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*Article*

# Microalgal Species Selection for Biodiesel Production Based on Fuel Properties Derived from Fatty Acid Profiles

**Muhammad Aminul Islam [1,*], Marie Magnusson [2,3,4], Richard J. Brown [1], Godwin A. Ayoko [1], Md. Nurun Nabi [1] and Kirsten Heimann [2,3,4,*]**

[1] Biofuel Engine Research facilities, Faculty of Science and Engineering, Queensland University of Technology, Brisbane, Queensland 4000, Australia; E-Mails: richard.brown@qut.edu.au (R.J.B.); g.ayoko@qut.edu.au (G.A.A.); nurun.nabi@qut.edu.au (M.N.N.)

[2] School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia; E-Mail: marie.magnusson@jcu.edu.au

[3] Centre for Sustainable Fisheries and Aquaculture, James Cook University, Townsville, Queensland 4811, Australia

[4] Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Townsville, Queensland 4811, Australia

* Authors to whom correspondence should be addressed; E-Mails: aminuliut@gmail.com (M.A.I.); kirsten.heimann@jcu.edu.au (K.H.); Tel.: +61-4-2381-9870 (M.A.I.); +61-7-4781-5795 (K.H.).

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**Abstract:** Physical and chemical properties of biodiesel are influenced by structural features of the fatty acids, such as chain length, degree of unsaturation and branching of the carbon chain. This study investigated if microalgal fatty acid profiles are suitable for biodiesel characterization and species selection through Preference Ranking Organisation Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) analysis. Fatty acid methyl ester (FAME) profiles were used to calculate the likely key chemical and physical properties of the biodiesel [cetane number (CN), iodine value (IV), cold filter plugging point, density, kinematic viscosity, higher heating value] of nine microalgal species (this study) and twelve species from the literature, selected for their suitability for cultivation in subtropical climates. An equal-parameter weighted (PROMETHEE-GAIA) ranked *Nannochloropsis oculata*, *Extubocellulus* sp. and *Biddulphia* sp. highest; the only species meeting the EN14214 and ASTM D6751-02 biodiesel standards, except for the double bond limit in the EN14214. *Chlorella vulgaris*

outranked *N. oculata* when the twelve microalgae were included. Culture growth phase (stationary) and, to a lesser extent, nutrient provision affected CN and IV values of *N. oculata* due to lower eicosapentaenoic acid (EPA) contents. Application of a polyunsaturated fatty acid (PUFA) weighting to saturation led to a lower ranking of species exceeding the double bond EN14214 thresholds. In summary, CN, IV, C18:3 and double bond limits were the strongest drivers in equal biodiesel parameter-weighted PROMETHEE analysis.

**Keywords:** *Nannochloropsis oculata*; cetane number; cold filter plugging point; kinematic viscosity; biofuel properties; Preference Ranking Organisation Method for Enrichment Evaluation-Graphical Analysis for Interactive Assistance

## 1. Introduction

Algae have recently received a lot of attention as a new biomass source for the production of renewable energy in the form of biodiesel and as a feedstock for other types of fuel [1,2]. Several biomass conversion processes have been explored for the production of renewable diesel from microalgae, such as hydrothermal conversion and gasification followed by Fisher-Tropsch synthesis [3]. While both process technologies can yield designer fuels thereby meeting the required specifications of different renewable fuels more easily (e.g., devoid of oxygen, nitrogen, sulphur, aromatics and degree of unsaturation is controlled through hydrogenation of double bonds), initial set up costs are high, the processes are typically more energy intensive, as they require heating to high temperatures and pressure, and the latter process has the added disadvantage of requiring dried biomass input (an additional energy cost) [3]. In contrast, transesterification-derived regular biodiesel, where fatty acids are converted to fatty acid methyl esters (FAMEs), is a conversion technology that can be economically applied at remote biomass production facilities for servicing production site and community energy and transport fuel demands today. The disadvantages of regular biodiesel production are: energy-expensive drying of biomass is required [4], limited storage time due to oxidative instability amongst others, and the reciprocal advantage and disadvantage of the long chain polyunsaturated fatty acid (PUFA) content on the cold temperature operability [cold filter plugging point (CFPP)] and the iodine value (IV), respectively [5]. Limitations can, however, be minimised by selecting a suitable algal species and manipulating the initial fatty acid profile by varying the growth conditions and extraction process.

Microalgae have been reported as one of the best sources of biodiesel [6]. They can produce up to 250 times the amount of oil per acre compared to soybeans [6]. In fact, producing biodiesel from microalgae may be the only way to produce sufficient automotive fuel to replace current petro-diesel usage [7]. Furthermore, unlike most vegetable oil sources currently used for biodiesel production, algae can be grown on non-arable land with different streams of wastewater and do not compete with the agricultural production of food crops [8]. Since different strains of algae can be grown in different conditions (e.g., some are freshwater strains while others tolerate brackish or even hypersaline conditions) [9], they are an attractive resource for liquid fuel production [6]. In addition to biomass and lipid productivities, lipid and oil content, quantitative and qualitative lipid and fatty acid compositions are regarded to be critical parameters for selecting algae species for large-scale

production [10]. Furthermore, a good biodiesel should meet the cetane number (CN) standard, which indicates good ignition quality, a suitable cold filter plugging point, low pollutants content and, at the same time, correct density, and viscosity [11].

Even though lipid content and FAME profiles can be variable for the same algal strain, algal species selection remains one of the most important steps to reduce cost and time for large-scale cultivation for biodiesel production [12,13]. Researchers have made efforts to find convenient and useful methods to predict key fuel properties from fatty acid profiles. For example, FAME composition was used to calculate CN [14] whereas other researchers used iodine—and saponification values to calculate the CN [15]. The Smittenberg relation was used to estimate the density of saturated methyl esters at 20 °C and 40 °C [16]. An empirical correlation of saturated and unsaturated FAMEs was proposed for estimating viscosity [17]. In this study, fatty acids were extracted from microalgae biomass and directly transesterified to FAMEs to investigate the suitability of microalgae FAMEs as biodiesel. These microalgae were ranked based on the calculated key fuel properties; CN, IV, CFPP, density (υ), kinematic viscosity ($\rho$), and higher heating value (HHV), derived from their FAME profiles to identify the most suitable microalgal species for biodiesel production. The FAME profiles of twelve additional microalgal species were sourced from the literature [12] and biodiesel properties were calculated for comparison to the nine species from this study. Selection of these microalgal species for both extraction and analyses in this study and for literature comparisons was based on their ability to grow in similar subtropical environments. As it was shown that growth phase and nutrient supplementation of microalgal cultures also affect FAME profiles, the effect of culture medium and growth phase was further investigated for *Nannochloropsis oculata* based on results published by [10].

Other biodiesel specifications, e.g., ester-, carbon-, sulphur-, water-, methanol- mono-, di- and triglyceride content, as well as free glycerin-, total glycerin- alkali-, earth-alkali- and free fatty acid contents listed in the B100 specifications of ASTM D6751-02 and EN14214 are also important but strongly influenced by biomass harvesting, processing, biomass actual oil content, extraction, conversion and purification efficiencies [18]. We, therefore, only list those biodiesel quality parameters as per EN 14214 and ASTM 6751-02 (See Table 3 in Section 3.3 of Results and Discussion) that can be calculated based on FAME profiles. Oxidative stability is a very important biodiesel criterion, as it results in the formation of gums, sedimentation and engine deposits and increases the viscosity of the fuel through the formation of allylic hydroperoxides and several secondary oxidation products such as aldehydes, alcohols and carboxylic acids [18]. Oxidative stability is influenced by the age of the biodiesel, the condition of storage and the degree of unsaturation of biodiesel FAMEs and can be improved by the addition of antioxidants [19]. Oxidative degradation is, however, additionally influenced by the FAME components with the presence of allylic and particularly bis-allylic double bond positions leading to greater oxidative instability [18]. Linolenate (C18:3) contains two bis-allylic groups and a limit of 12 wt% for this FAME has been set in the European B100 biodiesel standard (EN 14214), which also limits the amount of FAMEs with four or more double bonds to 1 wt%, while the ASTM D6751-02 contains no such restrictions [19]. Therefore, polyunsaturated fatty acid content of the biomass, as well as the weighted degree of unsaturation developed by Ramos *et al.* [14], and the predictive fuel stability calculated from only two FAME contents, linoleate (C18:2) and linolenate (C18:3) [20] can serve as indirect estimates of biodiesel oxidative stability. Taken the above into consideration, we applied a higher weighting to PUFA content compared to other FAME-derived

biodiesel properties in principal component analyses and additionally calculated the predictive oxidative stability as per [20] to evaluate the suitability of the microalgal FAME profiles. Where possible, biodiesel quality parameters that are not obtainable from FAME profiles have been sourced from available data for algae methyl esters from the literature and will be discussed in comparison to other feedstock for biodiesel as appropriate.

## 2. Materials and Methodology

### *2.1. Materials*

Nine microalgal species isolated from tropical Queensland, Australia, were used in this study and were selected for based on their proven ability to grow in tropical to subtropical climates. Isolates were established and grown at the North Queensland Algal Identification/culturing Facility (NQAIF) at James Cook University, Townsville, Australia (see Table 1 for a list of study species and their NQAIF accession numbers). Microalgal cultures were raised in a variety of growth media shown in Table 1.

