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PHYSIOLOGICAL RESPONSE OF *CHLORELLA VULGARIS* TO CADMIUM, PHOSPHORUS AND NITROGEN

(RESPOSTA FISIOLOGICA DE *CHLORELLA VULGARIS* SOB CONCENTRAÇÕES VARIÁVEIS DE CÁDMIO, FÓSFORO E NITROGÊNIO)

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Mathias Ahii Chia

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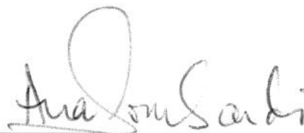

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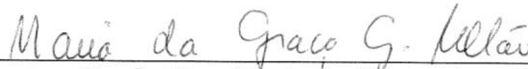

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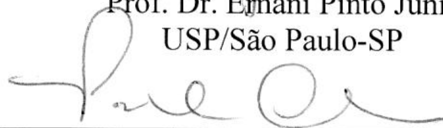

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Look deep, deep into nature, and then you will understand everything better – ***Albert Einstein***.

This work is dedicated to my lovely wife Estyluv for her unending love and to the King of all kings.

DECLARATION

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The experiments related to this study were carried out in the Plankton Laboratory, which is part of the Hydrobiology Department, UFSCar. These involved growth, biomass and biochemical composition determination of the microalga under different media type, nutrient (nitrogen and phosphorus) and cadmium concentrations. Biochemical analyses involved total proteins, carbohydrates and lipids. Lipid class determinations were done using the Iatroscan-TLC/FID system. Fatty acid composition kindly performed through the collaboration and supervision of Prof. Dr. Christopher C. Parrish at Memorial University of Newfoundland (Canada) in St John’s using gas chromatography (GC-FID).

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ABBREVIATIONS AND SYMBOLS

ω3 = Omega 3

μ = Specific growth rate

ANOVA = Analysis of variance

ABS = Absorbance

ALC = Free aliphatic alcohol(s)

AMPL = Acetone mobile polar lipid(s)

BBM = Bold basal medium

C = Carbon

$CaCl_2$ = Calcium chloride

Carb = Total carbohydrates

Cd = Cadmium

CDCA1 = Carbonic anhydrase

CH_2Cl_2 = Dichloromethane

Chl = Chlorophyll

CHU = Chu 10 medium

CoA = Coenzyme-A

Cu = Copper

DAG = Diacylglycerol(s)

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic acid

DTPA = Diethylenetriaminepentaacetic acid

DW = Dry weight

EDTA = Ethylenediamine tetracetic acid

FA = Fatty acids(s)

FAME = Fatty acid methyl ester

Fe = Iron

FFA = Free fatty acid(s)

FID = Flame Ionization detector

HC = Aliphatic hydrocarbons

HCl = Hydrochloric acid

HUFA = Highly-unsaturated fatty acid(s)

KE = Ketone(s)

L = Lipids

LC Oligo = LC Oligo medium

MeOH = Methanol

MUFA = Monounsaturated fatty acid(s)

N = Nitrogen

NaOH = Sodium hydroxide

NTA = Nitrilotriacetic acid

OD = Optical density

P = Phosphorus

PA = Phosphoric acid

Pb = Lead

PCA = Principal component analysis

PL = Phospholipid(s)

PPS = Partial pyrolysis scan

PR = Total proteins

PUFA = Polyunsaturated fatty acid(s)

Qv = Biomolecule/biomass productivity

RNA = Ribonucleic acid

SAFA = Saturated fatty acid(s)

SGR = Specific growth rate

ST = Free sterol(s)

TAG = Triacylglycerol(s)

UV-VIS = Ultraviolet-Visible light

WC = WC medium

WE/SE/ME = Wax ester(s)/Steryl ester(s)/Methyl ester

W/W = Wet weight

X = Biomolecule concentration

Zn = Zinc

ABSTRACT

Microalgae are capable of adapting themselves to changes in environmental conditions through the production of different biomolecules. This study investigated the effects of the metal, cadmium on the ecophysiological response of microalgae under varying nutrient conditions. In the first phase of the study, we examined the growth, biomass production and biochemical composition of *Chlorella vulgaris* using semi-continuous cultures employing three growth media (LC Oligo, Chu 10 and WC media) to enable the selection of the most appropriate growth medium. The highest cell density, chlorophyll *a,* carbohydrate, protein and lipid concentration were found in Chu and LC Oligo media. Due to the cost effectiveness of using LC Oligo medium, it was the chosen as the preferred medium among the three tested media.

The calorific values of *C. vulgaris* at different phosphorus (P) levels were investigated in the second phase of this study. Calorific value reported under replete concentration (13.78 kJ g^{-1}) was less than those found under P limitation (30.47-33.07 kJ g^{-1}). The highest calorific values without growth retardation were obtained at the 10^{-6} mol L^{-1} P.

The third phase of this research involved determinations of growth, biomass production and biochemical composition of *C. vulgaris* under varying cadmium (Cd), phosphorus (P) and nitrogen (N) concentrations. Three P (6.0×10^{-7}, 2.3×10^{-6} and 2.3×10^{-4} mol L^{-1}) and N (2.9×10^{-6}, 1.1×10^{-5}, 1.1×10^{-3} mol L^{-1}) concentrations were tested one at a time with or without the addition of Cd at 10^{-7} and 2.0×10^{-8} mol L^{-1}. Cadmium at 10^{-7} mol L^{-1} severely reduced growth and biomass production when P or N were limiting. Under N limitation total intracellular carbohydrates were accumulated most intensively compared to the P treatments with or without Cd. The presence of 2.0×10^{-8} Cd stimulated higher carbohydrate production when P was limiting. Protein productivity decreased with decreasing N and P concentrations. Total protein was highest in the control. Under nutrient replete conditions, the growth and biomass inhibitory effects of

Cd were significantly reduced. Combined P limitation and Cd exposure stimulated higher total lipid production than only P or N limitation.

