cyanide-resistant strain of *Scenedesmus* (*Desmodesmus*) *quadricauda*, which showed respiration up to concentrations as high as 0.1 M, though nitrate absorption was inhibited already at 10 μM. *Scenedesmus obliquus* was grown in culture media containing up to 400 mg L^{-1} of cyanide to evaluate its potential in removal and, though growth was significantly reduced already at 100 mg L^{-1} of cyanide, the alga did not die [44]. The loss of pigmentation observed at 400 mg L^{-1} (the only concentration studied) was similar to that observed during this work in *Scenedesmus* sp. 3PAV3 cultures grown in 50% and, to a lower extent, 25% grey water, though the concentration of cyanide in this work was about two orders of magnitude lower. *Nannochloropsis gaditana* showed inhibition of O_2 evolution at 250 μM KCN, and this inhibition was reversed by increasing the cell internal pool of carbon dioxide/bicarbonate [45]. It was not possible to establish which component of the

industrial grey water hampered *Scenedesmus* sp. 3PAV3 and delayed the onset of *Nannochloropsis* sp. F&M-M24 growth at the 50% concentration (about 550 and 8 mg L^{-1} of formate and cyanide, respectively). It likely was not the effect of a single molecule, but the combined effect of several wastewater components.

The use of wastewaters for microalgae cultivation has two main advantages: to provide a source of nutrients (mainly nitrogen and phosphorus) at low cost and to allow cultivation without competing for potable and irrigation water. Besides, this would allow to recycle a waste which has a negative input in the balance of municipalities, farmers and industries. The use of wastewater is seen by many authors as a necessity to permit significant cost reduction in the biofuel production process [2, 46-50]. In spite of the general accordance on this point, most of the data concerning algal growth in wastewaters derive from studies aimed at defining their removal ability for wastewater treatment. Studies of algae, particularly marine algae, aimed at biofuel production and grown in wastewaters are not numerous, also at laboratory scale [51-54]. The data presented in this study on the growth of *Nannochloropsis* sp. F&M-M24 (an alga on which interest is growing due to its ability to accumulate oil under stress) in wastewater is of particular interest, although obtained at laboratory scale.

4. Conclusions

The composition and the much increased amount of TAG after the starving process makes *Nannochloropsis* F&M-M24 one of the best candidates to provide large amounts of oil rich-biomass. The main drawback of this technology remains the high production cost of the starved biomass. In first generation GWP this cost was calculated to be higher than 11 € kg^{-1} [55]. Although algal biomass cost could be significantly reduced (to about 2 € kg^{-1}) by coupling open ponds to photobioreactors in a two-stage process, as already suggested [2, 12], further reduction of cost production in photobioreactors is necessary. Power consumption for mixing still represents one of the main limitations of air-bubbled and pump-mixed reactors in microalgae biomass production as biofuel feedstock. Reducing mixing rate in the GWP seems possible, especially by automatically tuning the mixing rate. Tuning the air-flow rate from 0.15 to 0.45 L L^{-1} min^{-1} according to hourly solar radiation impinging on GWP (at Florence latitude), could reduce the annual energy consumption for mixing of 40%, with respect to a constant air flow rate of 0.3 L L^{-1} min during the day and of 0.15 L $L^{-1}min^{-1}$ during night. The Net Energy Ratio (NER), expressing how efficiently the algae biomass is produced,

could be increased of 10%. The use of industrial, domestic or agricultural wastewaters could further improve energy and economic balance of algae production in GWP reactors. From our estimate (Bassi & Tredici, unpublished data) the use of wastewaters as source of nutrients could contribute to decrease the final biomass production cost of about 7% and to increase the final NER of 27% (considering an overall areal productivity [56] of 20 g m^{-2} d^{-1} and a production period of 200 days per year). However, this is not yet sufficient to make economically viable algae oil and further research is still necessary.

