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RESEARCH NOTE

Calorific values of *Chlorella vulgaris* (Trebouxiophyceae) as a function of different phosphorus concentrations

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SUMMARY

Quantification of the calorific content of microalgae is critical in studies of energy flow, trophic partitioning, plant/herbivore interactions in aquaculture and biomass production for biofuels. We investigated the calorific value and biochemical composition of *Chlorella vulgaris* at different phosphorus (P) concentrations (6.0×10^{-7}, 2.3×10^{-6} and 2.3×10^{-4} mol L^{-1} P). As expected, the control (2.3×10^{-4} mol L^{-1} P) supported better growth than P limited treatments. Biomolecules like total carbohydrates and lipids accumulated under P limitation, which significantly correlated with high calorific values. Lipid class composition showed that triacylglycerols were the most accumulated under P limited conditions. The calorific value reported under control conditions (13.78 kJ g^{-1}) was less than those obtained under P limitation (30.47–33.07 kJ g^{-1}). The highest calorific value with less growth retardation was obtained at 2.3×10^{-6} mol L^{-1} P.

Key words: biochemical composition, biomass, lipid class, microalgae, nutrient stress.

Microalgae represent the base of food chains in aquatic ecosystems and their calorific values can be a direct reflection of their food value. Energy flow within food chains can be monitored via changes in calorific values of organisms at different trophic levels (Lamare & Wing 2001). The basis of energy flow, trophic partitioning, plant versus herbivore interactions in aquatic systems are associated with the calorific content of algae (Vadas *et al*. 2000). Although it is important for the ecology of aquatic ecosystems, only few investigations have considered the use of bomb calorimeters in relation to biomolecule production (Illman *et al*. 2000; Lamare & Wing 2001; Scragg *et al*. 2002; Mallick *et al*. 2012).

Microalgae are considered as reliable sources of biological material for fuel production (Illman *et al*. 2000), which can be an alternatives to fossil fuels because they are more photosynthetically efficient than higher plants, and can be grown in a wide variety of media, domestic and industrial effluents (Sturm & Lamer 2011). Sturm and Lamer (2011) reported that direct combustion of algal biomass may be a more viable energy source than biofuel production, especially when the lipid content of the dry biomass is low. This demonstrates the applicability of microalgae calorific value of varying lipid content and composition for energy production.

It is known that phosphorus is required in copious amounts when a healthy microalgal population is needed, and any alteration in the amount of this nutrient is reflected by physiological changes in microalgae (Reynolds 2006; Villar-Argaiz *et al*. 2009; Bhola *et al*. 2011). Such changes can be monitored using parameters like growth rate, biomass production and biochemical composition of the cells. Calorific values of *Chlorella vulgaris* vary with changes in the concentration of major nutrients in the growth media (Illman *et al*. 2000; Bhola *et al*. 2011; Mallick *et al*. 2012), as well as other environmental factors (Lamare & Wing 2001). Illman *et al*. (2000) and Scragg *et al*. (2002) reported increased calorific value of *C. vulgaris* when grown in a low nitrogen medium. Bhola *et al*. (2011) reported calorific values of 17 kJ g^{-1} of *C. vulgaris* when grown in varying environmental conditions, which is lower than most results for nutrient limitation (approximately 28 kJ g^{-1}). The calorific content of the macroalgae *Macrocystis pyrifera* and *Ulva lactuca* have been shown to vary seasonally with changes in photoperiodicity and sea surface temperature (Lamare & Wing 2001).

Chlorella vulgaris is a microalga of vast importance in different industries that range from pharmaceutical to aquaculture. It is a microalga known for its high

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nutritional value and potential for bioenergy production when its biochemical composition is manipulated. Although the chemical composition of the microalga has been routinely examined and quantitatively determined under varying phosphorus concentrations, quantitative measures of calorific content using bomb calorimeters in relation to its biochemical composition have been less investigated. The biochemical composition is considered here as total carbohydrate, protein and lipid contents, in addition to lipid class composition. We measured changes in calorific values, protein, carbohydrate and lipid class composition of *C. vulgaris* under varying phosphorus concentrations. In this research, we used semi-continuous cultures that provide better growth conditions for microalgae (Lombardi & Maldonado 2011), and allow for consistency in the physiological status of the cells, and reproducible microalgal responses.