**Table 1.** Growth media, cultivation temperature, total lipid and total fatty acid content of nine microalgal species from this study and twelve green microalgal species from [12].

| Sample | Culture medium | Temp. (°C) | Total lipid (mg $g^{-1}$ dwt) | Total fatty acids * (mg $g^{-1}$ dwt) |
|---|---|---|---|---|
| ***Nine species from this study:*** | | | | |
| NQAIF034 *Amphidinium* sp. | L1 | 24 ± 1 | 189 | 141.0 |
| NQAIF272 *Biddulphia* sp. | f/2 | 24 ± 1 | 249 | 109.3 |
| NQAIF004 *Phaeodactylum tricornutum* | f/2 | 24 ± 1 | 217 | 187.3 |
| NQAIF284 *Picochlorum* sp. | L1 | 24 ± 1 | 305 | 274.8 |
| NQAIF283 *Nannochloropsis oculata* | L1 | 24 ± 1 | 410 | 267.1 |
| NQAIF254 *Extubocellulus* sp. | L1 | 24 ± 1 | 270 | 116.9 |
| NQAIF294 *Scenedesmus dimorphus* | Bold | 24 ± 1 | n.d. | 84.3 |
| NQAIF301 *Franceia* sp. | Bold | 24 ± 1 | n.d. | 79.7 |
| NQAIF303 *Mesotaenium* sp. | Bold | 24 ± 1 | n.d. | 76.5 |
| ***Twelve species from [12]:*** | | | | |
| *Ankistrodesmus falcatus* | LC | 25 ± 2 | 165 | 17.5 |
| *Ankistrodesmus fusiformis* | LC | 25 ± 2 | 207 | 27.3 |
| *Kirchneriella lunaris* | LC | 25 ± 2 | 173 | 30.5 |
| *Chlamydomonas* sp. | LC | 25 ± 2 | 151 | 14.1 |
| *Chlamydocapsa bacillus* | LC | 25 ± 2 | 135 | 19.2 |
| *Coelastrum microporum* | LC | 25 ± 2 | 206 | 49.1 |
| *Desmodesmus brasiliensis* | LC | 25 ± 2 | 180 | 37.0 |
| *Scenedesmus obliquus* | LC | 25 ± 2 | 167 | 4.4 |
| *Pseudokirchneriella subcapitata* | LC | 25 ± 2 | 284 | 36.7 |
| *Chlorella vulgaris* | CHU 13 | 25 ± 2 | 281 | 75.9 |
| *Botryococcus braunii* | CHU 13 | 25 ± 2 | 455 | 58.9 |
| *Botryococcus terribilis* | CHU 13 | 25 ± 2 | 490 | 16.7 |

n.d.: not determined; dwt: dry weight; * total fatty acid content (mg $g^{-1}$ dwt) for the twelve species from [12] was calculated based on information provided in Table 3 in [12].

Cultures were maintained in batch cultures (2 L Erlenmeyer flask) under indoor culture conditions at 50 μmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lights at 25 °C. Algal biomass was harvested in stationary phase, induced by nutrient depletion of the medium, by centrifugation at 3000 g for 20 min at room temperature. Harvested samples were analysed for total lipid content. The FAME composition and the total amount of FAME in mg $g^{-1}$ dry weight was analyzed by gas chromatography/mass spectrometry (GC/MS).

### *2.2. Lipid Content, Fatty Acid Methyl Ester Analysis*

A method modified from Folch *et al.* [21] and Somersalo *et al.* [22] with less toxic solvents, *i.e.*, hexane/methanol, was used to extract lipids from the microalgal samples. In addition to being less toxic, this solvent system (petroleum ether:MeOH [22]) was shown to yield similar total lipid content of plant tissue, and higher content of some phospholipids compared to $CHCl_3$:MeOH.

The biomass pellet was transferred to an 8 mL glass vial using 2 × 1 mL methanol:acetyl chloride (95:5 *v*/*v*). After adding 1 mL hexane, the vials were capped tightly and the lipids extracted at 100 °C for 60 min. After cooling to room temperature, 1 mL of water was added to facilitate phase separation and the content was transferred to a 15 mL centrifuge tube and centrifuged at 1800 g for 5 min at room temperature. The upper layer (hexane + lipid) was transferred to a new, pre-weighed 8 mL glass vial. The biomass was then extracted twice more with 1 mL of hexane. The combined hexane (3 mL) was evaporated under a gentle stream of $N_2$ and vials were weighed to 0.1 mg precision to determine the amount of lipids extracted.

For quantification and identification of fatty acids, 30 mg lyophilized biomass was extracted in triplicate with 2 mL of methanol-acetyl chloride (95:5 *v*/*v*). 300 μL C19:0 (nonadecanoic acid) was added as internal standard to the extraction mix and samples were heated at 100 °C for 60 min. The samples were subsequently cooled to room temperature and 1 mL of HPLC-grade hexane. Samples were then heated briefly again allowing the solvents to form a single phase before adding 1 mL Milli-Q water to facilitate phase separation. The upper layer was carefully collected and filtered through a 0.2 μm PTFE syringe filter (Pacific Laboratory Products, Melbourne, Australia) prior to analysis by GC/MS to determine fatty acid profiles as methyl esters. Butylated hydroxytoluene (BHT, 0.01%) was used as an antioxidant during the extraction.

FAME analysis was carried out as per [23] in scan-mode on an Agilent 7890 GC equipped with a flame ionization detector (FID) and connected to an Agilent 5975C electron ionisation (EI) turbo mass spectrometer (Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia). Separation was achieved on a DB-23 capillary column (15 μm cyanopropyl stationary phase, 60 m, 0.25 mm inner diameter). Helium was used as a carrier gas in constant pressure mode (approximately 230 kPa at 50 °C). Injector and FID inlet temperature were 150 °C and 250 °C, respectively (split injection, 1/50). Column temperature was programmed to hold at 50 °C for 1 min, then rise linearly at 25 °C $min^{-1}$ to 175 °C followed by a 4 °C $min^{-1}$ increase to 235 °C, and a 3 °C $min^{-1}$ increase to 250 °C as outlined in [24]. The quantity of fatty acids was determined by comparison of peak areas of external standards (Sigma Aldrich, Castle Hill, New South Wales, Australia) and was corrected for recovery of internal standard (C19:0). Total FAME content was determined as the sum of all FAMEs.

Previous analyses without the C19:0 internal standard confirmed this fatty acid is not a constituent of the fatty profiles of the study species, and was therefore an appropriate internal standard recovery.

### *2.3. Calculation of Fuel Properties from Fatty Acid Profiles*

The focus of this work was to screen suitable microalgal species for biodiesel production using published, simple, reasonable and reliable methods to minimise cost and time. In this study, several important biodiesel properties (CN, IV, CFPP, υ, $\rho$ and HHV) were calculated from the FAME composition. Fuel properties were calculated directly from FAME profiles [14,15,25,26]. In addition, CN was estimated using FAME profiles directly and using FAME-derived fuel properties SV and IV) [15], to investigate whether the two different approaches would yield different predictions of cetane values.

Along with the CN and chemical properties of biodiesel, some physical properties are also very important for biodiesel quality, such as υ, $\rho$, HHV, sulphur content, oxidation stability and so on. Here we used empirical equations to estimate three of the physical properties (υ, $\rho$ and HHV) of the FAME mixture, as proposed by [26].

CNs of vegetable oil methyl ester were calculated using the following equation [15]:

$$CN_1 = 46.3 + \left(\frac{5458}{Saponification\ Value}\right) - (0.225 \times Iodine\ value) \quad (1)$$

where $CN_1$ is the cetane number. The saponification value (SV) in mg $KOHg^{-1}$ and IV in g $I_2 100g^{-1}$ of fat are predicted by the following equations [27]:

$$SV = \sum_i \left(\frac{(560 \times N_i)}{Molecular\ weight\ of\ ith\ fatty\ acid}\right) \quad (2)$$

$$IV = \sum_i \frac{(254 \times D_i \times N_i)}{Molecular\ weight\ of\ ith\ fatty\ acid} \quad (3)$$

where $N_i$ is the percentage of each FAME; and $D_i$ is the number of double bonds of the *i*th FAME.

An equation proposed by [14] was used to calculate the degree of unsaturation (DU) based on the mass fraction of mono-unsaturated fatty acids (MUFA) and PUFA:

$$DU = \sum MUFA + (2 \times PUFA) \quad (4)$$

The long chain saturation factor (LCSF) and the CFPP in °C are also calculated based on [14]:

$$LCSF = (0.1 \times C16{:}0) + (0.5 \times C18{:}0) + (1 \times C20{:}0) + (2 \times C24{:}0) \quad (5)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (6)$$

Equation (1) estimates the *CN* based on the properties FAME molecular weights, saponification and iodine values. The CN can, however, also be calculated directly using the molecular weight and degree of unsaturation ($CN_2$), as shown in Equation (7) according to [26]:

$$CN_2 = \sum_i -7.8 + 0.302 \times M_i - 20 \times N \quad (7)$$

where $CN_2$ is the cetane number; $M_i$ is the molecular weight; and $N$ is the number of double bond in the *i*th FAME.