Triacylglycerols (TAG) was the most accumulated lipid class under P and N limitation and Cd stress among all the neutral and polar lipid classes. Increasing P and N limitation and Cd exposure resulted in higher SAFA and MUFA concentrations. 16:1n - 11 was an exception among the MUFA as its levels decreased at low N. Total PUFA and ω3 PUFA levels, and PUFA:SAFA ratios increased under healthy growth conditions (high P and N without Cd). An exception among the PUFA was 18:2n – 6 that responded to P treatments differently as its levels increased with increasing P and N limitation. A significant ($p<0.05$) positive relationship of PUFAs, AMPL and PL with P and N was observed. Total SAFA, 14:0, 18:1n – 9 and 18:2n – 6 were positively related to Cd and negatively correlated with P and N.

RESUMO

A geração de resíduos industriais e a possibilidade de produção biomassa de microalgas em águas residuais estimulam a pesquisa relacionada com os efeitos do cádmio na resposta fisiológica dos microorganismos. Este estudo investigou os efeitos de metais sob condições variaveis de nutrientes sobre a resposta ecofisiológica de *Chlorella vulgaris*. Na primeira fase do estudo, examinamos o crescimento, produção de biomassa e composição bioquimica de *Chlorella vulgaris* nos meios nutritivos LC Oligo, Chu 10 and WC para selecionar o meio mais adequado. A densidade celular, clorofila *a,* proteínas, carboidratos e lipídios determinados e os resultados mostraram maiores concentrações das biomoléculas nos meios Chu e LC Oligo. Devido à relação custo-eficácia do uso, o meio LC Oligo foi escolhido entre os três meios testados.

Esta segunda fase da pesquisa investigou o poder calorífico da *C. vulgaris* em concentrações diferentes do fósforo (P). O valor calorifico no controle (13.78 kJ g^{-1}) foi menor do que sob limitação do P (30.47-33.07 kJ g^{-1}). Os melhores valores caloríficos com menor retardo de crescimento foram obtidos no tratamento com $2.3x10^{-6}$ mol L^{-1} P.

A terceira fase deste estudo envolveu determinações de crescimento, produção biomassa e composição bioquímica da *C. vulgaris* na presença de cádmio (Cd) sob concentrações diferentes de fósforo (P) e nitrogênio (N). Três concentrações de P ($6.0x10^{-7}$, $2.3x10^{-6}$ and $2.3x10^{-4}$ mol L^{-1}) e N ($2.9x10^{-6}$, $1.1x10^{-5}$, $1.1x10^{-3}$ mol L^{-1}) foram testadas individualmente, com ou sem adição de Cd em 10^{-7} e $2.0x10^{-8}$ mol L^{-1}. Cádmio em concentração de 10^{-7} mol L^{-1} reduziu severamente o crescimento e produção biomassa, quando P ou N estavam limitando o crescimento. Sob limitação de N, carboidratos totais foram mais acumulados do que os tratamentos de P sem Cd. A presença de $2.0x10^{-8}$ mol L^{-1} Cd estimulou a produção de carboidratos quando P era limitante. A produtividade de proteína diminuiu com a diminuição das concentrações de N e P. Proteína total foi maior na combinação de $2.3x10^{-6}$ mol L^{-1} P e $2.0x10^{-8}$ mol L^{-}

^{1} Cd. Sob condições repleta de nutrientes, os efeitos inibitórios do Cd sobre o crescimento e produção de biomassa algal foram significativamente reduzidos. Limitação de P e estresse Cd combinados estimulou maior produção lipidica totais do que apenas N.

Triacilgliceróis (TAG) foi a classe de lipídios mais acumulada em limitação do P e N e com estresse de Cd, entre todas as classes de lipídios neutros e polares. Limitação do P e N, e a exposição ao Cd resultaram em maiores concentrações de SAFA e MUFA. 16:1n – 11 foi uma exceção entre os MUFA, uma vez que seus níveis diminuiram em baixo N. PUFA total e ω3 PUFA, e proporção de PUFA:SAFA aumentaram sob alta P sem Cd. Uma exceção entre os PUFA foi 18:2n – 6, cuja concentração aumentou com aumento da limitação do P e N. A relação ($p<0.05$) positiva de PUFA, AMPL e PL com P e N foi observada. Total de SAFA, 14:00, 18:1n – 9 e 18:2n – 6 foram correlacionadados positivamente com Cd e negativamente com P e N.

1. INTRODUCTION

The biosynthesis of biomolecules by microalgae is influenced by changes in physical and chemical conditions of their immediate environment. Abiotic variables like temperature, light intensity, pH, nutrients and pollutants affect the physiology, biomass production, and biochemical composition of algae (GRESSLER et al., 2011; PINTO et al., 2011). Thus, investigations into the physiology of microalgae involved in their biochemical composition generate important knowledge that can be used to manipulate and optimize the production of lipids, proteins or carbohydrates.

Contamination of aquatic ecosystems with trace metals has increased worldwide since the industrial revolution (RUANGSOMBOON and WONGRAT, 2006). Cadmium (Cd) is a potent environmental contaminant from batteries, anti-corrosive metal coating and pigment industry residues (BAJGUZ 2011) that can accumulate in food webs (CHAN et al., 2003). Cadmium toxicity to microalgae is well reported in literature. DUONG et al. (2010) showed a decrease in diatom cell density in Cd contaminated cultures. RUANGSOMBOON and WONGRAT (2006), BAUMANN et al. (2009) and MONTEIRO et al. (2011) showed that Cd inhibits growth, biomass production and affects microalgae biochemical composition. Although its nutritional role has not been confirmed for the majority of microalgae, Cd can substitute for Zn in *Thalassiosra weissflogii*, *T. pseudonana*, *Nitszchia cf. pusilla* and *Asterionellopsis glaciallis* (PARK et al., 2007). Cadmium has been proposed as a nutrient for marine diatoms with the evidence of a novel carbonic anhydrase (CDCA1) having Cd as its metal center (PARK et al., 2007).

The damage caused by reactive oxygen species (ROS) is known as oxidative stress (WU et al., 2009). Cd has been found to produce oxidative damage to lipids and nucleic acids (WATANABE et al., 2003). In response, algae have developed defense systems via non-enzymatic and enzymatic scavenging of cellular ROS to cope with oxidative stress (PINTO et al., 2003).

The water soluble ascorbate (AsA) and glutathione (GSH), and the water insoluble α-tocopherol and carotenoids are the non-enzymatic agents that scavenge ROS (MUNNÉ-BOSCH and ALEGRE, 2002).