The *C. vulgaris* strain used in this study was obtained from the Algae Collection Unit of the Departmento de Botânica, Universidade Federal de São Carlos, Brazil. The microalga was cultured in LC Oligo medium (AFNOR 1980) having the following composition: $Ca(NO_3)_2.4H_2O$ (1.7×10^{-4} M), KNO_3 (1.0×10^{-3} M), $MgSO_4.7H_2O$ (1.2×10^{-4} M), K_2HPO_4 (2.3×10^{-4} M), $CuSO_4.5H_2O$ (6.0×10^{-8} M), $(NH_4)_6Mo_7O_{24}.4H_2O$ (2.4×10^{-8} M), $ZnSO_4.7H_2O$ (1.0×10^{-7} M), $Mn(NO_3)_2.4H_2O$ (1.2×10^{-7} M), $H_3C_6H_5O_7.H_2O$ (1.4×10^{-7} M), H_3BO_3 (4.9×10^{-7} M), $C_6H_5FeO_7.5H_2O$ (1.5×10^{-6} M), $FeSO_4.7H_2O$ (1.1×10^{-6} M), $FeCl_2.4H_2O$ (1.6×10^{-6} M) and $NaHCO_3$ (1.79×10^{-4} M). Phosphorus limited cultures were grown in LC Oligo medium having K_2HPO_4 provided at 2.3×10^{-6} and 6.0×10^{-7} mol L^{-1}. Although cultures were not axenic, sterile techniques were used throughout to minimize contamination; all culture manipulations were performed under a flow of filtered and sterile air PA-PCR (Pachane, Brazil). The microalga was grown under controlled conditions of light intensity (160 μmol m^{-2} $s^{-1)}$, 16 : 8 h LD (light : dark) cycle, and temperature (22 ± 2°C). The reagents used in this study were of analytical grade and above.

Experiments were carried out in 1-L polycarbonate flasks having 250 mL of culture medium. Prior to beginning the experiments, *C. vulgaris* cells were acclimated to the specific treatment by transfers during the first half of exponential growth and further monitoring each new culture for its growth rate. After three statistically similar consecutive growth rates, the microalgae were considered to be acclimated, and their metabolism responding to each specific treatment condition. The acclimated cells were used for the experimental semi-continuous cultures at initial cell densities of approximately 10^5 cells mL^{-1}. The semi-continuous condition used for the experimental treatments was achieved through daily dilutions of the cultures (removal of culture and replacement with fresh and sterile culture media) in accordance with their growth rates. Prior to the daily dilutions, culture aliquots were taken to determine cell density and, consequently, growth rates. This guaranteed an almost constant cell density throughout the experiments. Optical density at 684 nm was measured using a HACH DR 5000 (HACH Company, Loveland, CO, USA) spectrophotometer. Based on equations 1 and 2 below (Lombardi & Maldonado 2011) specific growth rates (μ) were obtained.

$$\mu = \log\left(\frac{ABS(t2)}{a \times ABS(t1)}\right)(t2 - t1)^{-1} \quad (1)$$

Where

$$a = \frac{T_{vol} - R_{vol}}{Tvol} \quad (2)$$

and ABS = absorbance, t = time, T_{vol} = total volume and R_{vol} = removed/replaced volume.

Cell counts were done microscopically using an improved bright lined Neubauer haemocytometer. The semi-continuous cultures were maintained for 19 days, and on the 19th day, samples were taken for biochemical analysis. Three experimental replicates for all cultures were performed. Prior to algal dry weight determination, the filter papers were first dried at 60°C for 24 h and weighed. Algal dry weight was determined gravimetrically with the aid of a Sartorius MC21S analytical balance (Precision Weighing Balances, Bradford, MA, USA) with 1 μg readability, using 0.45 μM pore size cellulose acetate membrane filters (Millipore, Barueri, Sao Paulo, Brazil) with biomass retained after the filtration of 10 mL of culture, and drying at 60°C for 24 h.

Phosphate concentrations in the medium were determined according to the American Public Health Association (1998).

Chlorophyll *a* extraction and analysis were done in accordance with the procedure of Shoaf and Lium (1976). Chlorophyll concentration was computed using an equation described in Jeffrey and Humphrey (1975).