The υ, $\rho$ and HHV of each FAME can be calculated by using Equations (8)–(10), respectively, and summation of all FAME-derived fuel properties provides the final υ, $\rho$ and HHV of the biodiesel as published in [26]:

$$\ln(\upsilon_i) = -12.503 + 2.496 \times \ln(M_i) - 0.178 \times N \tag{8}$$

$$\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118 \times N \tag{9}$$

$$HHV_i = 46.19 - \frac{1794}{M_i} - 0.21 \times N \tag{10}$$

where ($\upsilon_i$ is the kinematic viscosity of at 40 °C in $mm^2/s$; $\rho_i$ is the density at 20 °C in $g/cm^3$; and $HHV_i$ is the higher heating value in MJ/kg of *i*th FAME.

Predictive oxidative stability was calculated, where possible, based on C18:2 and C18:3 content as suggested by [20], following Equation (11):

$$Y = \frac{117.9295}{X} + 2.5905 \ (0 < 100) \tag{11}$$

where $X$ is the content of linoleic and linolenic acids (wt%) ($0 < X < 100$); and $Y$ is the oxidation stability in hours.

The selection process took multiple criteria (biodiesel properties, FAME and lipid content of the biomass, C18:3 wt% and wt% of FAME with ≥ four double bonds) into account. Thresholds were set as per Table 3. A variety of multi-criteria decision analyses (MCDA) are available ranging from elementary to rather complex methods [28], such as ELECTRE, PROMETHEE and REGIME. A review of the MCDA literature revealed that Preference Ranking Organisation Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) has significant advantages (compared to other MCDA methods) because it facilitates rational decision making, *i.e.*, the decision vectors stretch towards the preferred solution [29]. This study applied the PROMETHEE-GAIA algorithm to rank microalgal species for suitability for biodiesel production. As importance of some of the biodiesel parameters vary from region to region, *i.e.*, CFPP is of low importance in subtropical and tropical climates and oxidative stability is of lesser importance in regions with fast turnaround (short storage times), the ranking was initially undertaken by giving equal weight to all biodiesel quality parameters. Following this, it was decided that the most suitable locations for biodiesel are subtropical and tropical regions, specifically with regards to microalgal biodiesel. Therefore, the weighting of the CFPP was not increased, but oxidative stability would be influenced by the storage temperature of the microalgal biodiesel. Hence, in addition to using C18:3 and ≥ four double bond wt% thresholds as per EN14214, PUFA content was used as a proxy for oxidative stability and the weighting of PUFA content was increased stepwise to saturation (the level where a further increase in weighting led to no further change in the ranking of the species).

## 3. Results and Discussion

### *3.1. Lipid Content*

The total lipid and fatty acid content of nine microalgal species were analyzed for biomass grown in three different culture media (L1, f/2, Bold), which were chosen based on optimal biomass production. Temperature and light were held constant (Table 1). The current study focused on using theoretical maximal yields of fatty acids to calculate fuel properties of biodiesel derived from a range of microalgae in order to rank the suitability of these species for further development. This enables to select the most suitable species for further characterization, including optimized growth and harvesting regimes to maximize yield of desirable fatty acids. Cultures were harvested in stationary phase, induced by nutrient limitation. Characterization and quantification of the fatty acid content in the separate fractions *i.e.*, triacylglycerides (TAGs or storage fats) and membrane lipids (phosphor- and glycolipids) would yield more information regarding the suitability of current industrial processing methods for production of biodiesel using oil from these algal species [30]. Lipid class content, however, varies depending on growth condition, nutrient provision and extraction solvent and process used and published results for these parameters are scarce, particularly with regards to comparable cultivation regimes.

Of the nine microalgal species from this study, the marine eustigmatophyte, *Nannochloropsis oculata*, had the highest total lipid content followed by the euryhaline chlorophyte *Picochlorum* sp., the marine diatoms *Extubocellulus* sp., *Biddulphia* sp., *Phaeodactylum tricornutum* and the marine dinoflagellate *Amphidinium* sp. Total lipid content was not determined for the freshwater chlorophytes *Scenedesmus dimorphus*, *Franceia* sp. and *Mesotaenium* sp. due to insufficient biomass. In contrast, *Picochlorum* sp. had a slightly higher total fatty acid content compared to *Nannochloropsis oculata*, while the fatty acid content of the other species were much lower and the lowest fatty acid contents were observed in the freshwater chlorophytes (Table 1). This result is not surprising, as freshwater chlorophytes do not store significant amounts of lipids and most fatty acids extracted are membrane-derived [31], in contrast to marine species like *Nannochloropsis oculata*, diatoms and dinoflagellates. In this regard, the high total lipid content of the euryhaline chlorophyte *Picochlorum* sp. is unusual. Based on the fatty acid content, which is the proportion of the total lipids that is useful for biodiesel production, *Picochlorum* sp. and *Nannochloropsis oculata* would be favorable, followed by *Phaodactylum tricornutum* and the dinoflagellate *Amphidinium* sp.

Fuel properties of twelve additional species, the trebouxiophycean strains *Chlorella vulgaris*, *Botryococcus braunii*, and *Botryococcus terribilis* and the chlorophyceaen strains *Ankistrodesmus falcatus*, *Ankistrodesmus fusiformis*, *Kirchneriella lunaris*, *Chlamydomonas* sp., *Chalmydocapsa bacillus*, *Coelastrum microporum*, *Desmodesmus brasiliensis*, *Scenedesmus obliquus*, and *Paseudokirchneriella subcapitata* acquired from [12], were also calculated for biomass produced under similar temperature regimes but other growth-optimized culture media (CHU 13 and LC Oligo) (Table 1). Of the twelve chlorophyte microalgal species [12], two of the three Trebouxiophyceae species, *Botryococcus braunii*, and *Botryococcus terribilis*, contained significantly more total lipids compared to *Chlorella vulgaris* (the other trebuxiophycean species) and the nine chlorophycean species. Total fatty acid contents were, however, much lower suggesting that a significant part of the total lipids are other non-polar

compounds, such as pigments. Only *C. vulgaris* had a total fatty acid content comparable to the green chlorophytes investigated in this study.

To investigate the effects of nutrients (cultivation media) and growth phase, lipid, FAME and FAME-derived biodiesel quality parameters were compared between *N. oculata* (this study) and data for *N. oculata*_RH, the latter investigating the impact of culture medium and growth phase for this species [10] (Table 4). *Nannochloropsis oculata* was selected for this comparison because this species is already cultivated on industrial-scale for its usefulness as an aquaculture feed (based on total lipid, fatty acid content and profile) and commercial-scale cultivation can be achieved in comparatively cheap open pond systems [raceways or high rate algal ponds (HIRAPs) yielding accurate and achievable year-round productivity estimates (20 g $m^{-2}$ $day^{-1}$) derived from decades of commercial-scale cultivation] [32]. The total lipid content of *N. occulata* varied with growth phase and culture medium used (Table 4; [10]). Total lipid content was generally higher than those reported for the chlorophycean microalgae, except for *P. subcapita*, and below the content achieved for *N. oculata* (this study) (Table 1). Total lipid content was highest in K medium, with content being higher in stationary (stat) compared to late logarithmic (LLog) phase, followed by stationary phase cultures raised in f/2 and L1 media, respectively, and then L1 and f/2 LLog, respectively. Lowest amounts of total lipids were observed for logarithmic (log) phase cultures in f/2 and L1 media, respectively. Even though growth phase clearly was a major factor affecting total lipid content, fertilization regime also had an effect, as the second highest total lipid content was observed in K medium-raised cultures, which could be due to supplementation of this medium with organic phosphate [33]. In contrast, L1 and f/2 cultivation media differ in trace elemental composition, which appeared to affect total lipid content to a lesser degree (Table 4).

### *3.2. FAME Composition*

A systematic analysis of the FAME composition and comparative fuel properties is very important for species selection for biodiesel production. The most common fatty acids of microalgae are Palmitic-(hexadecanoic-C16:0), Stearic-(octadecanoic-C18:0), Oleic (octadecenoic-C18:1), Linoleic-(octadecadienoic-C18:2) and Linolenic-(octadecatrienoic-C18:3) acids [34]. Most algae have only small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6), however, in some species of particular genera these PUFAs can accumulate in appreciable quantities depending on cultivation conditions [10]. In general, diatoms and eustigmatophytes make appreciable amounts of EPA, while dinoflagellates and haptophytes typically produce both EPA and DHA, with DHA being often dominant over EPA [35]. It has been suggested that, the higher the degree of unsaturation of the FAMEs of a biodiesel, the higher the tendency of the biodiesel to oxidize. There are, however, other parameters which also define the oxidation stability of the fuel, for example natural anti-oxidant and free fatty acid content [18,36,37]. A good quality biodiesel should have a 5:4:1 mass fatty acid ratio of C16:1, C18:1 and C14:0 , as recommended by Schenk *et al.* [38]. Of the nine micoalgal species investigated here, the FAME composition of *N. oculata* is closest to the recommended ratio with 5.1:3.5:1, but EPA is also present in appreciable quantities (fourth most dominant fatty acid) (Table 2).