5. References

[1] Chisti, Y. (2007). Biodiesel from microalgae. Biotecnol. Adv., 25, 294-306.

[2] Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng., 102, 100-112.

[3] Williams, P.J.B., Laurens, M.L. (2010). Microalgae as biodiesel & biomass feedstock: review & analysis of the biochemistry, energetic & economics. Energy Environ. Sci., 3, 554-590.

[4] Tredici, M.R. (2010). Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels,1, 143-162.

[5] Ben-Amotz, A. (2008). Bio-fuels and CO_2 capture by algae. 11th Congress of the International Society for Applied Phycology, Galway, Ireland, June 21-30, 2008.

[6] Kadam, K. (2001). Microalgae production from power plant flue gas: environmental implications on a Life Cycle Basis. NREL/TP-510-29417.

[7] Clarence, A.F., Resurreccion, E.P., White, M.A., Colosi, L.M. (2010). Environmental Life cycle comparison of algae to other bio-energy feedstock. Environ. Sci. Technol., 44, 1813-1819.

[8] Hassannia, J. (2009). A Project-Based Perspective: Algae Biofuels Economic Viability, Green Car Congress, 21 April 2009.

[9] Lardon, L., Helias, A., Salve, B., Stayer, J.P., Bernard, O. (2009). Life-Cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol., 43, 6475-6481.

[10] Stephenson, A.L., Kazamia, E., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G. (2010). Life-cycle assessment of potential algal biodiesel production in the United Kingdom: a comparison of raceways and air-lift tubular bioreactors. Energy Fuels, 24, 4062-4077.

[11] Tredici, M.R., Chini Zittelli, G., Rodolfi, L. (2010). Photobioreactors. In M.C. Flickinger, (Ed.), Encyclopaedia of Industrial Biothecnology. New York, NY: John Wiley & Sons, Inc.

[12] Chini Zittelli, G., Rodolfi, L., Bassi, N., Biondi, N., Tredici, M.R. (2012). Photobioreactors for microalgae biofuel production. In M. Borowitzka, N. Moheimani, (Eds.), Algae for biofuels and energy, (in press). Dodrecht: Springer.

[13] van Beilen, J.B. (2010). Why microalgal biofuels won't save the internal combustion machine. Biofuels Bioprod. Bioref., 4, 41-52.

[14] Bassi, N., Rodolfi, L., Chini Zittelli, G., DelBimbo, L., Tredici, M.R. (2010). "The Green Wall Panel": potential and limitation of a low-cost disposable photobioreactor. 4th Algal Biomass Summit (ABO), Phoenix, USA, September 26-30, 2010.

[15] Tredici, M.R., Rodolfi, L. (2004). Reactor for industrial culture of photosynthetic micro-organisms. PCT Patent WO 2004/074423 A2.

[16] Guillard, R.R.L., Ryther, J.H. (1962). Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. Can. J. Microbiol., 8, 229-239.

[17] Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol., 111, 1-61.

[18] Chini Zittelli, G., Pastorelli, R., Tredici, M.R. (2000). A Modular Flat Panel Photobioreactor (MFPP) for indoor cultivation of *Nannochloropsis* sp. under artificial illumination. J. Appl. Phycol., 12, 521-526.

[19] Marsh, J.B., Weinstein, D.B. (1966) Simple charring methods for determination of lipids. J. Lipid Res., 7, 574-576.

[20] Solórzano, L. (1969). Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr., 14, 799-801.

[21] Crumpton, W.G., Isenhart, T.M., Mitchell, P.D. (1992). Nitrate and organic N analyses with second-derivative spectroscopy. Limnol. Oceanogr., 37, 907-913.

[22] Ferree, M.A., Shannon, R.D. (2001). Evaluation of a second derivative UV/Visible spectroscopy technique for nitrate and total nitrogen analysis of wastewater samples. Water Res., 35, 327-332.

[23] Kreith, F., Kreider, J.F. (1978). Principles of solar engineering. New York, NY: McGraw Hill.