Intracellular carbohydrates were determined using the modified phenol-sulfuric acid method as described in Liu *et al*. (1973) with glucose as standard; and total proteins were determined according to the procedure described in Bradford (1976) with bovine serum albumin as protein standard. The extraction of total proteins was done following the protocol of Rausch (1981).

Lipid extraction was carried out using the modified Folch procedure (Parrish 1999) with dichloromethane and methanol in a 2:1 ratio. All samples were spiked with an internal standard (Hexadecan-3-one) just before extraction. Samples were homogenized with a glass rod and then sonicated for 2 min in two cycles of 1 min each. Samples were concentrated under pure

nitrogen gas and spotted onto silica gel-coated rods (Chromarods-SIII). Analysis of lipid class composition and concentrations was done using an Iatroscan TLC-FID MK6s system. Peak identification was done from calibration curves made with nine lipid standards: n-nonadecane (aliphatic hydrocarbon, HC), cholesteryl palmitate (wax esters/steryl ester, WE/SE), n-hexdecan-3-one (ketone), glyceryl tripalmitate (triacylglycerol, TAG), palmitic acid (free fatty acids, FFA), 1-hexadecanol (free aliphatic alcohol, ALC), cholesterol (free sterol, ST), 1-monopalmitoyl-rac-glycerol (acetone mobile phase lipids, AMPL) and 1,2, di-0-hexadecyl-sn-glycerol-3-phosphatidylcholine (phospholipids, PL). The lipid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). The analytical conditions used for the Iatroscan were: hydrogen flow of 173 mL min^{-1}, air flow 2 L min^{-1} and scan speed of 4 mm s^{-1}. All GF/C filters (0.45 μM pore size), glasswares, and metal materials that came into contact with the samples were previously baked overnight at 400°C. Teflon and glass materials were rinsed with acetone and then dichloromethane before use to reduce contaminations. All reagents used for lipid analyses were of chromatographic grade.

Calorific value of *Chlorella vulgaris* for each experimental treatment was determined using an IKA C200 bomb calorimeter (IKA, Heitersheim, Germany). Culture samples (150 mL) were filtered onto Sartorius cellulose acetate filters (0.45 μM pore size) to retain algal biomass and dried at 60°C for 24 h prior to analysis. These samples were then weighed before being combusted in the calorimeter. The calorific values of blank filters were obtained by combusting several filters without the microalga and the obtained value was subtracted from filters with algae (Illman *et al*. 2000). The instrument was calibrated according to the manufacturer's instructions using the pre-weighed tablets provided.

Analysis of variance (ANOVA) and Tukey's honestly significant difference multiple range comparison tests were used to test for significant differences between the means of analyzed parameters. A correlation matrix based principal components analysis (PCA) was used to determine the relationship between analyzed parameters. PCA scores were grouped by cluster analysis using a single linkage. All analyses were done at the 95% confidence interval. ANOVA and post hoc analyses were done using Statistica 8.0 (Stat Soft, Tulsa, OK, USA) software, PCA was done with PAST 2.09 for Windows (Hammer & Harper, Natural History Museum, Oslo, Norway) and cluster analysis with Minitab version 16.0 (Minitab Inc., State College, PA, USA).

Specific growth rate ranged from 0.45 to 0.85 day^{-1} according to the experimental treatments with the lowest P concentration having the lowest growth rate (Fig. 1, Table 1). Dry weight per cell increased with decreasing P, while cell density increased with increasing P (Table 1). The highest chlorophyll *a* concentration was recorded at 2.3×10^{-6} mol L^{-1} P, while the lowest was at 6.0×10^{-7} mol L^{-1} P. The biochemical composition of *C. vulgaris* was affected by phosphorus concentrations, and the calorific values increased with decreasing P concentrations. Protein decreased with decreased P concentrations, while carbohydrates and lipids increased under the same conditions. The highest protein value (12.12 pg $cell^{-1}$) was obtained at 2.3×10^{-6} mol L^{-1} P and the lowest (1.84 pg $cell^{-1}$) at 6.0×10^{-7} mol L^{-1} P.