**Table 2.** Fatty acid methyl ester (FAME) profile of nine microalgal species (mg $g^{-1}$ of dry biomass) (this study).

| FAME | *Amphidinium* sp. | *Biddulphia* sp. | *Phaeodactylum tricornutum* | *Picochlorum* sp. | *Nannochlopsis oculata.* | *Extubocellulus* sp. | *Scenedesmus dimorphos* | *Franceia* sp. | *Mesotaenium.* sp. |
|---|---|---|---|---|---|---|---|---|---|
| C8:0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| C10:0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C12:0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| C13:0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 14:0 | 1.1 | 23.4 | 6.1 | 1.5 | 15.3 | 7.6 | 0.4 | 0.4 | 0.4 |
| C 14:1 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 15:0 | 0.0 | 2.4 | 0.8 | 0.7 | 1.4 | 0.5 | 0.3 | 0.5 | 0.4 |
| C 15:1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 | 1.7 | 1.7 |
| C 16:0 | 49.6 | 25.7 | 43.8 | 45.9 | 85.4 | 29.4 | 13.1 | 10.0 | 10.1 |
| C16:1 (7) | 1.5 | 36.3 | 89.3 | 3.3 | 78.4 | 69.6 | 1.3 | 1.1 | 0.9 |
| C 16:1 (9) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 4.6 | 3.6 |
| C16:2 (7,10) | 0.0 | 1.9 | 2.6 | 13.9 | 0.0 | 3.1 | 1.7 | 1.2 | 1.7 |
| C16:2 (9,12) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.6 | 0.5 |
| C16:3 (cis 6,9,12) | 0.0 | 4.9 | 0.0 | 9.5 | 0.0 | 0.0 | 0.5 | 0.5 | 0.5 |
| C16:3 (7, 10, 13) | 0.0 | 0.0 | 7.9 | 0.0 | 0.0 | 0.0 | 1.3 | 1.0 | 1.4 |
| C16:4 (4,7,10,13) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 12.9 | 13.2 | 12.3 |
| C17:0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 0.3 | 0.4 | 0.0 |
| C17:1 | 0.0 | 0.0 | 0.0 | 0.8 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 18:0 | 5.7 | 0.8 | 1.5 | 9.3 | 2.6 | 1.1 | 0.5 | 0.4 | 0.4 |
| C 18:1 (9) | 26.9 | 1.6 | 6.7 | 42.4 | 53.3 | 3.7 | 5.8 | 3.4 | 4.3 |

**Table 2.** *Cont.*

| FAME | *Amphidinium* sp. | *Biddulphia* sp. | *Phaeodactylum tricornutum* | *Picochlorum* sp. | *Nannochlopsis oculata.* | *Extubocellulus* sp. | *Scenedesmus dimorphos* | *Francei a* sp. | *Mesotaenium.* sp. |
|---|---|---|---|---|---|---|---|---|---|
| C 18:1 (x) | 0.0 | 0.6 | 4.2 | 0.0 | 0.0 | 0.0 | 1.5 | 1.8 | 1.4 |
| C 18:2 (cis-9,12) | 0.0 | 0.0 | 0.0 | 97.6 | 3.4 | 0.0 | 10.6 | 6.6 | 8.9 |
| C18:3 all cis 6,9,12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 1.0 | 0.7 |
| C 18:3 (all cis-9,12,15) | 0.0 | 0.0 | 0.0 | 40.6 | 0.0 | 0.0 | 20.7 | 25.1 | 22.9 |
| C18:4 (6,9,12,15) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.9 | 2.9 | 2.3 |
| C 20:0 | 7.9 | 0.0 | 0.0 | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 20:2 (cis-11,14) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 |
| C 20:4 | 1.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 20:5 (all cis-5,8,11,14,17) | 16.6 | 10.5 | 22.5 | 0.0 | 21.9 | 0.0 | 0.0 | 0.7 | 0.6 |
| C 22:0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 |
| C 22:6 | 28.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C 24:0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.6 | 0.0 |
| C 24:1 (cis-15) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.0 | 0.0 |
| SFAs (%) | 46.3 | 48.8 | 28.1 | 23.7 | 40.5 | 33.6 | 18.9 | 15.9 | 15.4 |
| MUFAs (%) | 20.4 | 35.4 | 54.1 | 17.0 | 50.0 | 63.8 | 17.4 | 16.2 | 15.9 |
| PUFAs (%) | 33.3 | 15.8 | 17.8 | 59.2 | 9.5 | 2.7 | 63.7 | 67.9 | 68.7 |

Saturated fatty acids (SFA) play a significant role in fuel properties. The CN increases in fuels with high amounts of SFA [11]. *Biddulphia* sp. had the highest amounts of SFAs, followed by *Amphidinium* sp., *N. oculata*, *Extubocellulus* sp., *Phaeodactylum tricornutum* and *Picochlorum* sp. (Table 2). On the other hand, υ, CFPP and $\rho$ are largely influenced by the degree of unsaturation [39,40]. Therefore, both saturation and unsaturation of FAMEs should have an optimal balance for high biodiesel quality.

Culture growth phase and nutrient provision affect levels of SFAs, MUFAs and PUFAs in *N. oculata* [10], with higher amounts of SFAs and MUFAs observed in stationary phase, except when cultivated in K medium (LLog and stat growth phase concentrations are similar), the latter presumably due to organic carbon supplementation of K medium. In contrast, PUFA levels declined with growth phase, mainly due to the significant decrease in EPA which could be related to the accumulation of TAGs, and were observed to be only half of the stationary phase concentrations (L1 and f/2 cultures) in cultures raised in K medium (10.4% dwt) [10]. While SFA and EPA content of *N. oculata* (this study) were comparable to those published by [10], MUFA contents were ~10% higher and PUFA contents were 50% lower for *N. oculata* raised in L1 medium in this study. As growth conditions and strains used were identical, the FAME profile might suggest that nutrient status of the cultures were significantly different, *i.e.*, *N. oculata* could have been in an advanced state of nutrient starvation (one week into the stationary phase this study) compared to three days in [10]. Thus, in addition to culture growth phase, culture nutrient status, *i.e.*, degree of nutrient starvation, will likely affect biodiesel quality of *N. oculata*.

### *3.3. Fuel Properties*

CN is one of the most significant indicators for determining combustion behavior of diesel [41]. The CN of a fuel is related to the ignition delay time, which is the time between injection and ignition as referred in ASTM D613. The shorter the ignition delay time, the higher the CN, and *vice versa* [11]. According to the ASTM D6751-02 and EN14214 standard for biodiesel, the minimum CN should be 47.0 and 51.0, respectively, whereas the IV is set to a maximum of 120 g $I_2$/100 g fat. Biodiesel is most likely used with conventional petroleum diesel in different blend concentration depending on CN and density of the biodiesel. Therefore biodiesel with higher cetane numbers can be blended at higher concentrations with petroleum diesel. EN14214, ASTM D6751-02 and calculated CN, IV, SV, CFPP, LCSF, DU, υ, $\rho$ and HHV derived from the FAME compositions, C18:3 (wt%) and double bonds (≥4) (wt%), as well as oxidation stability calculated from C18:2 and C18:3 contents [20] of the nine microalgal species and from the published fatty acid profiles of the twelve published species [12] and preference for the PROMETHEE analyses (min/max) as well as values used in the analysis are presented in Table 3.

**Table 3.** Biodiesel properties calculated from the FAME profile of nine microalgal species (this study) and twelve species from [12].

| | Algae species | DU | LCSF | CFPP (°C) | IV (g $I_2$100g$^{-1}$ fat) | SV (mg KOHg$^{-1}$) | $CN_1$ | $CN_2$ | SFAs (%) | MUFA (%) | PUFA (%) | Kinematic viscosity (υ) (mm$^2$ s$^{-1}$) | Density (ρ) (g cm$^{-3}$) | HHV (MJ kg$^{-1}$) | C18:3 (wt%) | Db ≥ 4 (wt%) | Oxidation Stb. [a] (h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Biodiesel Standard EN 14214 | - | - | ≤5/≤−20 | ≤120 | - | ≥51 | ≥51 | - | - | - | 3.5–5.0 | 0.86–0.90 | NA | ≤12 | ≤1 | ≥ 6 |
| | Biodiesel Standard ASTM D6751−02 | - | - | NA | NA | - | ≥47 | ≥47 | - | - | - | 1.9–6.0 | NA | NA | - | - | - |
| | Min/max | max | max | max | max | max | min | min | min | max | max | max | max | min | max | max | |
| | Threshold value for PROMETHEE | - | - | 5 | 120 | - | 47 | 47 | - | - | - | - | 0.90 | - | 12 | 1 | - |
| | ***Nine species from this study:*** | | | | | | | | | | | | | | | | |
| *A* | *Nannochloropsis oculata* | 69 | 3.7 | −4.8 | 81 | 203 | 55.0 | 57.9 | 40.5 | 50.0 | 9.5 | 4.2 | 0.88 | 39.8 | 0 | 8.3 | 95.7 |
| *A* | *Extubocellulus* sp. | 69 | 3 | −7.0 | 65 | 209 | 57.8 | 60.9 | 33.6 | 63.8 | 2.7 | 3.92 | 0.89 | 40.1 | 0 | 0 | − |
| *A* | *Biddulphia* sp. | 67 | 2.7 | −7.9 | 88 | 210 | 52.5 | 54.6 | 48.8 | 35.4 | 15.8 | 3.71 | 0.89 | 40.0 | 0 | 9.6 | − |
| *B* | *Phaeodactylum tricornutum* | 90 | 2.8 | −7.8 | 114 | 204 | 47.3 | 50.3 | 28.1 | 54.1 | 17.8 | 3.74 | 0.89 | 39.8 | 0 | 12.1 | − |
| *C* | *Picochlorum* sp. | 136 | 5.5 | 0.7 | 135 | 195 | 44.0 | 48.9 | 23.7 | 17.0 | 59.2 | 3.99 | 0.89 | 39.9 | 14.9 | 0 | 4.9 |
| *C* | *Amphidinium* sp. | 87 | 11.3 | 19.1 | 159 | 188 | 39.5 | 42.9 | 46.3 | 20.4 | 33.3 | 4.13 | 0.9 | 40.3 | 0 | 32.1 | − |
| *C* | *Scenedesmus dimorphus* | 145 | 3.8 | −4.6 | 184 | 196 | 32.9 | 37.1 | 18.9 | 17.4 | 63.7 | 3.63 | 0.91 | 40.2 | 26 | 19.1 | 5.6 |
| *C* | *Franceia* sp. | 152 | 3.1 | −6.7 | 206 | 198 | 27.7 | 33.3 | 15.9 | 16.2 | 67.9 | 3.49 | 0.91 | 40.4 | 33.5 | 21.6 | 5.4 |
| *C* | *Mesotaenium* sp. | 153 | 1.6 | −11.4 | 202 | 200 | 28.3 | 33.4 | 15.4 | 15.9 | 68.7 | 3.45 | 0.91 | 40.2 | 31.4 | 20.3 | 5.3 |