The antioxidative responses to Cd stress are different between algal species (WU et al., 2009). Cd increased H_2O_2 concentrations, lipid peroxidation, and the activities of specific enzymes (APX, POX and CAT) in the marine microalga *Nannochloropsis oculata,* but decreased the activities of SOD and GR (LEE and SHIN, 2003). In the marine red macroalga *Gracilaria tenuistipitata,* Cd increased CAT activity but did not affect SOD or APX activities (COLLÉN et al., 2003). In the marine dinoflagellate *Gonyaulax polyedra,* acute exposure to Cd generated oxidative stress in chloroplasts while under chronic exposure, the antioxidant system was able to provide protection (OKAMOTO et al., 2001a,b; WU et al., 2009).

Nutrients are one of the most important factors regulating phytoplankton growth in natural and artificial systems. Changes in nutrient regimes can have drastic effects on aquatic ecosystems (CHIA et al., 2009, 2011a, 2011b); phosphorus is critical to the support of life and, excess phosphorus can cause eutrophication, which results in severe environmental impact in both fresh and marine ecosystems. It is well documented that algal blooms are caused by over abundance of nutrients and phosphorus, mostly in the form of phosphates, is often the reported as the limiting nutrient in the growth of algae (BUTZLER, 2002; CHIA et al., 2009). In biogeochemical cycles, phosphorus is considered as one of the most critical single factor that acts upon its maintenance.

Nitrogen is another important nutrient required in major amounts for the microorganisms. It enters phytoplankton based food chain mostly in the form of ammonium or nitrate ions (CHIA et al., 2011a). Nitrates released into tropical waters can come from sediments and/or ground water. Sewage from homes/industries and fertilizers from agricultural lands continuously increase the load of nitrogen and phosphorus into aquatic systems (CHIA et al., 2011a). Nutrients are usually spatially and temporally dynamic in terms of both

concentrations and structure in aquatic environments, and can be patchily distributed (BEARDALL et al., 2001).

As a response to nutrient availability and other environmental conditions, microalgal cells may undergo a series of metabolic acclimations that can be referred altogether as physiological plasticity. These often result in variations of the cellular composition, considering macromolecules such as carbohydrates, lipids, amino acids, nucleic acids, pigments and proteins (BERDALET et al., 1994; LATASA and BERDALET, 1994; ZHAO et al., 2009). Changes in these molecules and their specific ratios, including chlorophyll/protein, chlorophyll/RNA, RNA/DNA, protein/carbohydrate, carbohydrate/protein/lipid under different nutrient conditions can help to indicate the algal physiological state and nutritional status (LAI et al., 2011). Phytoplankton pigment abundance or composition has also been suggested as indicators of the physiological status of phytoplankton (STOLTE et al., 2000; LAI et al., 2011).

Thus, microalgae have attributes that can be used with success in biotechnological research, focusing on the production of specific molecules. This research focused on the physiological response of *Chlorella vulgaris*, a freshwater Chlorophyceae to several environmental stresses as to delineate its response through modifications of its biochemical composition. Cadmium was chosen as a toxic agent so we could infer its nutrient role as well.

1.1 Microalgae lipids

Microalgae synthesize fatty acids as building blocks for the formation of various types of lipids. Fatty acids are hydrocarbon derivatives, at about the same low oxidation state (highly reduced) as hydrocarbons in fossil fuels. They are primary metabolites of the acetyl-CoA pathway which is genetically determined, evolutionary very old, and therefore conservative (GUSHINA and HARWOOD, 2006a, 2009; PETKOV and GARCIA, 2007; HU et al., 2008) (FIGURE 1.1). The most commonly synthesized fatty acids by microalgae have chain lengths that range from C_{16} to C_{18}, similar to those of higher plants (OHLROGGE and BROWSE, 1995). Fatty acids can be either saturated or

unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone.

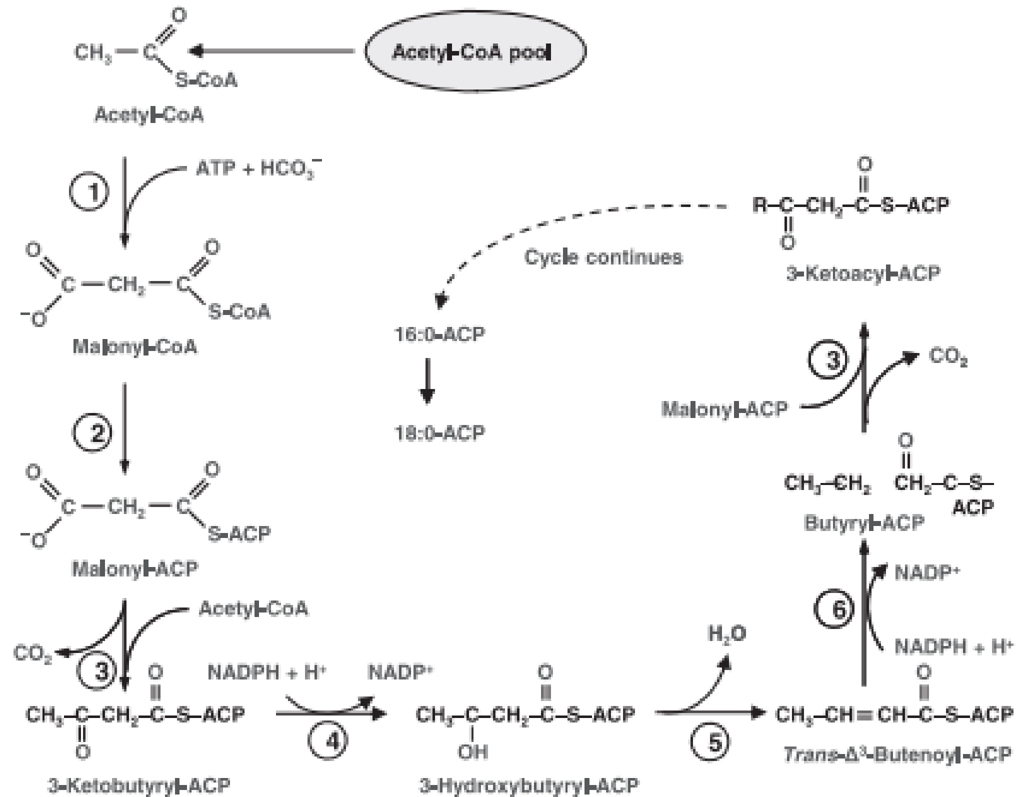

FIGURE 1.1 Fatty acid *de novo* synthesis pathway in chloroplasts. Acetyl CoA enters the pathway as a substrate for acetyl CoA carboxylase (Reaction 1) as well as a substrate for the initial condensation reaction (Reaction 3). Reaction 2, which is catalyzed by malonyl CoA:ACP transferase and transfers malonyl from CoA to form malonyl ACP. Malonyl ACP is the carbon donor for subsequent elongation reactions (adapted from HU et al., 2008).