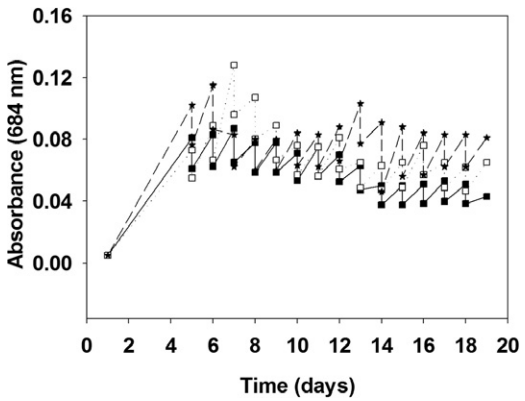

Fig. 1. Growth curve for *Chlorella vulgaris* at different P concentrations reported as absorbance (684 nm) vs time (days). Stars represent 2.3×10^{-4} mol L^{-1} P, empty squares 2.3×10^{-6} mol L^{-1} P, and filled squares 6.0×10^{-7} mol L^{-1} P.

Our results are similar to those of Khozin-Goldberg and Cohen (2006), which showed that under phosphorus limitation, cell division and chlorophyll synthesis of *Monodus subterraneus* were reduced. This confirms the important role phosphorus plays in microalgal metabolism, since the highest growth rate was obtained in the control. Optimum phosphorus concentrations result in high growth rates and biomass production in microalgae (Khozin-Goldberg & Cohen 2006; Chisti 2008; Widjaja *et al*. 2009; Bhola *et al*. 2011). Changes in dry weight and cell size depend on the concentration of nutrients in the algal surrounding environment. Under nutrient limitation, the rate of cell division by microalgae is reduced and cells tend to increase in size rather than divide as a survival strategy, which results in higher dry weight per cell. This is in agreement with the results of Chen *et al*. (2011), which showed that at low temperature and phosphorus concentration, *Scenedesmus obliquus* and *Microcystis aeruginosa* length or diameter and volume were higher. The present results showed that high calorific values are supported by the levels of total lipids and carbohydrates, which increased under P stress. This agrees with the results of other studies that showed that microalgae store carbohydrates and total lipids under stress conditions, thereby causing their

Table 1. Biomass, biochemical composition and calorific production of *Chlorella vulgaris* as a function of phosphorus concentrations

Parameter	Phosphorus concentration (mol L^{-1})		
	Control	2.3×10^{-6}	6.0×10^{-7}
Specific growth rate (d^{-1})	0.85 ± 0.05^{c}	0.67 ± 0.04^{b}	0.45 ± 0.04^{a}
Cell density ($\times 10^4$ cells mL^{-1})	$135 + 12.29^{c}$	$77 + 3.87^{b}$	$42 + 3.29^{a}$
Dry weight (pg $cell^{-1}$)	16.83 ± 2.17^{a}	41.74 ± 2.92^{b}	55.77 ± 4.93^{c}
Chlorophyll a (pg $cell^{-1}$)	1.12 ± 0.01^{b}	1.15 ± 0.08^{b}	0.73 ± 0.09^{a}
Carbohydrates (pg $cell^{-1}$)	2.73 ± 0.58^{a}	16.05 ± 2.29^{b}	18.77 ± 2.58^{b}
Protein (pg $cell^{-1}$)	2.07 ± 0.43^{a}	12.12 ± 0.41^{b}	1.84 ± 0.58^{a}
Total lipids (pg $cell^{-1}$)	1.44 ± 0.09^{a}	7.16 ± 0.88^{b}	9.10 ± 1.85^{b}
Calorific value (kJ g^{-1})	14.00 ± 1.66^{a}	30.47 ± 5.48^{b}	33.07 ± 3.98^{b}

Values represent plus or minus standard error for $n = 3$. Rows with the same alphabets are not significantly different at 95% significance level ($P < 0.05$).

Table 2. Lipid class composition (% total lipids) of *Chlorella vulgaris*. at different phosphate regimes (mol L^{-1})

Lipid class	Phosphorus concentration (mol L^{-1})		
	Control	2.3×10^{-6}	6.0×10^{-7}
HC	2.77 ± 0.48^{a}	5.29 ± 1.64^{b}	2.24 ± 0.38^{a}
WE/SE	2.63 ± 0.01^{a}	2.70 ± 0.99^{a}	–
TAG	2.63 ± 0.02^{a}	11.76 ± 5.05^{b}	57.64 ± 5.43^{c}
FFA	1.75 ± 0.01	–	–
ALC	1.86 ± 0.74^{a}	1.34 ± 0.51^{a}	0.89 ± 0.42^{a}
ST	3.44 ± 0.84^{b}	2.92 ± 0.57^{b}	1.12 ± 0.06^{a}
AMPL	57.49 ± 2.30^{c}	46.22 ± 2.31^{b}	18.72 ± 1.70^{a}
PL	35.04 ± 1.93^{c}	29.78 ± 5.46^{b}	19.75 ± 5.90^{a}

