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Oil production by the marine microalgae *Nannochloropsis* sp. F&M-M24 and *Tetraselmis suecica* F&M-M33

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ABSTRACT

Nannochloropsis sp. F&M-M24 and *Tetraselmis suecica* F&M-M33 were cultivated outdoors in Green Wall Panels under nutrient deficiency to stimulate oil synthesis. Under nitrogen deprivation, *Nannochloropsis* attained average biomass and lipid productivities of 9.9 and 6.5 g m^{-2} day^{-1}, respectively. Starved *Tetraselmis* cultures achieved a biomass productivity of about 7.6 g m^{-2} day^{-1} and a lipid productivity of 1.7 g m^{-2} day^{-1}. Lipids represented 39.1% and 68.5% of non-starved and starved *Nannochloropsis* biomass, respectively. Starvation did not increase lipid content in *Tetraselmis* biomass. Important differences in lipid classes and in fatty acid composition were observed under the different cultivation conditions for both microalgae.

1. Introduction

Biodiesel is currently produced from oils and fats of vegetable or animal origin. Biodiesel is constituted of fatty acid methylesters (FAMEs) that are normally obtained by means of a simple alkali catalyzed chemical reaction, from triglycerides and methanol, with parallel production of glycerol as a by-product. The general balance of this process, starting from 100 kg of neutral fat, is approximately 100 kg of biodiesel and 10 kg of glycerol, with a parallel consumption of 10 kg of methanol. The demand of natural oils for biodiesel production is constantly raising in Europe and worldwide (Subramaniam et al., 2010). For this reason and in order to avoid the competition with the food market, research is today focusing on alternative renewable feedstocks for biodiesel production.

Microalgal biodiesel is technically feasible. However, algal biomass is, at present, largely too expensive to compete with petrodiesel. Besides, the energy balance of algae biomass production is still not sufficiently positive (Tredici, 2010).

Microalgae have been identified as a possible source of new generation biofuels since they do not compete with food and feed crops, attain higher oil yields than currently available agricultural crops, and can be cultivated in seawater on non-arable land (Greenwell et al., 2010; Tredici, 2010). During last years several research projects have been launched and numerous papers have been published on this subject (Amaro et al., 2011; Gouveia and Oliveira, 2009; Griffiths and Harrison, 2009; Huerlimann et al., 2010; Mutanda et al., 2011; Scott et al., 2010). Oil-rich algae (the so-called oleaginous species) can be grown either autotrophically or heterotrophically. In the first mode, carbon dioxide (or bicarbonate) is used as the sole carbon source, which is incorporated by means of the photosynthetic process, while in the second mainly organic molecules are used for growth. The photosynthetic process needs light to take place and hence algal cultivation must be carried out with artificial light or under sunlight, while the heterotrophic process can take place in classical fermenters. Despite the metabolic flexibility of microalgae and the impressive progress achieved in these years by algal biotechnology, a long way must still be run before microalgae might be exploited for commercial biofuel production (Tredici, 2010).

A first crucial point to be cleared is the maximum oil yield attainable with microalgae cultures. This is strictly dependent on the selected microorganism, the geographical location of the production plant and the culture conditions (Hu et al., 2008). According to Rodolfi et al. (2009) and Studt (2010), the potential oil yield of microalgae cultures is from 5 to 20 times that of oil