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds. Based on the number of double bonds, individual fatty acids are named dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids (HU et al., 2008). In addition, relying on the position of the first double bond from the terminal methyl end (ω) of the carbon chain, unsaturated fatty acids are designated with an 'ω' or 'n' number. For example, a fatty acid may be an ω3 PUFA (i.e. the

third carbon from the end of the fatty acid) or an ω6 PUFA (i.e. the sixth carbon from the end of the fatty acid: HU et al., 2008). Studies on major algal groups show that the predominant PUFAs are 20:5n – 3 and 22:6n – 3 in Bacillarilophyceae; 18:2 and 18:3n – 3 in Chlorophyceae; 18:2 and 18:3n – 3 in Euglenophyceae; 20:5, 22:5 and 22:6 in Chrysophyceae; 18:3n – 3, 18:4 and 20:5 in Cryptophyceae; 20:3 and 20:4n – 3 in Eustigmatophyceae; 18:3n – 3 and 20:5 in Prasinophyceae; 18:5n – 3 and 22:6n – 3 in Dinophyceae; 18:2, 18:3n – 3 and 22:6n – 3 in Prymnesiophyceae; 18:2 and 20:5 in Rhodophyceae; 16:3 and 20:5 in Xanthophyceae; and 16:0, 18:2 and 18:3ω3 in Cyanobacteria (COBELAS and LECHADO, 1989; BASOVA, 2005; PETKOV and GARCIA, 2007; HU et al., 2008). Unlike what is observed in higher plants where there is constancy in fatty acid composition, significant variations in fatty acid composition are found in algal taxa (HU et al., 2008; HARWOOD and GUSHINA, 2009).

Different lipid classes are produced by microalgae ranging from biogenic hydrocarbons to phospholipids. Hydrocarbons are often thought of as being indicators of pollution. However, hydrocarbons can account for a significant proportion of lipids in aquatic organisms. An important example is seen in *Botryococcus braunii*, a fresh- and brackish water organism, which is predisposed to hydrocarbon synthesis. It can produce hydrocarbons in amounts between 20 and 80% of its dry mass (HU et al., 2008). Generally, the amount of this lipid class is found in small quantities ($\leq$2%) in most algae (LOMBARDI and WANGERSKY, 1991, 1995; HU et al., 2008).

Free fatty acids are those fatty acids that exist in chemically uncombined form (‘free’) in nature. They are not just an artifact that may be caused by the action of lipases released from damaged cells (PARRISH 1988, LOMBARDI and WANGERSKY, 1995), but they constitute a normal part of tissue lipid pool. The amounts of this lipid class in phytoplankton range from 5 to 25% of total lipids.

Triacylglycerols, diacylglycerols and monoacylglycerols are important classes of algal lipids. Triacylglycerols act as energy reservoirs, buoyancy control and/or as thermal insulators (BASOVA, 2005). The exact proportion of triacylglycerols in the cell is regulated by nutrient availability (MERZLYAK et al., 2007). Mono- and diacylglycerols are usually minor constituents in cells, but they are important intermediates in anabolic and catabolic fatty acid ester pathways (PARRISH, 1988).

Chloroplast/membrane lipids include pigments, glycolipids, sulpholipids and phospholipids (HU et al., 2008). Phospholipids are important constituents of all cell membranes, while the other pigmented (acetone mobile polar lipids – AMPL) and non-pigmented lipids are peculiar to cells capable of photosynthesis (PARISH, 1988). These lipids are actively involved in the mechanisms of photosynthesis. Chlorophyll *a* is the principal pigment in all photosynthetic plants cells, and monogalactosyl diglyceride, digalactosyl diglyceride, and sulphoquinovosyl diglyceride are the principal glycolipids and sulpholipids in plant cells (PARRISH, 1988; GUSHINA and HARWOOD, 2006a). Phospholipids are mixed esters of fatty acids and phosphoric acid with an alcohol, usually glycerol. They are continuously being broken down and re-synthesized in living cells (PARRISH, 1988). Phosphatidic acid, the simplest of the phospholipids, is the precursor of not only more complex phospholipids but also of triacylglycerols. Phospholipids, triacylglycerols and diacylglycerols are closely linked biochemically (PARRISH, 1988; GUSHINA and HARWOOD, 2006a; HU et al., 2008) (FIGURE 1.2).

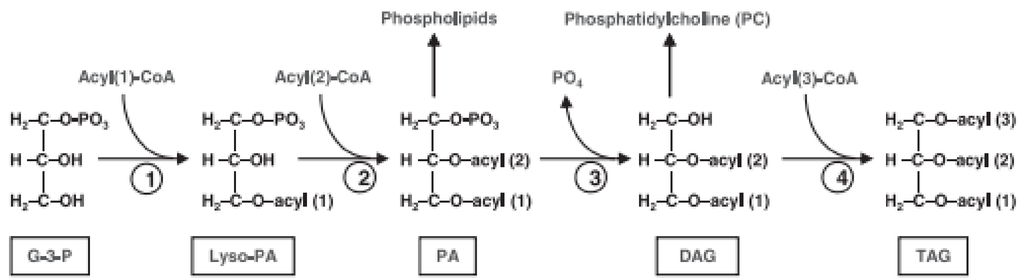

FIGURE 1.2: Simplified schematic figure showing the triacylglycerol biosynthesis pathway in algae. (1) Cytosolic glycerol-3-phosphate acyl transferase, (2) lyso-phosphatidic acid acyl transferase, (3) phosphatidic acid phosphatase, and (4) diacylglycerol acyl transferase (adapted from HU et al., 2008).