[24] Giannelli, L., Scoma, A., Torzillo, G. (2009). Interplay between light intensity, chlorophyll concentration and culture mixing on the hydrogen production in sulfur-deprived *Chlamydomonas reinhardtii* cultures grown in laboratory photobioreactors. Biotechnol. Bioeng., 104, 76-90.

[25] Hu, Q., Richmond, A. (1996). Productivity and photosynthetic efficiency of *Spirulina platensis* as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. J. Appl. Phycol., 18, 139-145.

[26] Merchuk, J.C., Garcia Camacho, F., Molina Grima, E. (2007). Photobioreactor design and fluid dynamics. Chem. Biochem. Eng. Quart., 21, 345-355.

[27] Hu, Q., Gutterman, H., Richmond, A. (1996). A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. Biotechnol. Bioeng., 51, 51-60.

[28] Richmond, A. (2004). Biological principles of mass cultivation. In A. Richmond, (Ed.), Handbook of microalgal culture: Biotechnology and Applied Phycology, (pp. 125-177). Oxford: Blackwell Science.

[29] Weissman, J.C., Goebel, R.P., Benemann, J.R. (1988). Photobioreactor design: mixing, carbon utilization, and oxygen accumulation. Biotechnol. Bioeng., 31, 336-344.

[30] Richmond, A., Wu, Z. (2001). Optimization of a flat plate glass reactor for mass production of *Nannochloropsis* sp. outdoors. J. Biotech., 85, 259-269.

[31] Tredici, M.R., Chini Zittelli, G. (1997) Cultivation of *Spirulina* (*Arthrospira*) *platentis* in flat plate reactors. In A. Vonshak, (Ed.), *Spirulina* (*Arthrospira*) *platentis*: Physiology, cell-biology and biotechnology, (pp 117-130). Taylor & Francis, London.

[32] Bondioli, P., Della Bella, L.., Rivolta, G., Casini, D., Prussi, M., Chiaramonti, D., Chini Zittelli, G., Bassi, N., Rodolfi, L., Tredici, M.R. (2010). Oil production by the marine microalga *Nannochloropsis* sp. F&M-M24. Proceedings of the 18th European Biomass Conference and Exhibition, May 3-7, 2010, Lyon, France, (pp. 538-541).

[33] Tracy, N.I., Crunkleton, D.W., Price, G.L. (2010) Gasoline production from phytol. Fuel, 89, 3493-3497.

[34] Hu, H., Gao, K. (2006) Response of growth and fatty acid compositions of *Nannochloropsis* sp. to environmental factors under elevated CO_2 concentration. Biotechnol. Lett., 28, 987-992.

[35] Khozin-Goldberg, I., Boussiba, S. (2011) Concerns over the reporting of inconsistent data on fatty acid composition for microalgae of the genus *Nannochloropsis* (Eustigmatophyceae). J. Appl. Phycol., 23, 933-934.

[36] Ohta, S., Miyamoto, K., Miura, Y. (1987). Hydrogen evolution as a consumption mode of reducing equivalents in green algal fermentation. Plant Physiol., 83, 1022-1026.

[37] Allison, R.K., Skipper, H.E., Reid, M.R., Short, W.A., Hogan, G.L. (1954). Studies on the photosynthetic reaction. II. Sodium formate and urea feeding experiments with *Nostoc muscorum*. Plant Physiol., 29, 164-168.

[38] Igamberdiev, A.U., Lea, P.J. (2002). The role of peroxisomes in the integration of metabolism and evolutionary diversity of photosynthetic organisms. Phytochem., 60, 651-674.

[39] Igamberdiev, A.U., Bykova, N.V., Kleczkowski, L.A. (1999). Origins and metabolism of formate in higher plants. Plant Physiol. Biochem., 37, 503-513.