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palm, generally recognized as the most productive crop at industrial scale. Another interesting challenge is related to the energy balance. It is often ignored that novel and fascinating algal technologies and widely advertised photobioreactors may require more energy than they can provide (Tredici, 2010). This is one of the reasons why the old raceway pond mixed with low energy input paddle wheels is still the main culture system used to commercially grow microalgae. Useful data to draw an economic balance have been recently published (Ratledge and Cohen, 2008). The authors estimated that it would be difficult to produce algae oil for less than €5000 per tonne, a cost approximately ten times higher than that of biofuels in the current conditions of fossil oil price. Among the possible strategies to reduce costs of algae biofuels there are: (1) increase productivity and oil content; (2) use of natural seawater or wastewaters; (3) reduce energy requirements for cultivation (e.g. mixing and cooling) and harvesting and (4) avoid drying of the biomass before extraction. An important issue to be solved in order to improve the microalgae-to-biofuel chain is the high water content of the collected biomass. After harvesting (e.g. by centrifugation) the biomass has a moisture of 70% or higher and, in the best case, an oil content of 50% (on dry weight basis). Therefore the oil content of wet algal biomass is comparable to that of an oil seed containing approximately 15% of oil, such as an olive fruit. If algae could be processed such as olives and oil extracted using a wet technology, the high costs of drying could be saved. In this regards, it is worth mentioning that similar lipid yields have been obtained with hexane and supercritical carbon dioxide extraction from wet paste (30% solids) and dry biomass of a marine *Chlorococcum* (Halim et al., 2011). Nevertheless, this approach may jeopardize the valorisation of the residual biomass (as for example for feed use). In this case it will be necessary, after oil extraction, to drastically reduce the water content of the residual biomass to avoid microbiological degradation. A key and often ignored fact is that algae grown under normal, nutrient sufficient, conditions can synthesize an interesting amount of lipids, but often these lipids are constituted of little amounts of glycerides or free fatty acids and of large amounts of polar lipids such as glycolipids and phospholipids (Hu et al., 2008). These lipid categories cannot be extracted using the standard crushing oil techniques and cannot be transformed in biodiesel using the existing facilities, even after partial modification. To solve this problem, Johnson and Wen (2009) suggested to carry out the direct trans-esterification of algal biomass by means of an *in situ* reaction. This solution is interesting, but the amount of chemicals to be used for biodiesel production is so high to make it practically unfeasible. An interesting and economically attractive alternative comes from the starvation technique to obtain oil rich algae biomass (Rodolfi et al., 2009). It is well known that in some species, removing nutrients such as nitrogen from the growth medium, slows down the cell division and induces a "stress" behavior in which cell size increases and neutral lipid accumulates in the cytoplasm as observed in *Chlorella vulgaris* (Stephenson et al., 2010). The potential as a source of renewable oil of the marine microalgae *Nannochloropsis* sp. F&M-M24 and *Tetraselmis suecica* F&M-M33, among a number of different algae strains, has been the focus of a recent work (Rodolfi et al., 2009). In a two-phase cultivation process (a nutrient sufficient phase to produce the inoculum followed by a nitrogen – or nitrogen and phosphorus – deprived phase to boost lipid synthesis) the crude lipid content of *Nannochloropsis* biomass increased up to 60% and the culture attained a much higher lipid productivity compared with a nutrient sufficient single-phase process. The oil production potential of this microalga in the Mediterranean climate was projected up to 20 tonnes per hectare per year. The main limitations of the technology appeared to be the high energy requirement for culture mixing and cooling. In the same work, *T. suecica* F&M-M33 was shown not to be able to accumulate large amount of lipids under nitrogen starvation confirming the results of Becker (1994). The characteristics of the lipids accumulated in the starved biomass of the two strains were not determined. These aspects are considered in the present work together with biomass and lipid productivity under nutrient deficient conditions in outdoor cultures.

2. Methods

2.1. Organisms and culture conditions

Nannochloropsis sp. F&M-M24 was cultivated outdoors under nitrogen deprivation during the summer in a 590-L, 10 m-long, 1 m-high, Green Wall Panel (GWP) (Tredici and Rodolfi, 2004). The reactor was placed facing south. The cultivation was carried out for seven days in a semi-continuous mode adopting a 44% daily dilution rate, with the exception of the first and the last two days of the experiment in which the culture was not diluted. Samples were daily withdrawn for determination of dry weight and lipid content. At the end of the experiment, the culture was harvested by centrifugation and the biomass stored at −20 °C for lipid characterization. A nutrient sufficient culture (control) was grown in a smaller Green Wall Panel operated outdoors to provide standard biomass for comparison.

Artificial seawater (Adriatic Sea Aquarium & Equipment, Rimini, Italy) at 30 g L^{-1} salinity, filtered (through 10 and 1-μm pore size filters) and added with *f* medium nutrients (Guillard and Ryther, 1962) without the nitrogen source, was used as growth medium. Nitrogen content in the medium at the beginning of the experiment was 8.2 mg L^{-1} because of nitrate brought with the inoculum, but it was quickly consumed.