1.2 Effect of nutrients on microalgal biochemical composition

Under nutrient limitation microalgae exhibit variations in biochemical composition, which depend on the type of limiting nutrient and degree of limitation (KILHAM et al., 1997). Phosphorus and nitrogen limitations result in higher carbohydrate and lipid production as storage products than in non-nutrient limited cells (KILHAM et al., 1997; ALCOVERRO et al., 2000; GRANUM et al., 2002; URBANI et al., 2005; BHOLA et al., 2011). Production of carbohydrates and lipids by microalgae are known to be highly variable depending on the species, growth stage and environmental conditions (KILHAM et al., 1997; URBANI et al., 2005; GUSHINA and HARWOOD, 2006a; MATA et al., 2010). While some species increase their lipid content under nutrient limitation, others may have opposite behavior. MATA et al. (2010) showed that *Dunaliella bardawil* and *Dunaliella salina* presented a 10% decrease of its lipid fraction (W/W_{DW}), and a metabolic shift was observed towards carbohydrate synthesis and storage.

PRATT and JOHNSON (1963) reported that the accumulation of lipids in excess of about 25% of total dry weight is indicative of waning vigor in cultures of *Chlorella*, and a lipid content that equals or exceeds the protein content is indicative of extreme senescence. Microalgal lipid class compositions and proportions vary with changes in nutrient concentrations. Under nutrient replete

conditions (nitrogen and phosphorus) the polar lipids in the form of acetone mobile polar lipids (AMPL) and phospholipids (PL) make up the major lipid classes in most green algae (LOMBARDI and WANGERSKY, 1995; KHOZIN-GOLDBERG and COHEN, 2006). When one or more important nutrients become limiting, microalgae resort to accumulation of neutral lipids (mainly TAGs) resulting in an increased total lipid production (MUTLU et al., 2011).

The overall lipid yield by microalgae depends not only on the concentration of biomass, but also on the oil content of the individual cells (AMARO et al., 2011). Lipid productivity and content exhibit a negative relationship with each other (KHOZIN-GOLDBERG and COHEN, 2006; RODOLFI et al., 2009). According to ILLMAN et al. (2000), lipid content of *Chlorella vulgaris* grown under nitrogen-sufficient conditions ranges from 14 to 30%, while SCOTT et al. (2010) showed values up to 70%. In addition, *Chlorella emersonii* and *Chlorella minutissima* increased their lipid content by 63% and 56%, respectively, under nitrogen limitation (AMARO et al., 2011). The general principle is that when there is insufficient nitrogen for the protein synthesis required for growth, excess carbon from photosynthesis is channeled into such storage molecules such as triacylglycerols or starch (SCOTT et al., 2010). RODOLFI et al. (2009) proposed a two step process that led to 0.2 kg oil m^3 d^{-1} in the case of photosynthetic microalgae; in their setup, cells were first grown under nitrogen-sufficient conditions to enhance biomass accumulation, and only subjected to nutrient deprivation afterwards for lipid synthesis (SCOTT et al., 2010). However, STEPHENSON et al. (2010) claimed that the most effective strategy to achieve high lipid contents in *C. vulgaris* was to allow cells to naturally deplete the nitrogen source in the medium, rather than transferring them into one devoid of that element. *Isochrysis* sp. accumulates higher fractions of lipids comprising *ca*. 25% of the cell weight following 10 d of nitrogen starvation (DE MORAIS and COSTA, 2007

Phosphorus (P) is an essential nutrient for algal nucleic acid and ATP production among other essential molecules, and it is often the growth limiting nutrient for phytoplankton in aquatic systems (SPIJKERMAN, 2008; SPIJKERMAN and WACKER, 2011). One of the responses to a P-limitation in plants and algae is membrane lipid remodelling: upon P-deficiency, a significant portion of membrane phospholipids is replaced by non-P galactolipids and sulfolipids (ANDERSSON et al., 2003; VAN MOOY et al., 2009). Although the fatty acid (FA) composition of lipids differs between different algal species (DIJKMAN and KROMKAMP, 2006), membrane lipid classes in the green alga typically are characterized by unsaturated FAs such as 16:3, 16:4, 18:1, 18:2, 18:3 and 18:4 (GIROUD et al., 1988). As a result of the changes in lipid class composition, P-limited phytoplankton will have a different FA composition than the P-saturated (AHLGREN et al., 1997; SPIJKERMAN and WACKER, 2011). For example, total FAs in P-limited *Scenedesmus quadricauda* had a lower percent of polyunsaturated fatty acids (PUFAs) than P-replete cells, mainly as a result of the lower content of 18:3 (AHLGREN et al., 1998). Additionally, under P-limitation growth decline, and reduced photosynthetic rates are observed, which results in triacylglycerol accumulation (GUSCHINA and HARWOOD, 2009). Changes in the biochemical composition of phytoplankton have important ecological consequences. It is known that changes in the cellular P quota and FA composition affect the food quality for herbivores (MULLER-NAVARRA, 1995; VILLAR-ARGAIZ et al., 2009), and a decreased PUFA content in algae affect the growth and health status of higher level organisms that depend on algae (TOCHER, 2003; ARTS and KOHLER, 2009; SPIJKERMAN and WACKER, 2011).

1.3 Effect of trace metals on the physiology of microalgae

A number of trace metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions and catalyze enzymatic reactions (ASH and STONE, 2003). For instance, copper (Cu), zinc (Zn) and iron (Fe) are essential as constituents of the catalytic sites of several enzymes (ALLAN, 1997; TORRES, et al. 2008). Other metals, however, such as lead (Pb), mercury (Hg) and cadmium (Cd) may displace or substitute for essential trace metals and interfere with proper functioning of enzymes and associated cofactors (TORRES et al., 2008; QIAN et al., 2009; BAJGUZ, 2011). These metals however occur at low or very low (~ 10^{-9} - 10^{-12} mol L^{-1}) concentrations in aquatic ecosystems. The exposure of microalgae to trace metals occurs in mainly chronic exposure, e.g, exposure to low concentrations of trace metal for long periods of time. This can lead to metabolism modification/adaptation and persistence of the cells, enabling them to survive or eliminate the algae that are unable to adapt themselves to the new conditions. Finally, this will affect the biodiversity of the environment.