[40] Shiraishi, T., Fukusaki, E., Miyake, C., Yokota, A., Kobayashi, A. (2000). Formate protects photosynthetic machinery from photoinhibition. J. Biosci. Bioeng., 89, 564-568.

[41] Stemler, A.J. (1997). The case for chloroplast thylakoid carbonic anhydrase. Physiol. Plant., 99, 348-353.

[42] Österlind, S. (1951). Anion absorption by an alga with cyanide resistant respiration. Physiol. Plant., 4, 528-534.

[43] Österlind, S. (1952). Inhibition of respiration and nitrate absorption in green algae by enzyme poisons. Physiol. Plant., 5, 292-297.

[44] Gurbuz, F., Ciftci, H., Akcil, A., Gul Karahan, A. (2004). Microbial detoxification of cyanide solutions: a new biotechnological approach using algae. Hydrometall., 72, 167-176.

[45] Huertas, E., Colman, B., Espie, G.S. (2002). Mitochondrial-driven bicarbonate transport supports photosynthesis in a marine microalga. Plant Physiol., 130, 284-291.

[46] Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J., 54, 621-639.

[47] Li, Y., Horsam, N., Wu, N., Lan, C.Q., Dubois-Calero, N. (2008). Biofules from microalgae. Biotechnol. Prog., 24, 815-820.

[48] Mata, T.M., Martins, A.A., Caetano, N.S. (2010). Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy. Rev.14, 217-232.

[49] Park, J.B.K., Craggs, R.J., Shilton, A.N. (2011). Wastewater treatment high rate algal ponds for biofuel production. Biores. Technol., 102, 35-42.

[50] Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx. U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. (2008). Second generation biofuels: High-efficiency microalgae for biodiesel production. Bioenerg. Res., 1, 20-43.

[51] An, J.Y., Sim, S.J., Lee, J.S., Kim, B.W. (2003). Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. J. Appl. Phycol., 15, 185-191.

[52] Mandal, S., Mallick, N. (2011). Waste utilization and biodiesel production by the green microalga S*cenedesmus obliquus*. Appl. Environ. Microbiol., 77, 374-377.

[53] Pittman, J.K., Dean, A.P., Osundeko, O. (2011). The potential of sustainable algal biofuel production using wastewater resources. Biores. Technol., 102, 17-25.

[54] Woetz, I., Feffer, A., Lundquist, T., Nelson, Y. (2009). Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. J. Environ. Eng., 135, 1115-1122.

[55] Bassi, N. (2010). Energetic and economic assessment of a disposable panel reactor for *Nannochloropsis* sp. biomass production. PhD thesis, University of Florence.

[56] Tredici, M.R. (2004) Mass production of microalgae: photobioreactors. In A. Richmond, (Ed.), Handbook of microalgal culture: Biotechnology and Applied Phycology, (pp. 178-214). Oxford: Blackwell Science.

Table 1 - Influence of air-flow rate and solar radiation on volumetric productivity of *Nannochloropsis* sp. F&M-M24 cultured in N-S facing, vertical 20-L GWPs. Volumetric productivity is reported as mean value ± standard deviation. Different letters represent significant differences between treatments within each level of irradiance.

Solar radiation range (MJ m^{-2} [reactor surface] d^{-1})	**No. days**	**Average solar radiation** (MJ m^{-2} [reactor surface] d^{-1})	**Volumetric productivity** ($g\ L^{-1}\ d^{-1}$) **Air-flow rate** ($L\ L^{-1} min^{-1}$)		
			0.45	**0.15**	**0.05**
2-8	3	4.3	0.073±0.07[a]	0.051±0.03[a]	0.087±0.081[a]
8-16	15	13.2	0.159±0.06[a]	0.171±0.077[a]	0.161±0.074[a]
16-24	23	19.8	0.205±0.085[a]	0.188±0.056[a]	0.141±0.071[b]