T. suecica F&M-M33 was cultivated outdoors in autumn in three 16-L (0.5-m-long, 1-m-high) Green Wall Panel reactors, which were placed facing south. The cultivation was carried out for 14 days in a semi-continuous mode applying a 45% dilution rate every 2–3 days with growth medium prepared as described above. Three different nutrient conditions were tested: (i) nitrogen and phosphorus sufficient medium (control); (ii) nitrogen deprived medium and (iii) nitrogen and phosphorus deprived medium. Samples were daily withdrawn for determination of dry weight. At the end of the experiment the cultures were harvested by centrifugation and the biomasses stored at −20 °C for biochemical composition and lipid characterization.

Culture overheating was prevented by automatically spraying water on the reactor surfaces when the temperature exceeded 25 °C for *Nannochloropsis* and 28 °C for *Tetraselmis*. pH was maintained at values of about 7.6–7.8 by injecting pure CO_2 into the air stream during daylight hours.

2.2. Analytical procedures

2.2.1. Crude lipid extraction

A modified Bligh and Dyer (B&D) procedure was used (Christie, 2003). Approximately 10 g of wet algal biomass, typically containing 65% of water, were added to 60 mL of a mixture chloroform–methanol 2:1 (v/v) and treated for 15 min in a Ultraturrax homogenizer (IKA – Staufen, Germany) at 11,000 rpm at ambient temperature. After the treatment the sample was centrifuged at 4000 rpm for 15 min in a IEC CL 10 Centrifuge (Thermoscientific – Rodano, Italy). The supernatant was transferred into a 250-mL pre-weighed flask after filtration and the solid material treated three times more as described above. The pooled filtered extract (crude lipid extract) was evaporated under vacuum in a rotary evaporator and the residual solvent traces eliminated by flushing the residue with

nitrogen. The biomass moisture was evaluated by weight loss in a thermostatic oven (105 ± 2 °C).

2.2.2. Supporting the B&D extract on silica gel

Approximately 1 g of B&D extract was dissolved in 20 mL of methanol. After dissolution, 12.5 g of silica gel (Merck No. 8834, 40–230 mesh – Darmstadt, DE) were added and the sludge was stirred for 15 min. The sample was finally dried and methanol carefully removed under vacuum in a rotary evaporator. The complete methanol removal was checked by means of mass balance.

2.2.3. Chromatographic fractionation of the crude extract in lipid classes

Approximately 400 mg of the B&D extract supported over 5 g of silica gel were introduced in a chromatography column (15-mm diameter), packed with 10 g of the same silica gel suspended in chloroform. Three different fractions containing neutral lipids (200 mL of chloroform), polar lipids (200 mL of mixture acetone–methanol, 90:10 v/v) and phospholipids (200 mL of methanol) were collected respectively and carefully weighed after solvent removal.

2.2.4. Chromatographic fractionation of neutral lipids

Approximately 400 mg of the B&D extract, supported over 5 g of silica gel were introduced in a chromatography column (25 mm diameter), filled with 15 g of the same silica gel in hexane. Four different fractions containing non polar constituents such as hydrocarbons and esters of fatty acids with monoalcohols (150 mL of a mixture hexane–diethylether 98:2 v/v), triglycerides (150 mL of a mixture hexane–diethylether 90:10 v/v), diglycerides, free fatty acids and sterols (150 mL of a mixture hexane–diethylether 50:50 v/v) and monoglycerides (150 mL of diethylether) were separately collected, and weighed after solvent elimination. The efficiency of the chromatographic separation was checked by TLC (eluent system hexane–diethylether 90:10 v/v; detection with 2,7 dichlorofluorescein + UV light).