It is recognized that stress-induced changes at the ecosystem level are of concern. However, such changes are generally too complex and are often omitted from the list of indicators used for early detection and prediction of environmental stress (DEPLEDGE et al., 1993). A probable solution to this problem lies in the effective characterization of distress signals at the molecular and cellular levels (biomarkers) that can provide early warning prognostics of reduced performance (MOORE et al., 2004; TORRES et al., 2008). Doing this, possible linkages to higher ecological levels are possible and precise prediction of its consequences can be made. Typically, biomarkers are defined as quantitative measures of changes in the biological system that can be related to exposure to the toxic effect of environmental chemicals (TORRES et al., 2008). According TORRES et al. (2008) the use of the term *biomarker* or *biomarker response* is often restricted to cellular, biochemical, molecular or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism that are indicative of environmental stress. An observation of

biochemical composition variation like total proteins, carbohydrates and lipids will implicate the effect of metals on microalgae. Metal toxicity is usually accompanied by an inhibition of protein production by algae (KUMAR et al., 2010). An increased production of carbohydrates is also common when microalgae are exposed to toxic metal concentrations (PISTOCCHI et al. 2000). However, the production of proteins and carbohydrates by microalgae when exposed to different metal concentrations vary depending on their degree of tolerance and species.

Changes in lipid class and fatty acid composition occur with varying concentrations of metals in the environment. A study by EINICKER-LAMAS et al. (1996) showed that 2 mg L^{-1} ($1.8x10^{-5}$ $mol.L^{-1}$) Cd was capable of increasing total lipid content of *Euglena gracilis* in autotrophic, heterotrophic and mixotrophic cultures. They showed that among the membrane lipids, sterol content was lower in Cd-treated cells cultivated under illumination. However, no apparent changes were noticed in total phospholipid content, although the cardiolipin levels were altered in all three culture types.

It has generally been shown that exposure to metals leads to increases in 18:1 content, alteration of the relative proportions of 18:2 and 18:4 (with the changes being metal-specific) in microalgae (GUSHINA and HARWOOD, 2006a; PINTO et al., 2011).

1.4 Justification

It is known that microalgae respond with physiological alterations in media composition where they grow (VALENZUELA-ESPINOZA et al., 2002; SCRAGG et al., 2002). This behavior can be viewed as a biotechnological attribute that can be manipulated in order to control the algae biochemical composition and growth, focusing on specific compounds and higher productivity. Therefore, the selection of inexpensive and promising media so as to improve microalgae production yield is of general interest. HARRISON and BERGES (2005) proposed that three main experimental categories, culture

maintenance, algal biomass yield and physiological/growth experiments are a good way to search for ideal production conditions.

It is known that growth rates give a general index of algal health status and physiological condition in the cultures since it reflects algal metabolism, as a response to all of its cellular cycles (LOMBARDI and MALDONADO, 2011). From this rationale, it is clear that batch cultures can offer adequate growth conditions for microalgae only during a short period of time, after which cell metabolism begins to collapse and photosynthesis is reduced (LOMBARDI and MALDONADO, 2011). Thus, physiological investigations with microalgae are better performed using continuous or semi-continuous cultures, which offer more constant and stable growth conditions than batch cultures.

Most studies have considered the influence of either metals or nutrients on the growth, biomass production and biochemical composition of microalgae. Very few investigations consider both together, metals at sub-lethal concentrations and limitation of major nutrients. It is known that the availability and uptake of trace metals, including Cd, are influenced by the concentrations of nutrients like phosphorus and nitrogen (SIVAKUMAR et al., 2010). According to SIVAKUMAR et al. (2010) phosphorus (as phosphate) and nitrogen (as nitrate) are the most common reactive forms in aquatic ecosystems. It has been shown that there is an association of high levels of Cd with high nutrient levels, e.g., artificial eutrophication. The presence of nutrient replete conditions aids microalgae to cope with the toxicity of trace metals in the environment (SATHYA and BALAKRISHNAN, 1988; SERRA et al., 2010).

Metals can be complexed with ligands both extracellularly and intracellularly involving chelation by peptide ligands such as metallothioneins (MTs) and phytochelatins (PCs) (PAL and RAI, 2010). Phytochelatins (PCs) are enzymatically synthesized peptides present in heavy metal detoxification and accumulation processes. These molecules have been detected in plants grown in sites contaminated with metals. PCs have been identified in a wide variety of plants including monocots, dicots, gymnosperms, and algae. This suggests that as

trace metal binding ligands, the PCs and their producing pathway evolved very early in order to maintain the endurance of plants and algae growing under potentially toxic environments (PAL and RAI, 2010).

In many aquatic systems, growth of phytoplankton is not only phosphorus and/or nitrogen limited (or in excess), but also controlled by the presence of trace metals (SPIJKERMAN and WACKER, 2011). This in turn poses a risk to the survival of herbivores that depend on phytoplankton, which is due to P limitation, fatty acid limitation and Cd toxicity (LUKAS et al., 2011). Therefore there is a need for studies that contribute to the limited knowledge about the combined effect of trace metals and major nutrients, such as nitrogen and phosphorus on the physiology of freshwater microalgae.

Most investigations relating physiology with metal ions focus on the toxicity of the metal, not the biochemical composition of the algae. There is one study that has taken into consideration the effect of phosphorus, Pb and Cu on lipid composition of cultured lichen photobionts (GUSHINA and HARWOOD, 2006b). This investigation has considered the effects of nitrogen and Cd, and phosphorus and Cd on the biochemical composition of *C. vulgaris*, analyzing lipid class and fatty acid profiles, as well as carbohydrates and proteins, and calorific values of the cells.