**Table 3.** *Cont.*

| Algae species | DU | LCSF | CFPP (°C) | IV (g $I_2$100g$^{-1}$ fat) | SV (mg KOHg$^{-1}$) | $CN_1$ | $CN_2$ | SFAs (%) | MUFA (%) | PUFA (%) | Kinematic viscosity (υ) (mm$^2$ s$^{-1}$) | Density (ρ) (g cm$^{-3}$) | HHV (MJ kg$^{-1}$) | C18:3 (wt%) | Db ≥ 4 (wt%) | Oxidation Stb. [a] (h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Twelve species from literature [12]:*** | | | | | | | | | | | | | | | | |
| *Ankistrodesmus falcatus* | 85 | 4.38 | −2.7 | 96 | 191 | 53.2 | 49.3 | 41.4 | 28.4 | 30.2 | 3.68 | 0.82 | 36.6 | 26.86 | 0 | 6.7 |
| *Ankistrodesmus fusiformis* | 99 | 3.75 | −4.7 | 108 | 189 | 50.8 | 47.4 | 37.3 | 22.4 | 40.2 | 3.65 | 0.82 | 36.9 | 26.5 | 0 | 5.6 |
| *Kirchneriella lunaris* | 111 | 3.53 | −5.4 | 130 | 192 | 45.4 | 45.5 | 32.1 | 23.1 | 44.8 | 3.70 | 0.85 | 38.2 | 39.6 | 0 | 5.3 |
| *Chlamydomonas* sp. | 27 | 10.8 | 17.6 | 26 | 206 | 66.9 | 62.4 | 78.6 | 14.6 | 6.8 | 3.93 | 0.81 | 36.5 | 2.76 | 0 | 20.2 |
| *Chlamydocapsa bacillus* | 100 | 3.93 | −4.1 | 109 | 187 | 51.0 | 48.0 | 35.7 | 23.6 | 40.7 | 3.69 | 0.83 | 37.1 | 25.45 | 0 | 5.6 |
| *Coelastrum microporum* | 86 | 4.02 | −3.8 | 84 | 195 | 55.4 | 57.2 | 45.9 | 38.0 | 16.1 | 4.15 | 0.86 | 38.8 | 11.1 | 0 | 8.6 |
| *Desmodesmus brasiliensis* | 87 | 4.43 | −2.6 | 83 | 195 | 55.6 | 57.9 | 34.5 | 44.1 | 21.4 | 4.18 | 0.86 | 39.0 | 9.43 | 0 | 8.1 |
| *Scenedesmus obliquus* | 36 | 8.95 | 11.6 | 34 | 204 | 65.5 | 63.2 | 70.8 | 21.7 | 7.5 | 4.04 | 0.83 | 37.5 | 2.83 | 0 | 18.5 |
| *Pseudokirchneriella subcapitata* | 82 | 4.23 | −3.2 | 79 | 194 | 56.7 | 57.5 | 35.4 | 47.4 | 17.3 | 4.14 | 0.85 | 38.3 | 9.87 | 0 | 9.4 |
| *Chlorella vulgaris* | 56 | 8.04 | 8.8 | 50 | 189 | 63.8 | 63.3 | 52.2 | 37.5 | 10.3 | 4.28 | 0.84 | 38.1 | 1.57 | 0 | 14.3 |
| *Botryococcus braunii* | 99 | 1.51 | −11.7 | 90 | 188 | 55.1 | 58.7 | 9.9 | 79.6 | 10.5 | 4.39 | 0.86 | 39.2 | 5.34 | 0 | 13.8 |
| *Botryococcus terribilis* | 67 | 5.08 | −0.5 | 64 | 184 | 61.7 | 59.0 | 43.2 | 44.3 | 12.6 | 4.13 | 0.82 | 37.3 | 7.22 | 0 | 12.2 |

$CN_1$: Cetane number [15]; $CN_2$: Cetane number [26]; Db: Double bond; A: entirely within both biodiesel standards (EN 14214; ASTM D6751-02) [42] except the number of double bond ≥ 4; B: Within biodiesel standard ASTM D6751-02 [42]; C: not compliant with any of the two biodiesel standards; [a]: Oxidation stability was not considered for PROMETHEE analysis.

Most of the nine microalgal species investigated here were within the range of standard values for CN and IV, except for the three chlorophyte freshwater species *S. dimorphus*, *Franceia* sp. and *Mesotaenium* sp. (Table 3). Based on this, the primary selection process can exclude these three species from further analyses. Furthermore, biodiesel must have an appropriate kinematic viscosity (υ) to ensure that an adequate fuel supply reaches injectors at different operating temperatures [26]. Since υ is inversely proportional to temperature, it also affects the CFPP for engine operation at low temperatures. Kinematic viscosity limits are set to 1.9–6.0 $mm^2 s^{-1}$ and 3.5–5.0 $mm^2 s^{-1}$ as per ASTM 6751-02 and EN 14214. All microalgal species listed in Table 3 were in the prescribed viscosity range with 3.44–4.20 $mm^2 s^{-1}$, therefore meeting both standards. The fuel injection system supplies fuel by volume not by mass which means denser biodiesel will be injected with greater mass in to the combustion chamber consequently affecting the stoicheometric ratio of air and fuel [43,44]. Therefore, density ($\rho$), for which a standard value has been set at 0.86–0.90 g $cm^{-3}$ according to EN 14214, is another important parameter for biodiesel quality. FAME profile-derived $\rho$-values of five microalgal species *N. oculata*, *Extubocellulus* sp., *Biddulphia* sp., *P. tricornutum* and *Picochlorum* sp. were within this range and the range was slightly exceeded (0.899–0.915 g $cm^{-3}$) by the four other species (Table 3). In contrast, nine of the chlorophyte microalgae were slightly below the range and only *Coelastrum microporum*, *Desmodesmus brasiliensis* and *Botryococcus braunii* barely met the specification (0.86 g $cm^{-3}$) (Table 3).The FAME-derived HHVs of all microalgal species investigated were found to comply with the set range (39.8–40.4 MJ $kg^{-1}$) of regular biodiesel, which is normally 10% to 12% less than obtained for petroleum-derived diesel (46MJ $kg^{-1}$) [26]. As C18:3 is the precursor for the synthesis of EPA and DHA, it was not surprising that high EPA and/or DHA microalgae like *N. oculata*, *Extubocellus* sp., *Biddulphia* sp. *Phaoedactylum tricornutum* and *Amphidinium* sp., had no detectable amounts of this fatty acid (Table 3). For the green algal species, C18:3 content varied greatly (Table 3). All green microalgae from this study but only four of the twelve species derived from the literature exceeded the EN14214 threshold of 12 wt% C18:3. In general, chlorophytes had 0 wt% of ≥ four double bonds, except for *S. dimorphus*, *Franceia* sp. and *Mesotaenium* sp. from this study which had a high content exceeding that of the diatoms *Extubocellulus* sp., *Biddulphia* sp. and *Phaeodactylum tricornutum* and the eustigmatophyte *N. oculata*. Highest values were found in the dinoflagellate *Amphidinium* sp. (Table 3). Where possible, oxidation stability was calculated based on C18:2 and C18:3 contents, but this was not possible for species where these fatty acids were absent from the fatty acid profile (Table 3). Most of the oxidative stability values calculated here are within or slightly below or above the range reported for algal methyl esters (8.5–11 h) [19] except for *N. oculata* (Table 3). It must be pointed out that these estimates must be taken with caution, as values much exceeding the set time frame of 6 h are likely a function of low contents of these fatty acids (Tables 2 and 3). Based on microalgal FAME profiles (Table 2) and the estimated oxidative stability (Table 3), it appears that the formula developed by Park *et al.* [20] for higher plants, where other long chain polyunsaturated acid contents are low, would have to be altered for FAME profiles of microalgae to provide an appropriate weighting of other dominant fatty acids with a high degree of unsaturation (EPA, DHA, *etc.*). This would require to measure oxidative stability of the microalgal biodiesel directly.