2.2.5. Determination of fatty acid composition by gas chromatography

The samples obtained from different silica gel column separations were transformed into the corresponding methylesters according to ISO 12966-2:2011 procedure 4.4 with boron trifluoride. The gas chromatographic analysis was carried out according to ISO 5508:1990, using a TRACE (Thermo Finnigan – Rodano, Italy) instrument, with split injector (split ratio 100:1, temperature 250 °C), FID detector (temperature 275 °C) with a CP WAX 52 CB capillary column (Varian, Middleburg – NL) 30 m length × 0.32 mm ID, film thickness 0.25 μm. Carrier gas was helium at a constant flow of 1 mL min^{-1}. Oven temperature programme: 140 °C (3 min) → 240 °C (5 °C/min) → 240 °C (5 min). The identification of different methylester peaks was carried out by comparison of the retention time of pure compounds.

2.2.6. Iodine value

Iodine values of methylesters fractions were calculated from fatty acid composition, as determined by gas chromatography, using the procedure reported in EN 14214:2008 +A:2009, annex B. Values of uncommon (for biodiesel) FAMEs were stoichiometrically obtained.

2.2.7. Carbohydrate and protein content

Carbohydrates and proteins were extracted and quantified according to Dubois et al. (1956) and Lowry et al. (1951), respectively, starting from lyophilized biomass.

2.2.8. Lipid content during growth

Lipid content of samples collected during growth was determined according to Marsh and Weinstein (1966).

2.2.9. Dry weight

To monitor algal growth, dry biomass concentration (expressed in g L^{-1}) was measured according to Chini Zittelli et al. (2000).

All chemicals used for analyses were of analytical grade.

3. Results and discussion

3.1. Growth and lipid accumulation in nitrogen starved cultures of Nannochloropsis sp. F&M-M24

Nannochloropsis sp. F&M-M24 was grown outdoors in a 10-m^2 GWP in nitrogen-deprived medium. Biomass productivity increased during the first three days from 6.7 to 17.2 g m^{-2} day^{-1}, then decreased to zero on the 7th day (Fig. 1). The average productivity of the whole period was 9.9 g m^{-2} day^{-1}. On day 7th, the culture was harvested by centrifugation and the biomass was stored at −20 °C for analyses. The initial crude (B&D) lipid content of the biomass was about 30% and stayed at that value during the first two days, then it increased gradually up to 68.5% at the end of the cultivation. Lipid productivity for the whole period averaged 6.5 g m^{-2} day^{-1} (Fig. 1). The data clearly show the effect of nitrogen deprivation on lipid accumulation and confirm previous results with the same strain (Rodolfi et al., 2009).

As already discussed, the nature of accumulated lipid is crucial for biodiesel production. It is well known that *Nannochloropsis*, as other oleaginous algae grown in nutrient sufficient conditions, mainly produce polar lipids that cannot be processed by existing biodiesel industrial plants. For biodiesel production a neutral oil, mainly constituted of triglycerides (TAGs) and partial glycerides that can be easily converted into the corresponding FAMEs by means of an alkali catalyzed reaction with methanol, is necessary. Free fatty acids can also be used as feedstock for biodiesel production, but in this case an acidic catalytic system or a pre-esterification step is required. To investigate the suitability of

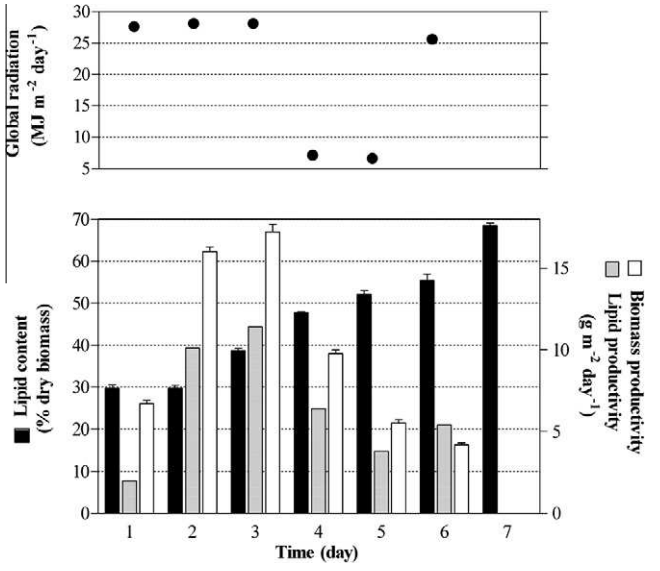

Fig. 1. Lipid content and biomass and lipid productivity (grams per square meter of illuminated reactor surface per day) of *Nannochloropsis* sp. F&M-M24 grown outdoors in a Green-Wall Panel reactor under nitrogen starvation. Global radiation is also reported.