1.5 Research hypotheses

The research hypotheses for this study were that:

1. Cd influences the growth and biochemical composition of *Chlorella vulgaris.*

2. P and N with structural function for the cells control the growth and biochemical composition of *C. vulgaris.*

3. Cd interacts with N and P to determine the growth, biomass production and biochemical composition of *C. vulgaris.*

1.6 Objectives

The objectives of this study were to:

1. Select for the least expensive growth media that would still result in high growth rates. Hence, we have tested the effect of media composition on growth rate, biomass production, lipid classes, carbohydrates and proteins in *Chlorella vulgaris* grown in semi-continuous cultures;
2. Evaluate the influence of Cd and P on the growth rate, biomass production, biochemical (lipid classes, carbohydrates and proteins) and fatty acid compositions in *C. vulgaris*;
3. Determine the influence of Cd and N on the growth rate, biomass production, biochemical (lipid classes, carbohydrates and proteins) and fatty acid compositions in *C. vulgaris*;
4. Evaluate the calorific value of *C. vulgaris* under varying phosphorus (P) concentrations in relation to its biochemical composition.

2. MATERIALS AND METHODS

2.1 Unialgal species

The alga used for this study was *Chlorella vulgaris.* Stock cultures were maintained in LC Oligo medium (table 2.1) (AFNOR 1980) in batch system. Culture media sterilization was performed through autoclaving at 121°C for 20 min, which was done 24 h before use. Cultures were kept under controlled conditions of light intensity (150 µmol m^{-2} s^{-1}), light/dark cycle (16:8 hours), and temperature (20 $\pm$ 2 °C). The reagents used for culture media preparations were of analytical grade.

2.2 The effect of media composition

This experimental phase was carried out by culturing *C. vulgaris* in three different growth media: half-strength Chu 10 (Chu) (NALEWAJKO and O'MAHONY, 1989), WC medium (WC) (GUILLARD and LORENZEN, 1972), and LC Oligo medium (LC Oligo) (AFNOR 1980). The different media compositions used are shown in table 2.1. The experiments were performed in 500 mL polycarbonate Erlenmeyer flasks containing 200 mL of culture kept semi-continuously (see section 2.4).

The choice of the different growth media was due to their use in culture of a wide array of microalgal species including *Chlorella.* In addition, the LC Oligo medium is recommended in Brazil (ABNT) for ecotoxicity evaluation with phytoplankton. The culture media investigated had varied composition such as the presence of metal complexing ligands (EDTA and citrate) and vitamins, which are required for microalgal growth. However, the presence of EDTA and vitamins increases the cost of cultivation. Therefore, the selection of cheap and promising media as to improve microalgae production yield is of general interest.

TABLE 2.1: Growth media composition for LC Oligo, WC and half-strength Chu 10 culture media used in this study. Final concentrations are reported in mol L^{-1}.

LC Oligo		WC		Chu 10	
Reagent	Concentration	Reagent	Concentration	Reagent	Concentration
$Ca(NO_3)_2 . 4H_2O$	$1.7 x 10^{-4}$	$NaNO_3$	$1.0 x 10^{-3}$	$Ca(NO_3)_2$	$1.2 x 10^{-4}$
KNO_3	$1.0 x 10^{-3}$	$CaCl_2.2H_2O$	$2.5 x 1^{-4}$	K_2HPO_4	$1.4 x 10^{-5}$
$MgSO_4 \cdot 7H_2O$	$1.2 x 10^{-4}$	$MgSO_4.7H_20$	$1.5 x 10^{-4}$	$MgSO_4.7H_2O$	$5.1 x 10^{-5}$
K_2HPO_4	$2.3 x 10^{-4}$	$NaHCO_3$	$1.5 x 10^{-4}$	Na_2CO_3	$9.4 x 10^{-5}$
$CuSO_4.5H_2O$	$6.0 x 10^{-8}$	$Na_2SiO_3.9H_2O$	$1.0 x 10^{-4}$	Na_2SiO_3	$1.0 x 10^{-4}$
$(NH_4)_6Mo_7O_{24}.4H_2O$	$2.4 x 10^{-8}$	K_2HPO_4	$5.0 x 10^{-5}$	$FeCl_3$	$2.5 x 10^{-6}$
$ZnSO_4.7H_2O$	$1.0 x 10^{-7}$	$Na_2EDTA.2H_20$	$1.2 x 10^{-5}$	H_3BO_3	$4.0 x 10^{-8}$
$Mn(NO_3)_2.4H_2O$	$1.2 x 10^{-7}$	$FeCl_3.6H_2O$	$1.2 x 10^{-5}$	$MnSO_4.H_2O$	$8.7 x 10^{-9}$
$H_3C_6H_5O_7.H_2O$	$1.4 x 10^{-7}$	$CuSO_4.5H_2O$	$4.0 x 10^{-8}$	$ZnSO_4.7H_2O$	$8.0 x 10^{-10}$
H_3BO_3	$4.9 x 10^{-7}$	$ZnSO_4.7H_2O$	$7.7 x 10^{-8}$	$CuSO_4.5H_2O$	$4.0 x 10^{-10}$
$C_6H_5FeO_7.5H_2O$	$1.5 x 10^{-6}$	$CoCl_2.6H_2O$	$4.2 x 10^{-8}$	$(NH_4)_6Mo_7O_{24}.4H_2O$	$5.7 x 10^{-11}$
$FeSO_4.7H_2O$	$1.1 x 10^{-6}$	$MnCl_2.4H_2O$	$9.1 x 10^{-7}$	$Co(NO_3)_2.6H_2O$	$4.8 x 10^{-10}$
$FeCl_2.4H_2O$	$1.6 x 10^{-6}$	$Na_2MoO4.2H_2O$	$2.5 x 10^{-8}$	Vitamin B1	$1.5 x 10^{-7}$
$NaHCO_3$	$1.79 x 10^{-4}$	H_3BO_3	$1.6 x 10^{-5}$	Biotin	$1.0 x 10^{-8}$
		Vitamin B1	$3.0 x 10^{-7}$	Cyanocobalamin	$1.8 x 10^{-9}$
		Biotin	$2.1 x 10^{-9}$		
		Cyanocobalamin	$3.7 x 10^{-10}$		

2.3 Cadmium, phosphorus and nitrogen experiments

These experiments were performed using the selected culture medium, LC Oligo. Phosphorus (P) was provided as K_2HPO_4 at $6.0x10^{-7}$, $2.3x10^{-6}$ and $2.3x10^{-4}$ mol L^{-1} (control) (table 2.3), and nitrogen (N) at $2.9x10^{-6}$, $1.1x10^{-5}$, $1.1x10^{-3}$ mol L^{-1} (control) (table 2.4) concentrations. Because the LC Oligo medium N is provided in four different reagents, for the experiments nitrogen concentrations were varied using $Ca(NO_3)_2.4H_2O$ (~ 10^{-3} mol L^{-1}) and KNO_3 (~ 10^{-4} mol L^{-1}). Due to their higher concentrations, the concentrations of the other two sources, $Mn(NO_3)_2.4H_2O$ and $(NH_4)_6Mo_7O_{24}.4H_2O$ that are ~ 10^{-7} and 10^{-8} mol L^{-1} respectively, are considered insignificant compared to the other two. Nitrogen and P were altered one at a time, not simultaneously, and when pertinent, Cd was added as $Cd(NO_3)_2$ at $2.0x10^{-8}$ and $1.0x10^{-7}$ mol L^{-1} (table 2.3 and 2.4).