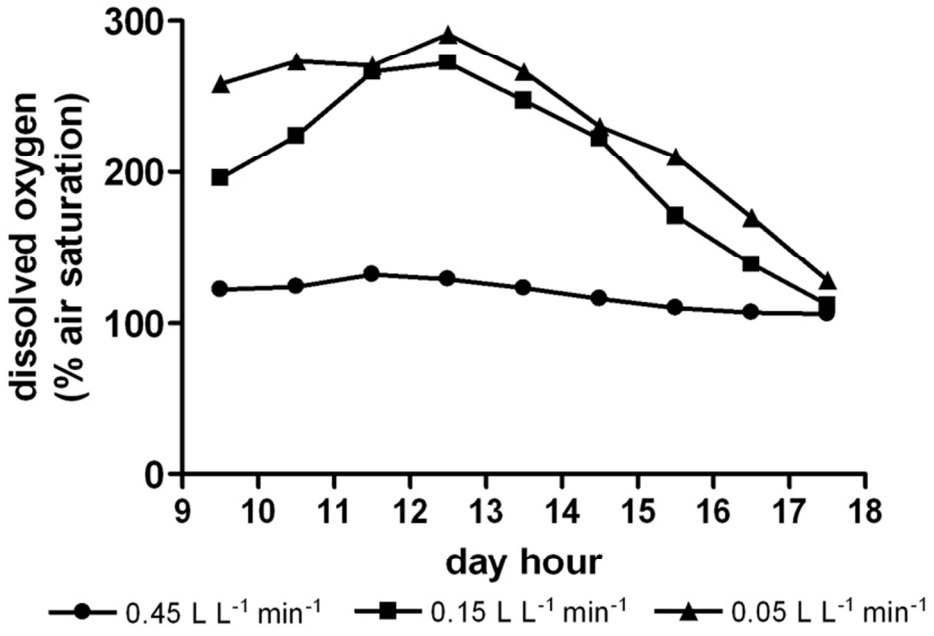

Fig. 1 Diurnal variation of dissolved oxygen in Nannochloropsis sp. F&M-M24 cultures grown in GWPs at different air-flow rates. Values were measured in a winter sunny day (19 MJ m-2 [reactor surface] d-1).
75x52mm (300 x 300 DPI)