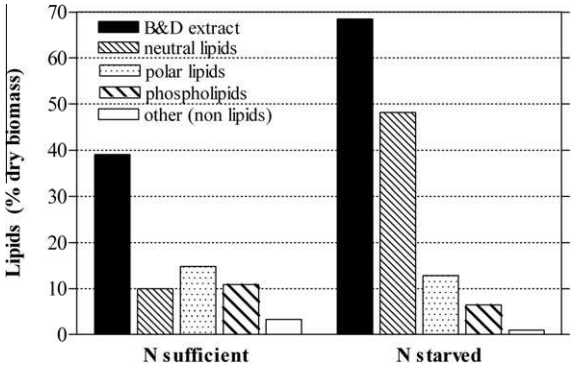

Fig. 2. Total (crude) lipid content and lipid class distribution (% dry biomass) of *Nannochloropsis* sp. F&M-M24 grown outdoors in a Green Wall Panel under nitrogen starvation and in a control culture. N starved: culture grown under nitrogen starvation; N sufficient: control culture.

Table 1
Fatty acid composition (%) of three different lipid classes (neutral-, polar- and phospho-lipids) of nitrogen starved *Nannochloropsis* sp. F&M-M24 biomass.

Fatty acid	Lipid class		
	Neutral	Polar	Phospho
C14:0	4.39	5.36	2.51
C16:0	44.49	30.10	22.20
C16:1, n-7	30.54	21.36	9.28
C18:0	1.37	1.56	1.80
C18:1, n-7 + n-9	12.83	13.73	23.62
C18:2, n-6	1.10	4.05	6.68
C18:3, n-3	0.23	0.00	1.26
C18:3, n-6	0.00	0.78	0.32
C20:2, n-?	0.02	0.00	0.00
C20:3, n-6	0.32	0.00	0.78
C20:4, n-6	1.39	1.40	12.29
C20:5, n-3	3.32	21.65	19.26
Total saturated	50.25	37.02	26.51
Total monounsaturated	43.37	35.09	32.90
Total polyunsaturated	6.38	27.88	40.59
Iodine Value[g I_2/100 g]	61.5	129.2	164.0

Nannochloropsis for biodiesel production, the lipid classes of two samples, one taken from the starved culture on day 7th of the experiment, and the second taken from the nutrient sufficient culture (control), were determined (Fig. 2). Both the total (39% of the dry biomass) and neutral lipid content (26% of the total lipid and 10% of the dry biomass) of *Nannochloropsis* grown under nutrient sufficient conditions were higher than the values typically reported for this alga suggesting that the culture was not in the active growth phase or that it was suffering from nutrient limitation. As a consequence of nutrient deprivation, the total lipid in the nitrogen starved culture reached 68.5% of the dry biomass and the neutral lipid increased to 70% of the total lipid (48% of the dry biomass) (Fig. 2). It is to remark that the increase of neutral lipids cannot be attributed solely to the mobilization of the other lipid classes. In fact, following nitrogen starvation, polar lipids (including phospholipids) decreased from 26 to 19% of the dry biomass. The data clearly show that under nitrogen starvation in *Nannochloropsis* F&M-M24, neutral lipids are mostly produced by *de novo* synthesis as already suggested (Rodolfi et al., 2009). Similar results were obtained by Suen et al. (1987) with *Nannochloropsis* sp. QII. Under nitrogen deficient conditions, this strain reached a total lipid content of 55% (against 24–30% of the culture grown in nitrogen sufficient conditions) and the major lipids represented were TAGs, polar lipids and hydrocarbons (79%, 9% and 2.5% of total lipids, respectively). These observations confirm the assumption that under nutritional conditions limiting growth, oleaginous algae may react by storing surplus energy in form of neutral fat, without an important decrease in structural lipid components (Rodolfi et al., 2009).