As elucidated above, growth phase had a major impact on FAME profiles in *N. oculata*; it is thus not surprising that CNs and IVs were also tightly linked with growth phase (Table 4).

**Table 4.** Effect of growth phase and cultivation media on biodiesel properties calculated from the FAME profile, total lipid, saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) contents of *Nannochloropsis oculata*.

| Algae species | Growth media and growth phase | DU | LCSF | CFPP (°C) | IV (g $I_2$100g$^{-1}$ fat) | SV (mg KOHg$^{-1}$) | $CN_1$ | SFAs (%) | MUFA (%) | PUFA (%) | Total lipid (mg g$^{-1}$ dwt) | C18:3 (wt%) | Db ≥ 4 (wt%) | Oxidation Stb. (h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Min/max | - | max | max | max | max | max | min | min | max | max | min | max | max | min |
| Threshold value for PROMETHEE analysis | - | - | - | 5 | 120 | - | 47 | - | - | - | - | 12 | 1 | 6 |
| * *N. oculata* | L1_Stat | 69 | 3.7 | −4.8 | 81 | 203 | 55.0 | 40.5 | 50.0 | 9.5 | 410 | 0 | 8.23 | 95.4 |
| *N. oculata*_RH | L1_Log | 107 | 4.0 | −4.0 | 175 | 195 | 35.0 | 31.9 | 28.6 | 39.4 | 213 | 0 | 37.10 | 56.2 |
| *N. oculata*_RH | L1_LLog [a] | 96 | 4.1 | −3.7 | 155 | 198 | 39.9 | 37.7 | 29.1 | 33.4 | 313 | 0 | 31.1 | 56.2 |
| *N. oculata*_RH | L1_Stat | 75 | 4.9 | −1.1 | 107 | 200 | 49.5 | 44.8 | 35.2 | 20.1 | 327 | 0 | 18.6 | 81.2 |
| *N. oculata*_RH | f/2_Log | 102 | 4.1 | −3.5 | 163 | 196 | 37.4 | 33.9 | 29.8 | 36.2 | 219 | 0 | 34.1 | 58.7 |
| *N. oculata*_RH | f/2_LLog | 97 | 4.5 | −2.2 | 154 | 208 | 38.0 | 40.2 | 28.7 | 31.3 | 292 | 0.1 | 19.1 | 56.2 |
| *N. oculata*_RH | f/2_Stat | 74 | 5.1 | −0.4 | 105 | 200 | 49.9 | 45.9 | 34.1 | 20.0 | 332 | 0 | 18.4 | 76.3 |
| *N. oculata*_RH | K_LLog | 70 | 4.5 | −2.5 | 83 | 203 | 54.3 | 40.2 | 47.1 | 12.6 | 357 | 0.1 | 11.7 | 150 |
| *N. oculata*_RH | K_Stat | 79 | 4.5 | −2.4 | 113 | 200 | 48.0 | 40.8 | 48.8 | 10.4 | 378 | 0.1 | 9.4 | 120.5 |

*: species from this study; Log: logarithmic growth phase; LLog: late logarithmic growth phase; Stat: stationary growth phase; RH, species from [10]; and [a]: average of two samples.

Cultures in stationary phase met the specifications of both biodiesel quality parameters irrespective of medium, while those in Log and LLog phase (except for cultures raised in K medium) exceeded and were below the prescribed levels for IV and CN, respectively. As *N. oculata* has a low C18:3 content, as C18:3 is immediately used for the synthesis of EPA, C18:3 content limits did not affect the ranking, however, culture growth phase and fertilization had a large impact on ≥ 4 double bond content, with cultures raised in K (*N. oculata*_RH K_stat) and L1 medium (this study) in stationary growth phase having the lowest content (Table 4). The FAME profile of *N. oculata* exceeded the EN14214 ≥ 4 double bond threshold under all cultivation conditions. Nonetheless, these results indicate, that the decision making process for microalgal species selection for large-scale biodiesel production must take growth phase and nutrients (*i.e.*, provision of organic carbon in K medium) into account.

### *3.4. Selection of Suitable Algae Species for Biodiesel*

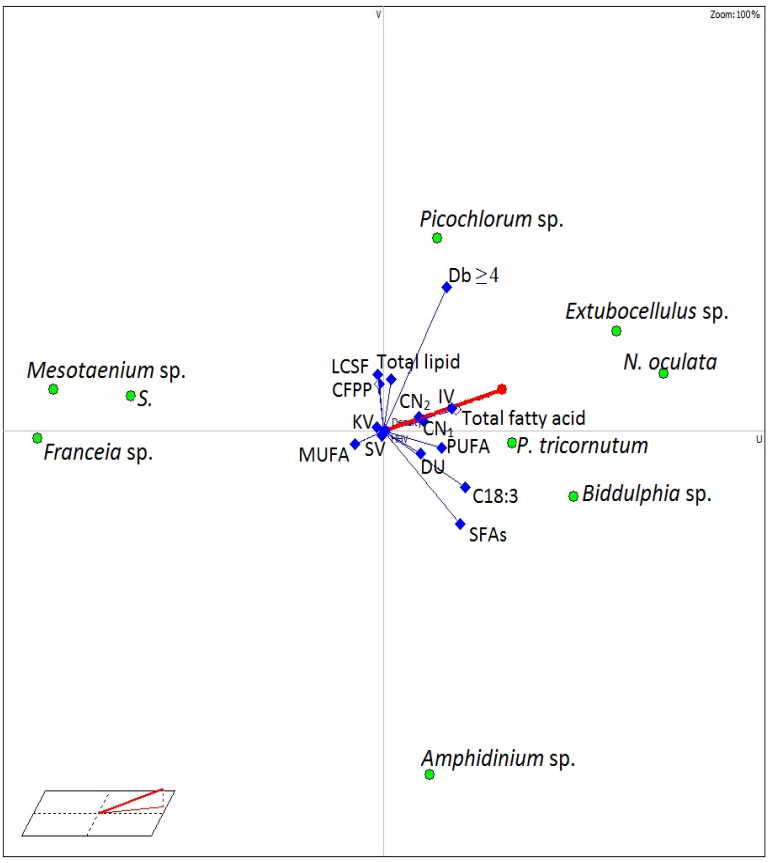
To be an ideal source of sustainable biodiesel, selected microalgal species should contain sufficient lipid with suitable fatty acids for good biodiesel properties. The three freshwater chlorophyte species *S. dimorphus*, *Franceia* sp., *Mesotaenium* sp. were identified to have poor biodiesel properties. A multi-criteria decision method (MCDM) software PORMETHEE-GAIA was used to make objective selections for large-scale production. Suitable microalgal species were selected from the nine species (Figure 1) and twelve additional microalgal FAME profiles sourced from the literature ([12]; Figure 2) based on twelve estimated biodiesel characteristics: IV, LCSF, CFPP, DU, CN1, CN2, υ, $\rho$, HHV; SFAs, MUFA and PUFA, and EN14214 C18:3 and ≥ four double bond thresholds as well as total lipid and fatty acid contents, with all components receiving an equal weighting. In addition, where possible oxidative stability was calculated based on C18:2 and C18:3 contents as per [20], which was, however, not included in the analyses, as it could not be calculated for some species and the reliability for algae with high EPA and DHA contents but low C18:2 and C18:3 contents is questionable. In Figure 1, two axes explain 83.3% of the total variability.

The preference functions of criteria (fuel properties) were modeled as Min (*i.e.*, lower values are preferred for good biodiesel) or Max (higher values are preferred for good biodiesel) per Table 3. The length of the criteria vectors and their directions indicate the influence these criteria have on the decision vector (red line in Figure 1a) and preference to the species (Figure 1a). For example the CN is at the maximum for the species *Extubocellulus* sp. *Nannochloropsis oculata* and *Biddulphia* sp. whereas IV is at the minimum for these species. On the other hand, *Picochlorum* sp., *N. oculata*, *and P. tricornutum* had the maximum amount of total fatty acids, whereas *S. dimorphus*, *Mesotaenium* sp. and *Franceia* sp. represented the minimum according to Figure 1a.

A decision vector that is long and not orthogonal (at right angle) to the GAIA plane is preferred for strong decision making [45]. The decision vector indicates the most preferable species, *i.e.*, those that align with the direction of this vector and the outermost criteria in the direction of the decision vector are the most preferable [46]. In general, the criteria which lie close to (±45°) are correlated, while those lying in opposite directions (135–225°) are anti-correlated, and roughly in orthogonal direction have no or less influence [45]. For example CN, IV, DU, PUFA, Db ≥ 4, C18:3 and total fatty acid in Figure 1a) were correlated, whereas MUFA was anti-correlated with these criteria and total lipid and SFAs had no or little influence on these. The length of the criteria vectors indicate their influence

on the decision vector and therefore the ranking [46]. Very short criteria vectors ($\rho$, $\upsilon$ and HHV) indicate that the microalgal species showed little to no variance in these important biodiesel quality parameters (Table 3), thus they do not influence the length and direction of the decision vector (Figure 1a). It can be concluded that removal of important biodiesel quality parameters $\rho$, $\upsilon$ and HHV will not change the ranking of microalgal biodiesel and these are therefore, at least in this case, not effective components for microalgal species selection for biodiesel production. In contrast, Db ≥ 4, SAFs, and C18:3 were highly variable criteria (Table 3) and they had a strong effect on the decision vector. According to Figure 1a and the calculated outranking flows, the most suitable species are *N. oculata*, *Extubocellulus* sp., *Biddulphia* sp., *P. tricornutum* and *Picochlorum* sp. (Figure 1b). For further suitability analysis of microalgal species for biodiesel production, the nine species investigate here were compared with twelve chlorophyte microalgal species from the literature which were grown in a similar subtropical climate (eutrophic lagoon located at Salvador City, Bahia, Brazil of similar latitude as Townsville, Australia) [12].