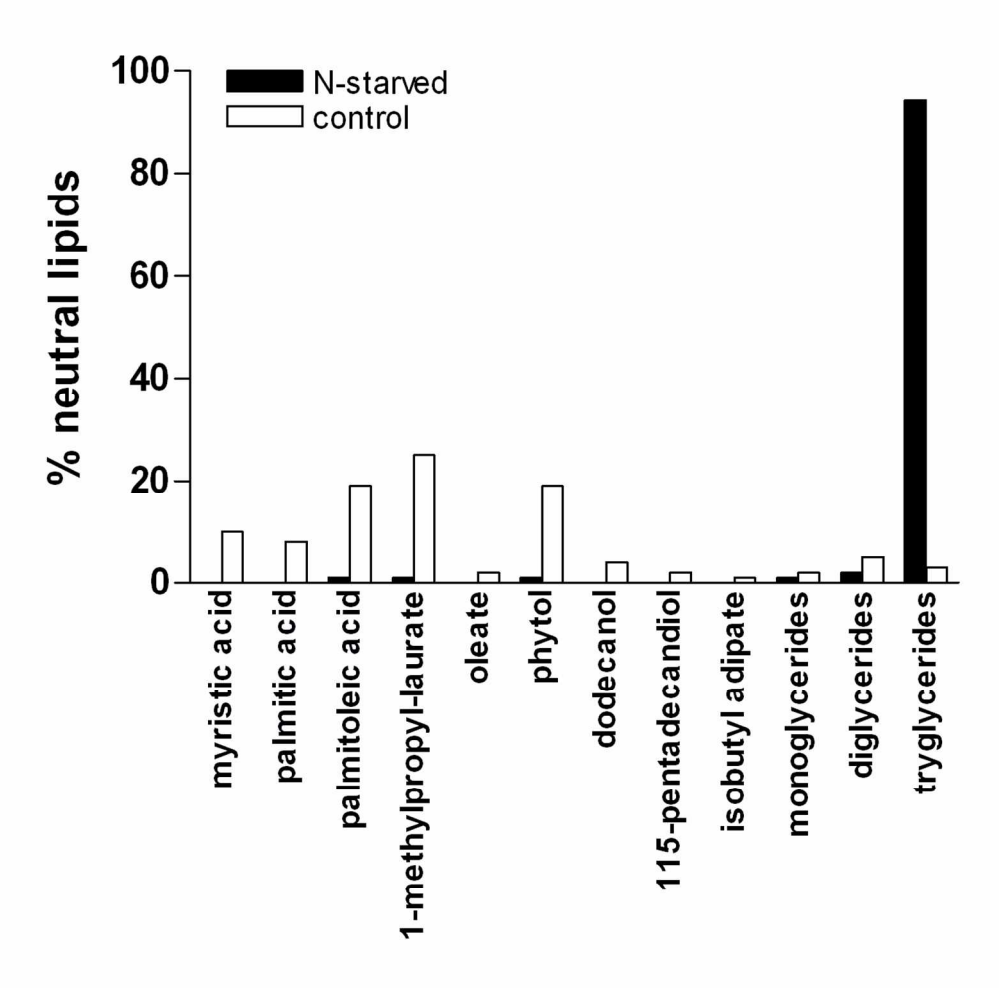

Fig. 2 Composition of the neutral lipid fraction of Nannochloropsis sp. F&M-M24 grown under nitrogen deprived and nitrogen sufficient conditions (control).
116x114mm (300 x 300 DPI)