Table 1 shows the fatty acid composition of the different lipid fractions of the starved *Nannochloropsis* F&M-M24 biomass together with the corresponding iodine value. Large differences were found in the relative fatty acid content among the different lipid classes. In all the fractions, 16-carbon fatty acids were predominant, being as high as 75% of the total fatty acids in neutral lipids, 51% in polar lipids and 31% in phospholipids. Unsaturated fatty acids largely prevail in polar lipids (63%) and particularly in phospholipids (73.5%). Neutral lipids contain an almost equivalent amount of unsaturated and saturated fatty acids. Eicosapentaenoic acid (EPA, 20:5 n3), which represents 19–22% of the fatty acids in both phospholipids and polar lipids, is 3.3% in the neutral lipid fraction. The content of C16:0, C16:1, n-7 and C18:1, n-9 found in the neutral lipid fraction of starved *Nannochloropsis* F&M-M24 is in good agreement with the values observed in the TAGs fraction of *Nannochloropsis* by Khozin-Goldberg and Boussiba (2011). Lower amounts of these fatty acids together with higher amount in EPA in the TAGs fraction were found in *Nannochloropsis* by Hodgson et al. (1991), probably because of the mild nitrogen starvation conditions applied. As expected, docosahexaenoic acid (DHA, C22:6, n-3) was not detected in any of the lipid classes.

It is to mention that an iodine value of 61.5 calculated for the neutral lipid fraction of starved *Nannochloropsis* biomass, is significantly lower than the limit established by the EN 14214: 2008+A1:2009 of 120 g I_2/100 g of substance. Although the neutral lipid fraction of starved *Nannochloropsis* biomass fulfills biodiesel requirements for iodine value, this is not the case for its polyunsaturated fatty acid (PUFA) content, which exceeds the established limit of 1% (EN 14214 Standard). There are two possibilities to solve this problem: (i) dilution with vegetable or animal oil and (ii) separation of biodiesel fractions by means of fractional distillation. The separation of C16 and C18 methyl esters is feasible by vacuum fractional distillation and in this case the PUFA concentration can be reduced close to zero (Dieckelmann and Heinz, 1988; Johnson and Fritz, 1989).

This unit operation could also provide additional benefits in terms of unsaponifiable reduction, with the contemporary preparation of a PUFA/unsaponifiable rich fraction very attractive for cosmetic applications. If the culture of *Nannochloropsis* will ever be industrially exploited in the future, a side stream of complex lipid based products could be attained that might find application in the cosmetic/nutraceutical market, where selling volumes are low, but added values are important. Among the omega-3 polyunsaturated fatty acids, interesting is, for its high commercial value, the presence of EPA found mainly in polar lipids and phospholipids.

For a further characterization of the neutral lipids obtained from nitrogen starved *Nannochloropsis*, a chromatographic fractionation of the crude lipid extract was carried out. Four different fractions were obtained: (i) hydrocarbons, waxes and fatty acid esters with monohydric alcohols (fraction 1); (ii) triglycerides (fraction 2); (iii) diglycerides, free sterols, free fatty acids (fraction 3) and (iv) monoglycerides (fraction 4). These four fractions represented 1.5, 44.6, 3.6 and 0.4% of the dry biomass, respectively, in good agreement with the value of 48.2% of the dry biomass obtained for total neutral lipids. In conclusion, approximately 50% of *Nannochloropsis* starved biomass (namely fraction 2, 3 and 4) could be converted into biodiesel with only a small portion of the neutral lipids (fraction 1) consisting of non-polar lipids unsuitable for biodiesel production. During the separation of the less polar fractions a red extract, likely containing carotenoids, was also isolated.

3.2. Growth and lipid accumulation in nutrient deficient T. suecica F&M-M33 cultures

T. suecica F&M-M33 was grown outdoors in a 0.5 m^2 GWP under nitrogen or nitrogen and phosphorus deprivation. A nutrient sufficient culture was also setup as control. During the first week average productivities of 7.4, 7.8 and 7.4 g m^{-2} day^{-1} were attained with the control, the nitrogen starved and the nitrogen and phosphorus starved cultures, respectively. In the second week, the productivity of the control did not change (achieving an average of 7.8 g m^{-2} day^{-1}) while that of the starved cultures decreased to 2.2 g m^{-2} day^{-1} (nitrogen starved culture) and 0.7 g m^{-2} day^{-1} (nitrogen and phosphorus starved cultures). All the cultures were harvested at the end of the 14th day and the biomasses were stored at −20 °C for analyses.