Values are mean plus or minus standard error for $n = 3$. Rows with the same alphabets are not significantly different at 95% significance level ($P < 0.05$). ALC, free aliphatic alcohol; AMPL, acetone mobile phase lipids; FFA, free fatty acids; HC, aliphatic hydrocarbon; PL, phospholipids; WE/SE, wax esters/steryl ester; ST, free sterol; TAG, triacylglycerol.

calorific values to increase (Illman *et al*. 2000; Scragg *et al*. 2002; Yin 2011). This is because carbohydrates and lipids are high energy yielding biomolecules. The calorific value obtained in the present research for the control (14 kJ g^{-1}) is lower than those reported in other studies (17–21 kJ g^{-1}) for *Chlorella* spp. when grown under healthy growth conditions (Illman *et al*. 2000; Scragg *et al*. 2002; Bhola *et al*. 2011). However, the values we recorded (30–33 kJ g^{-1}) under P limitation are higher than those reported by Illman *et al*. (2000) and Scragg *et al*. (2002) (approximately 28 kJ g^{-1}). Our results show that the lower growth retardation (0.67 day^{-1}) at 2.3×10^{-6} mol L^{-1} P and high calorific value obtained at this P concentration may be a promising combination for biomass production directed towards biofuel extraction. This is because at this P concentration the volume specific calorific value (KJ mL^{-1}) was highest. Illman *et al*. (2000), Scragg *et al*. (2002), Bhola *et al*. (2011) and Mallick *et al*. (2012) pointed out that for microalgae to be viable as diesel replacement, they need to have high calorific value coupled with a compromise of high growth rates. To date the highest value reported for *Chlorella vulgaris* optimized using a combination of five factors (glucose, N, P, Fe and incubation period) with a central composite rotary design (CCRD) is approximately 38 kJ g^{-1} (Mallick *et al*. 2012). However, these authors did not show the calorific value changes from healthy P to limited P conditions. The calorific values obtained by the authors and those obtained in this study are lower than that of petroleum diesel, that is approximately 42 kJ g^{-1}. This lower value is probably due to the differences in the chemical composition and the presence of oxygen molecule (10% of weight) in the molecular structure of biodiesel (Mallick *et al*. 2012).

Lipid class analysis showed that the storage lipid classes HC, WE/SE and TAG increased at low P concentrations. At the lowest P (6.0×10^{-7} mol L^{-1}) the TAG component was the most accumulated lipid among all lipid classes. Aliphatic hydrocarbons and ST content decreased at low P (Table 2). The polar lipids composition showed that AMPL and PL were lower under P limitation (Table 2). Principal components analysis showed that P was positively associated ($P < 0.05$) with PL, ST, AMPL, cell density, chlorophyll *a,* and growth rates (Fig. 2). However, a negative relationship ($P < 0.05$) was observed for P concentration with TAG, dry weight, total lipids, carbohydrates and calorific values. The PCA showed that 77.33% of the accounted variations were within the first two components.