**Figure 1.** (**a**) Graphical Analysis for Interactive Assistance (GAIA) plot of nine microalgal species from the present study showing 16 criteria (14 biodiesel properties from Table 3, total lipid and fatty acid content from Table 1) and decision vector; and (**b**) corresponding ranking of species based on their outranking flow.




(**a**)

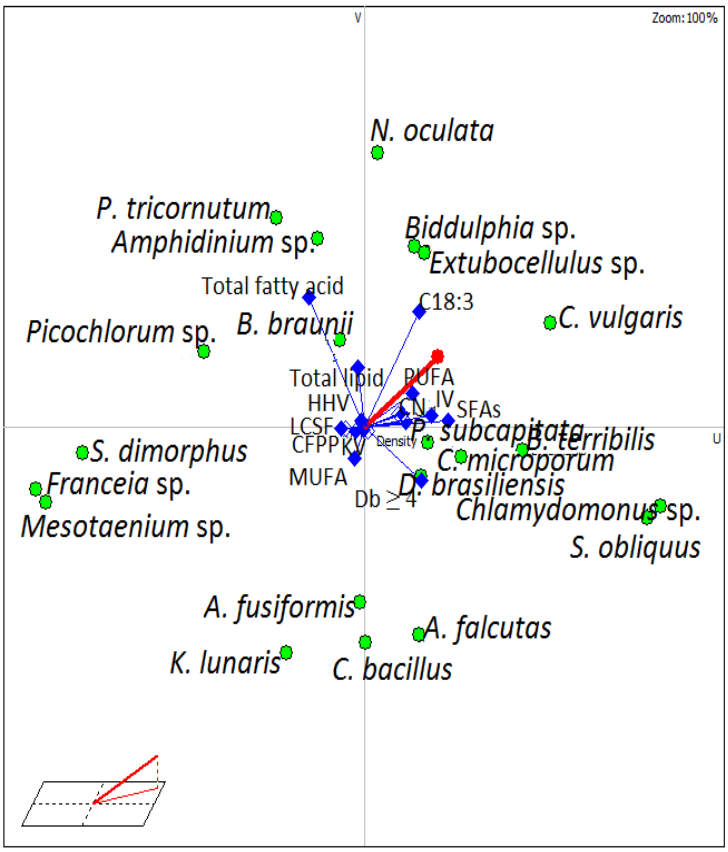
| Rank | Species | Phi |
|---|---|---|
| 1 | *N. oculata* | 0.27 |
| 2 | *Extubocellulus* sp. | 0.20 |
| 3 | *Biddulphia* sp. | 0.18 |
| 4 | *P. tricornutum* | 0.09 |
| 5 | *Picochlorum* sp. | 0.06 |
| 6 | *Amphidinium* sp. | −0.07 |
| 7 | *S. dimorphus* | −0.21 |
| 8 | *Mesotaenium* sp. | −0.25 |
| 9 | *Franceia* sp. | −0.28 |

(**b**)

A GAIA plane of 21 species (nine from this study and twelve from [12]) is shown in Figure 2a, where the two axes explain 75.1% of the variability.

Inclusion of the twelve chlorophyte microalgae changed the suitability ranking of the nine investigated microalgae, with the green microalgae *Chlorella vulgaris* being ranked highest when all criteria received equal weighting and *N. oculata*, *Extubocellulus* sp. and *Biddulphia* sp. maintained their high ranking (ranked 2nd 3rd and 4th, respectively) for biodiesel quality (Figure 2b). *Picochlorum* sp. and *P. tricornutum*, which ranked highly when only the nine microalgal species were considered, lost significant ground now ranking 11th and 13th amongst the 21 investigated species (Figure 2b). *S. dimorphus*, *Mesotaenium* sp. and *Franceia* sp. remained their low ranking and are the least suitable species for biodiesel production.

**Figure 2.** (**a**) GAIA plot of nine microalgal species from the present study and twelve from [12] showing 16 criteria (14 biodiesel properties from Table 3, total lipid and fatty acid content from Table 1) and the decision vector; and (**b**) Corresponding outranking flow. *: this study.




(**a**)

| Rank | Species | Phi |
|---|---|---|
| 1 | *C. vulgaris* | 0.17 |
| 2 | **N. oculata* | 0.16 |
| 3 | **Extubocellulus* sp. | 0.16 |
| 4 | **Biddulphia* sp. | 0.13 |
| 5 | *B. terribillis* | 0.11 |
| 6 | *chlamydomonas* sp. | 0.10 |
| 7 | *S. obliquus* | 0.09 |
| 8 | *C. microporum* | 0.07 |
| 9 | *B. braunii* | 0.06 |
| 10 | *P. subcapitata* | 0.05 |
| 11 | **P. tricornutum* | 0.02 |
| 12 | *D. brasiliensis* | 0.01 |
| 13 | **Picochlorum* sp. | −0.01 |
| 14 | *A. falcatus* | −0.05 |
| 15 | *A. fusiformis* | −0.05 |
| 16 | **Amphidinium* sp. | −0.08 |
| 17 | *C. bacillus* | −0.09 |
| 18 | *K. lunaris* | −0.12 |
| 19 | **S. dimorphus* | −0.21 |
| 20 | **Mesotaenium* sp. | −0.24 |
| 21 | **Franceia* sp. | −0.26 |

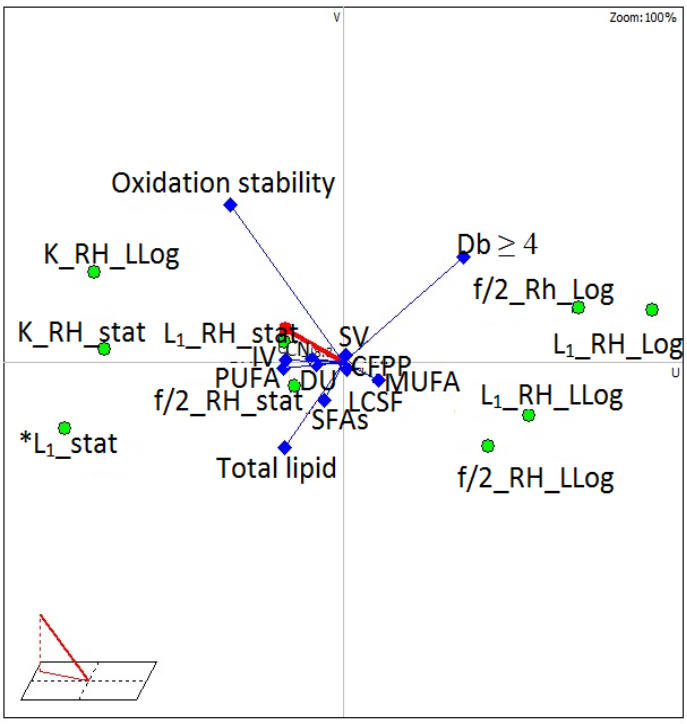
(**b**)

As *N. oculata* ranked first and second in the previous PROMETHEE-GAIA analyses with an equal weighting of all parameters, the impact of nutrient provision (culture medium) and growth phase on biodiesel quality was investigated for *N. oculata* in a PROMETHEE analysis with equal ranking of all parameters (Figure 3). Data obtained for *N. oculata* raised in L1 medium through to stationary growth phase (Stat; this study) were compared to FAME-derived data obtained for *N. oculata* grown in L1, f/2 and K medium for growth phases logarithmic (Log), late logarithmic (LLog) and Stat [10] using the biodiesel characteristics, min/max and thresholds as per Table 4 and including estimated oxidative stability as [20], as an additional parameter, as reliability of the estimate is less of

concern for this single species (low or no amounts of C18:3 and C18:2) culture condition impact study on predicted biodiesel quality.

The two axes in Figure 3a explained 96% of the variability. Estimated oxidation stability, IV, PUFA, CN, DU and organic carbon provision were highly correlated with the decision vector, while total lipid, SFA had little or no effect and Db ≥ 4 MUFA and LCSF were anti correlated (Figure 3a). The anti-correlation of Db ≥ 4 is not surprising, as the set limit of 1 wt% was exceeded by this organism under all culture conditions used (Table 4). *Nannochloropsis oculata*_K_RH−LLog ranked highest followed by **N. oculata*_L1_Stat (this study) and *N. oculata*_RH_L1_Stat (Figure 3b). Inspection of the FAME profiles showed that culture conditions for the first ranked species resulted in substantially lower concentrations of EPA and arachidonic acid (AA) and almost doubled amounts of palmitoleic acid (C16:1 n−7) [10].