A very different response to nutrient starvation was observed in *T. suecica* F&M-M33 compared to *Nannochloropsis* F&M-M24. In fact, the crude lipid content (B&D extract) of *Tetraselmis* (22% at the beginning of the experiment) remained stable for more than one week in all the cultures. At the end of the experiment the crude lipid content was 22% in the nitrogen starved culture, 27% under combined nitrogen and phosphorus deprivation and 29% in the nutrient sufficient culture (Fig. 3). The higher lipid content and lipid productivity of the control culture do not have a clear explanation. Lipid productivity in the control culture raised from 1.6 g m^{-2} day^{-1} in the first week to 2.4 g m^{-2} day^{-1} in the second. In both the starved cultures a maximum lipid productivity of 1.7 g m^{-2} day^{-1} was achieved during the first week.

In *T. suecica* F&M-M33 nitrogen or nitrogen plus phosphorus deprivation caused a dramatic decrease of proteins (from 46% to less than 10% of dry biomass), which was compensated by a similar increase of carbohydrates (from 15% to more than 50%) (Fig. 3).

Despite the similarity in the lipid content among the different culture conditions, important differences were observed in lipid composition (Fig. 4). Following cultivation under nutrient deprivation neutral lipids reached about 1.8 (under nitrogen starvation) and 2.5 (under nitrogen and phosphorus starvation) folds higher contents than under nutrient sufficiency, while both polar lipids and phospholipids decreased of more than 50%.

As in *Nannochloropsis* F&M-M24, in *T. suecica* F&M-M33 the neutral lipid content significantly rises as a consequence of nutrient starvation, but the main metabolic change caused by N and P shortage is the diversion towards carbohydrate synthesis. Feng et al. (2011) investigated the influence of nitrate supply on lipid production in *Isochrysis zhangjiangensis.* Under nitrate depletion

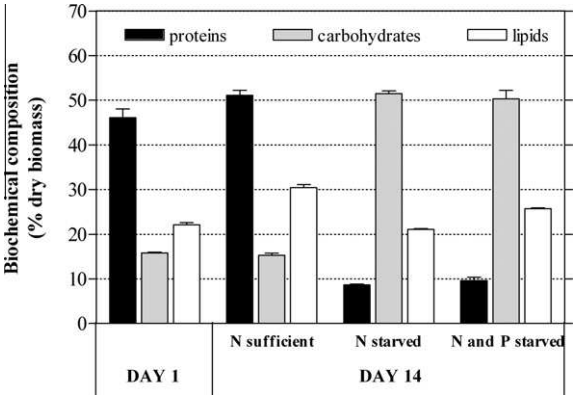

Fig. 3. Biochemical composition of *Tetraselmis suecica* F&M-M33 grown under nutrient sufficient and nutrient deprived conditions. N starved: culture grown under nitrogen starvation; N and P starved: culture grown under nitrogen and phosphorus starvation; N sufficient: control culture.

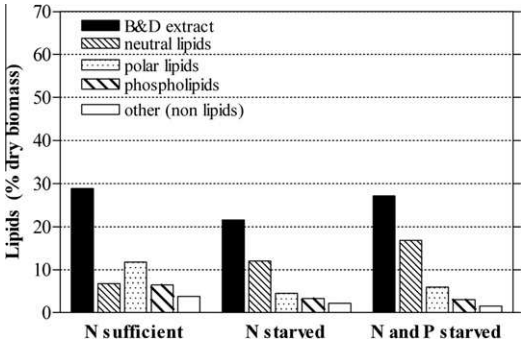

Fig. 4. Total (crude) lipid content and lipid class distribution (% dry biomass) of *Tetraselmis suecica* F&M-M33 grown outdoors in Green-Wall Panels under nutrient sufficient and nutrient deprived conditions. N starved: culture grown under nitrogen starvation; N and P starved: culture grown under nitrogen and phosphorus starvation; N sufficient: control culture.

the alga did not accumulate lipids, but carbohydrates (up to about 50% of dry biomass), thus showing a *T. suecica*-type behavior. At very high nitrogen concentrations, on the contrary, lipids were accumulated up to more than 50% of dry biomass. It was not clear why under heavy nutrient stress, which suppressed growth, *Isochrysis* responded either with carbohydrate (nitrogen depletion) or lipid (nitrogen excess) accumulation.