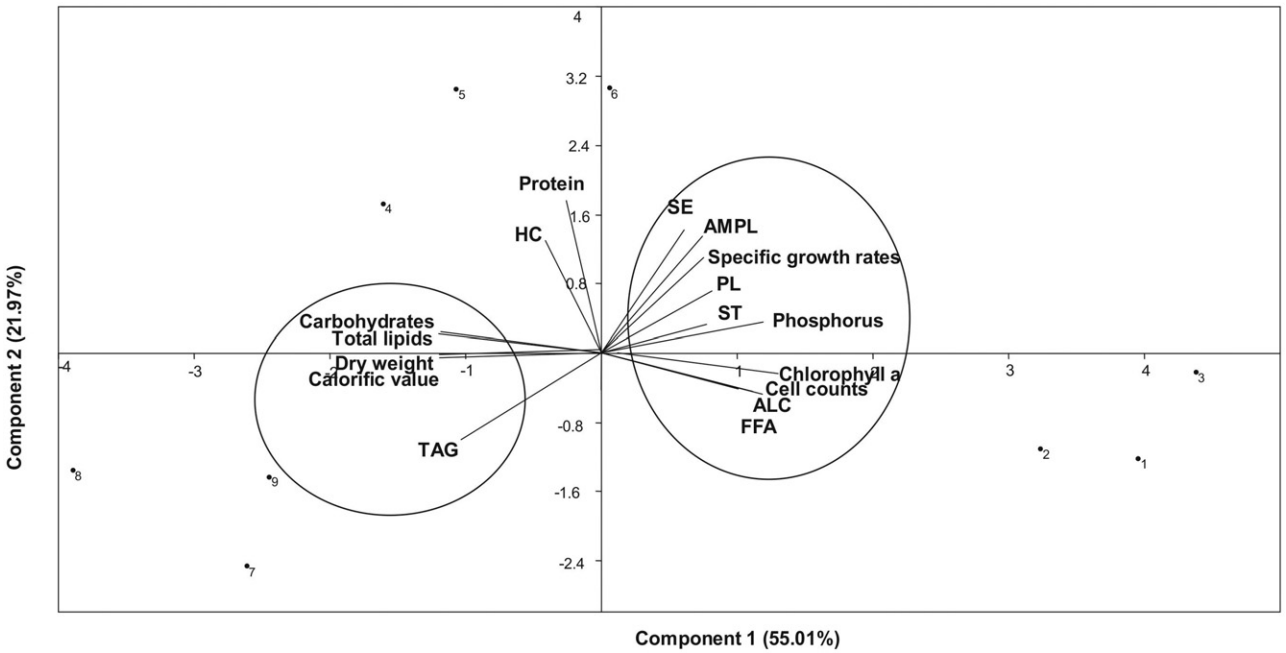

Fig. 2. Principal component analyses to show the relationship between and within phosphate levels and physiological parameters analyzed for *Chlorella vulgaris* grown as a function of different phosphate concentrations (mol L^{-1}).

When P is deficient in an environment, microalgae tend to accumulate more of TAG in place of other storage lipid classes (Hu *et al*. 2008; Goutx *et al*. 2009). Under limited P conditions, *C. vulgaris* showed an increased TAG/PL ratio, which is indicative of nutrient stress conditions (Guschina & Harwood 2006). This is further supported by the fact that under stress conditions, the production of TAG progresses with precedence over that of PL. This change in lipid composition directed towards higher neutral lipid production also supports the higher calorific value reported under P limitation. The significant association we obtained between phosphorus and phospholipids is explained by the important role phosphorus plays in the PL molecule. The increase of triacylgycerols and decrease of polar lipids to total lipids we obtained under phosphorus limitation is in agreement with the results of Lynn *et al*. (2000) and Hu *et al*. (2008). These authors reported that up to 80% TAG can be produced by microalgae under nutrient stress.

The biochemical composition for example, protein and lipid content of the grazed algae are suggested as the primary determinants of their nutritional value, and any changes in their composition and quantity may be detrimental to the ecological balance (Lamare & Wing 2001). The possession of high calorific value by microalgae does not mean they will be ideal for the optimum growth, survival, biomass production and reproduction of zooplankton and other herbivores that graze on them. As we have demonstrated, the conditions that supported higher calorific values also supported higher carbohydrate and lipid content (TAG). However, the conditions that promote higher polar lipids (e.g. phospholipids) production are crucial for the survival of higher trophic level organisms that depend on microalgae, and this condition was associated with lower calorific values in the present research. It is known that changes in cellular P quota and lipid composition of algae affect their value as food for herbivores/grazers (Villar-Argaiz *et al*. 2009), and a decreased PUFA content, which was associated with lower polar lipid content in *C. vulgaris* will affect the growth, biomass production and reproduction of organisms of higher trophic levels that depend on the microalga as food source (Spijkerman & Wacker 2011).

The calorific value of *C. vulgaris* under P limiting concentrations was improved. High concentrations of biochemical parameters such as carbohydrate and total lipid, and biomass (dry weight per cell) concentrations were positively correlated with high calorific values. Increasing the severity of P limitation decreases growth, and supports the accumulation of storage lipids (TAGs) over structural lipids. The best calorific values with less growth retardation were obtained at 2.3×10^{-6} mol L^{-1} P concentration.

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