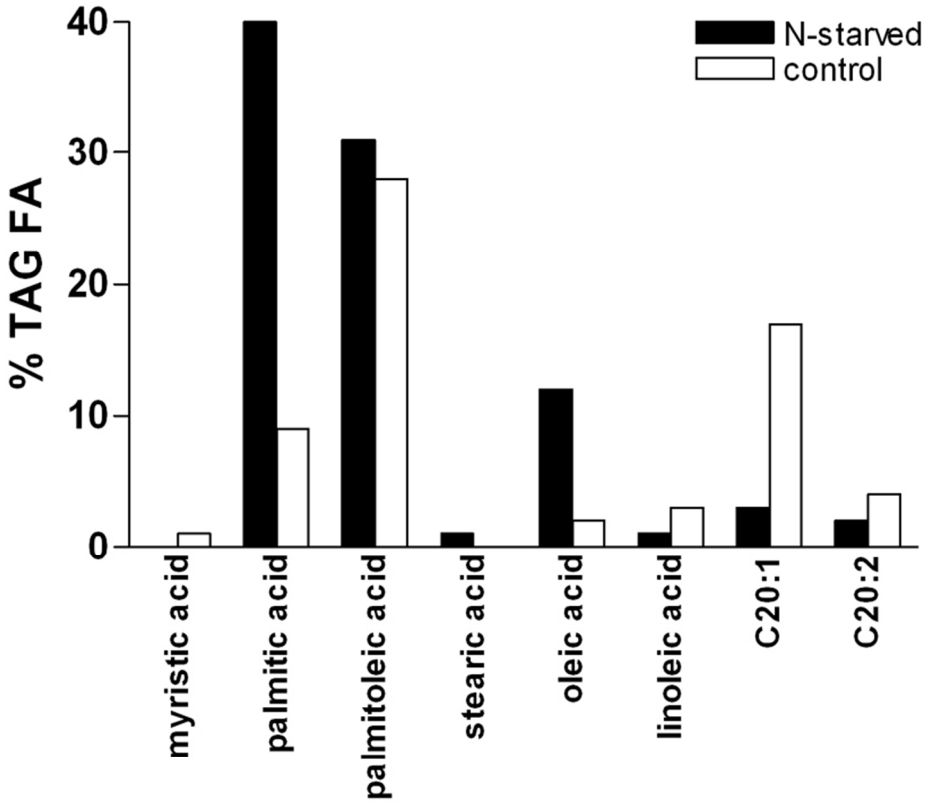

Fig. 3 Fatty acids composition of the TAG fraction of Nannochloropsis sp. F&M-M24 biomass obtained in nitrogen starved and sufficient conditions.
75x68mm (300 x 300 DPI)

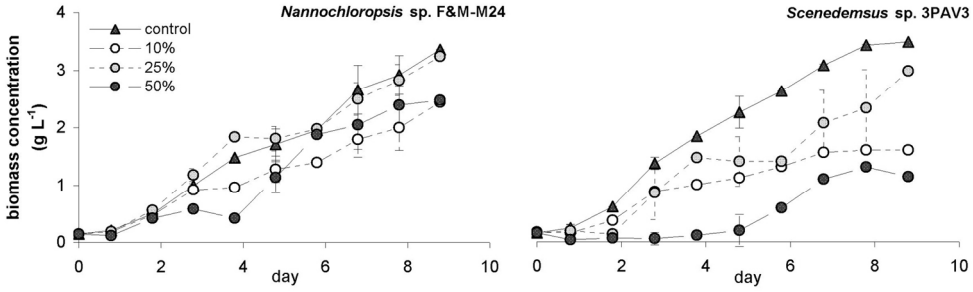

Fig. 4 Growth of Nannochloropsis sp. F&M-M24 and Scenedesmus sp. 3PAV3 in bubbled tubes at different concentration of grey water and in the control.
150x46mm (300 x 300 DPI)

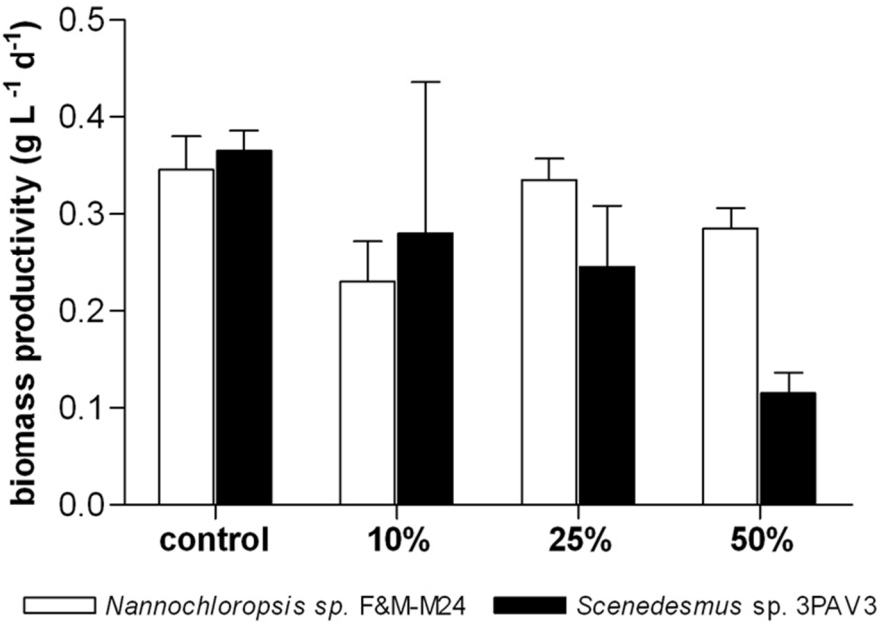

Fig. 5 Biomass productivity of Nannochloropsis sp. F&M-M24 and Scenedesmus sp. 3PAV3 in bubbled tubes at different concentration of grey water and in the control.
75x52mm (300 x 300 DPI)