As in *Nannochloropsis* F&M-M24, important differences in fatty acid distribution within the different lipid classes were recorded under the different culture conditions. In Table 2 the fatty acid profile of the neutral-, polar- and phospho-lipids of *T. suecica* F&M-M33 cultivated under nitrogen and phosphorus starvation is reported. The most important fatty acids were palmitic (C16:0), oleic (C18:1, n-9), α-linolenic acid (C18:3, n-3) particularly abundant in polar lipids, and EPA (C20:5, n-3) mainly present in phospholipids. In the phospholipid fraction all fatty acids of the unsaturated C20 series were detected, with the exception of C20:2. The fatty acid composition of the neutral lipid fraction is suitable for biodiesel production, except for the presence of C20 unsaturated fatty acids, a limitation already observed in the neutral lipid fraction of *Nannochloropsis*. A number of fatty acids could not be identified in this

Table 2
Fatty acid composition (%) of three different lipid classes (neutral-, polar- and phospho-lipids) of *Tetraselmis suecica* F&M-M33 grown under nitrogen and phosphorus starvation.

Fatty acid	Lipid class		
	Neutral	Polar	Phospho
N.I.	0	4.30	27.02
N.I.	0	12.19	1.26
C16:0	31.34	19.32	0.88
N.I.	0	6.03	1.66
C16:1	2.85	0	0.66
N.I.	0.72	0	1.11
N.I.	0.59	0	7.13
C16:3	0.92	0	0.00
C16:4	4.30	0	18.78
C18:0	1.42	0	3.01
C18:1, n-7 + n-9	43.06	22.21	5.39
C18:2, n-6	4.78	6.39	4.95
C18:3, n-3	6.51	21.43	0.00
C18:4, n-3	0.94	0	1.49
C20:1	1.07	1.86	1.22
C20:3, n-6	0	0	24.29
C20:4, n-6	0.25	0	27.02
C20:5, n-3	1.24	6.26	1.26
Total saturated	32.76	–	–
Total monounsaturated	46.98	–	–
Total polyunsaturated	18.94	–	–

Table 3
Neutral lipid fractions (% of dry biomass) present in the biomass of *Tetraselmis suecica* F&M-M33 grown outdoors in Green Wall Panel reactors under different nutrient conditions.

Sample	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Nutrient sufficiency	0.4	0.1	3.7	2.6
N starvation	1.5	9.2	1.2	0.1
N and P starvation	0.5	14.3	1.9	0.1

Fraction 1: hydrocarbons, waxes and fatty acid esters with monohydric alcohols.
Fraction 2: triglycerides.
Fraction 3: diglycerides, free sterols, free fatty acids.
Fraction 4: monoglycerides.

work, and for this reason the iodine value of the neutral lipid fraction of *Tetraselmis* was not calculated.

The fractionation of neutral lipids in four fractions of different polarity, showed that *Tetraselmis* grown under nutrient sufficient conditions practically does not synthesize TAGs that, on the contrary, constitute the greatest proportion of the neutral fraction (on average 81%) after nutritional stress, particularly when both nitrogen and phosphorus are lacking (Table 3).

4. Conclusions

Nannochloropsis sp. F&M-M24 has a large potential as a renewable biofuel feedstock. Under nitrogen deprivation, the alga accumulates neutral lipids up to 50% of the dry biomass, with triglycerides representing the most abundant component, and produces oil that, with the exception of a high PUFA content, fulfills biodiesel feedstock requirements.

In *T. suecica* F&M-M33 nutrient-starved cultures, the synthesis of triglycerides is negligible and carbohydrates (with potential in bioethanol production) are accumulated, instead.

To develop an economically viable algae biofuel production process, the valorization of co-products (e.g., polar- and phospholipids and other non lipidic constituents) is necessary.

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