**Figure 3.** (**a**) GAIA plot of the effect of nutrients (media L1, f/2, K) and growth phase [Logarithmic (Log), Late Logarithmic (LLog), and stationary (Stat)] on **N. oculata* (present study) and from [10] biodiesel quality showing ten criteria (twelve biodiesel properties and total lipid content from Table 4) and the decision vector; and (**b**) corresponding outranking flow.




(**a**)

| Rank | Species | Phi |
|---|---|---|
| 1 | *N. oculata*_K_RH_LLog | 0.119 |
| 2 | **N. oculata*_L1_Stat | 0.089 |
| 3 | *N. oculata*_L1_RH_Stat | 0.062 |
| 4 | *N. oculata*_K_RH_Stat | 0.053 |
| 5 | *N. oculata*_f/2_RH_Stat | 0.046 |
| 6 | *N. oculata*_L1_RH_LLog(a) | −0.058 |
| 7 | *N. oculata*_f/2_RH_Log | −0.094 |
| 8 | *N. oculata*_f/2_RH_LLog | −0.100 |
| 9 | *N. oculata*_L1_RH_Log | −0.117 |

(**b**)

Although *N. oculata*_K_Stat showed similar effects of fertilization on the FAME profile, it was ranked fourth, most likely due to the combined effects of IV and PUFA over estimated oxidation stability (Table 4). It can be concluded, that growth phase and, to a lesser extent fertilization regime, *i.e.*, organic carbon provision, are important drivers for biodiesel quality for this species, which, given the impact on EPA and AA levels, would also affect oxidation stability.

The importance of some fuel properties depend on the country and place where it will be used and stored. As this study investigated the potential use of microalgal-derived biodiesel for onsite and

community use in tropical/sub-tropical regions, where industrial-scale cultivation for biofuels is predicted to occur, CFPP was not considered to be of importance here. Elevated temperatures of these regions are, however, likely to affect oxidative stability of the biodiesel. Given the impact of a high degree of unsaturation on this parameter, PUFA content was used as a proxy for oxidation stability in PROMETHEE where the weighting was increased from 1 (equal to all other parameters) to 50 (level of saturation, where further increases in the PUFA weighting led to no further change in the ranking of the nine species (this study) and the twelve species derived from the literature) [12] (Table 5). This weighting led to a significant change in predicted suitability of species with regards to the predicted quality of the biodiesel, as this weighting selected for species that also were well within the limits of Db ≥ 4, C18:3 and IV values. The diatom *Extubocellulus* sp. was ranked highest followed by the chlorphytes *Chlamydomonas* sp. and *Scenedesmus obliquus*. However, the heavy weighting of PUFA content changed the ranking of the previously first and second ranked species, *C. vulgaris* and *N. oculata* only slightly, as they remained in the top six of the 21 species investigated. In contrast, *Biddulphia* sp. dropped from rank 4 to 8, as *B. braunii* and *B. terriblis* moved to 6th and 7th place, with the largest improvement in ranking observed for *Chlamydomonas* sp. and *Scenedesmus obliquus* (Table 5). PUFA weighting did not change the ranking of species from 10th (*P. subcapitata*) to 21st (*Franceia* sp.) except *Mesotaenium* sp. and *Franceia* sp. traded positions. Given that *N. oculata* remained in 4th position, even under heavy weighting of PUFA content, it should be considered a suitable species for biodiesel production. This conclusion is also based on the proven ability for industrial cultivation in tropical/subtropical climates and the well-established year round average productivities of 20 g dry weight $m^{-2}$ $day^{-1}$ derived from several decades of production in highly economical race way outdoor operations, parameters that are yet to be established for the three highest ranked species.

**Table 5.** Ranking of nine microalgae species from the present study and twelve from [12] based on PUFA weightings of 1, 10, 30, 40, and 50. All other fuel properties were ranked as 1. A PUFA weighting of > 50 no longer affected rank order, indicating weighting saturation for this parameter.

| Species | Comparative Rank shift with different PUFA weighting | | | | | |
|---|---|---|---|---|---|---|
| | All weight = 1 | PUFA weight = 10 | PUFA weight = 30 | PUFA weight = 40 | PUFA weight = 50 | Direction of rank shift |
| *C. vulgaris* | 1 | 3 | 4 | 5 | 5 | ↓ |
| **N. oculata* | 2 | 2 | 3 | 3 | 4 | ↓ |
| **Extubocellulus* sp. | 3 | 1 | 1 | 1 | 1 | ↑ |
| **Biddulphia* sp. | 4 | 7 | 8 | 8 | 8 | ↓ |
| *B. terribillis* | 5 | 6 | 6 | 7 | 7 | ↓ |
| *chlamydomonas* sp. | 6 | 4 | 2 | 2 | 2 | ↑ |
| *S. obliquus* | 7 | 5 | 5 | 4 | 3 | ↑ |
| *C. microporum* | 8 | 9 | 9 | 9 | 9 | ↑ |
| *B. braunii* | 9 | 8 | 7 | 6 | 6 | ↑ |
| *P. subcapitata* | 10 | 10 | 10 | 10 | 10 | - |
| **P. tricornutum* | 11 | 11 | 11 | 11 | 11 | - |
| *D. brasiliensis* | 12 | 12 | 12 | 12 | 12 | - |
| **Picochlorum* sp. | 13 | 18 | 18 | 18 | 18 | - |
| *A. falcatus* | 14 | 13 | 13 | 13 | 13 | - |
| *A. fusiformis* | 15 | 15 | 15 | 15 | 15 | - |
| **Amphidinium* sp. | 16 | 14 | 14 | 14 | 14 | - |
| *C. bacillus* | 17 | 16 | 16 | 16 | 16 | - |
| *K. lunaris* | 18 | 17 | 17 | 17 | 17 | - |
| **S. dimorphus* | 19 | 19 | 19 | 19 | 19 | - |
| **Mesotaenium* sp. | 20 | 20 | 21 | 21 | 21 | ↓ |
| **Franceia* sp. | 21 | 21 | 20 | 20 | 20 | ↑ |

Red arrows: a large decline in ranking for the microalgal species ranked highest under equal weighting of the biodiesel quality parameters; Blue arrows: a large increase in species ranking to top six species rank at a PUFA weighting of 50; black arrows: slight changes in ranking at a PUFA weighting of 50; Hyphen: no ranking change for a PUFA weighting of 50.

## 4. Conclusions

In this study, nine microalgal species were cultivated and their total lipid and FAME profiles analysed, the latter was then used to estimate biodiesel properties [CN, IV, kinematic viscosity (υ), cold filter plugging point, density (ρ), higher heating values, SFAs, MUFA, and PUFA]. An equal parameter weighted PROMETHEE analyses established that the marine microalgae *Nannochloropsis oculata*, *Extubocellulus* sp. and *Biddulphia* sp. outranked the other six microalgal specieswhile the three freshwater chlorophytes (*Scenedesmus dimorphus*, *Franceia* sp., *and Mesotaenium* sp.) did not meet the ASTM D6751-02 and EN14214 standards. Since fatty acid composition determines the physical and chemical properties of biodiesel, and the amount of total fatty acid is a vital factor for commercial biodiesel production, both should be given priority for the selection of microalgal species for commercial biodiesel production.

Equal weighted PROMETHEE-GAIA analysis of FAME-derived biodiesel properties, C18:3 and double bond thresholds as per EN14214 of the nine microalgal species with twelve published FAME profiles of chlorophyte species, chosen based on similar subtropical climatic conditions, ranked *N. oculata* second but with only marginal differences to the first ranked species, *Chlorella vulgaris*.

The effect of nutrient provision (cultivation media) and growth phase was evaluated for calculated biodiesel properties of *N. oculata.* It was established that growth phase affected biodiesel quality to a greater extent compared to fertilization (nutrients), as a better ranking was achieved by stationary phase cultures; however, organic carbon provision in K medium also had an effect. *Nannochloropsis oculata* raised in K medium and harvested in late logarithmic growth phase achieved the best ranking for biodiesel quality due to the decline in PUFA (primarily driven by the decline of EPA and AA) and therefore better suited CN and IV values, followed by stationary phase *N. oculata* raised in L1.

As oxidative stability of biodiesel is affected by temperature and the main production sites of microalgae for biodiesel production will be tropical/subtropical areas with low population densities, a further analysis applied a saturated PUFA weighting as a proxy for oxidative stability of the biodiesel. In this analysis, *N. oculata* ranked fourth among the species. However, except for *Chlorella vulgaris*, a species that is incredibly difficult to extract [47], industrial-scale production has not yet been performed with any of the higher ranked species. Thus, unlike for *N. oculata*, no long term year-round average data on biomass and lipid productivities exist, which requires investigation before a final recommendation regarding these species can be made.

In summary, this study derived biodiesel quality parameters from FAME profiles and showed that CN, IV, C18:3 and double bond limits were the strongest drivers in equal biodiesel parameter-weighted PROMETHEE analysis. Using *N. oculata* as an example, it is clearly shown that stationary phase and, to a lesser extent, nutrient provision positively affect FAME profiles and thus biodiesel quality parameters. Application of a PUFA weighting to saturation proved important, as it led to a lower ranking of species exceeding the double bond EN14214 thresholds.

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## Conflicts of Interest

The authors declare no conflict of interest.

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