The chosen N and P concentrations reflect conditions of natural environments that range from eutrophic to nutrient limited environments. The Cd concentrations used here are environmentally relevant as the lowest concentration ($2.0x10^{-8}$ mol L^{-1}) approximates to the maximum allowed level of Cd in water. However, prior to the selection of these concentrations, laboratory trials were made with the microalga to determine concentrations within which their growth was inhibited.

Free Cd^{2+} ions concentrations were obtained through calculations using the chemical equilibrium software MINEQL$^+$ 4.62.3 (Environmental Research Software, Hallowell, ME, USA). These results showed that approximately 98% of the added Cd remained as free Cd^{2+} ions in the cultures (table 2.2).

The biochemical composition, e.g., the determination of lipid classes, total carbohydrates and proteins in the cells, as well as growth rates, biomass (chlorophyll *a*, dry weight, optical density and cell counts) were determined for each treatment.

TABLE 2.2: Cd speciation in the growth media as calculated using MINEQL software. Where Cd1 = 2.0×10^{-8} mol L^{-1} Cd and Cd2 = 10^{-7} mol L^{-1} Cd, P = phosphorus, N = nitrogen, pCd and pLabile are negative natural log values for free Cd ion and labile Cd concentrations.

Treatment	Cd speciation		
		pCd2+	pLabile Cd
Control	No Cd	18.01	
	Cd1	7.71	9.37
	Cd2	7.01	9.93
P 2.3×10^{-6}	No Cd	28.01	
	Cd1	7.71	10.63
	Cd2	6.71	8.43
P 6.0×10^{-7}	No Cd	18.01	
	Cd1	7.71	9.27
	Cd2	7.01	9.43
N 1.1×10^{-5}	No Cd	18.01	
	Cd1	7.71	10.00
	Cd2	7.01	9.13
N 2.9×10^{-6}	No Cd	18.01	
	Cd1	7.70	9.95
	Cd2	7.01	8.93

TABLE 2.3: Experimental combinations of Cd and P to which *Chlorella vulgaris* was exposed.

		Cadmium (mol L^{-1})	
P (mol L^{-1})	No Cd	$2.0x10^{-8}$ (Cd1)	$1.0x10^{-7}$ (Cd2)
$2.3x10^{-4}$ (P1)	P1	P1Cd1	P1Cd2
$2.3x10^{-6}$ (P2)	P2	P2Cd1	P2Cd2
$6.0x10^{-7}$ (P3)	P3	P3Cd1	P3Cd2

TABLE 2.4: Experimental combinations of Cd and N to which *Chlorella vulgaris* was exposed.

		Cd (mol L^{-1})	
N (mol L^{-1})	No Cd	2.0 x 10^{-8} (Cd1)	1.0 x 10^{-7} (Cd2)
1.1 x 10^{-3} (N1)	N1	N1Cd1	N1Cd2
1.1 x 10^{-5} (N2)	N2	N2Cd1	N2Cd2
2.9x 10^{-6} (N3)	N3	N3Cd1	N3Cd2

2.4 Experimental cultures

Experimental cultures were carried out in 1 L polycarbonate bottles (Nalgene, U.S.A.) containing 250 mL of sterile culture media. 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). All plastic, glassware and polycarbonate materials used for the experiments were washed with neutral detergent and rinsed with deionized water. Further, they were left for 7 days in

10% HCl and finally rinsed with deionized water and ultrapure water (18.2 mΩ-cm, EasyPure II, Thermo Scientific, USA) before use.

Algal cells were acclimated to the specific N or P concentrations that would be used for the experimental treatments prior to the beginning of the experiments. This was performed through culture transfers at exponential growth phase (~ 10^6 cell mL^{-1}). This acclimation process is important to assure that algae metabolism reflects the culture media condition. During each acclimation, growth rates were measured for each new culture, and after 3 statistically similar and consecutive growth rates, *Chlorella vulgaris* was considered to be acclimated and reflecting the specific N or P condition to be tested.

For the experiments, performed in semi-continuous cultures, an inoculum was obtained from the acclimated batch cultures and daily dilutions through culture removal and replacement with fresh and sterile culture media were made according to the growth rates, so cell density was kept within a known range throughout the experiments. These daily dilutions continued for about 16-19 days, when samples were obtained for the biochemical composition determination.

2.5 Growth and biomass determinations

2.5.1 Absorbance

On each sampling day, aliquots were taken for biomass and growth kinetics determinations before daily dilutions were made. Monitoring of growth was done using optical density at 684 nm using a HACH DR 5000 (HACH Company, USA) spectrophotometer. Specific growth rates (μ) were determined according to equations 1 and 2, as described in LOMBARDI and MALDONADO (2011).

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

Where

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

Note: ABS = absorbance at 684 nm, t = time, T_{vol} = total volume and R_{vol} = removed/replaced volume.

Three experimental replicates were performed for each treatment.

2.5.2 Dry Weight

Dry biomass was determined using either Millipore or Sartorius cellulose acetate filter (0.45 µM pore size) that were dried to constant weight at 60°C in an oven (FANEM – Orion 515, Brazil) for 24 h and weighed on a Sartorius MC21S micro analytical balance with 1 µg readability (Precision Weighing Balances; Bradford, MA). Twenty milliliters of algal culture was filtered to retain algal biomass using a Millipore Swinnex® filter holder. Afterwards the filters were dried at 60°C for 24 h, removed and placed in a dessicator for at least 10 min and weighed to the nearest microgram (µg).

2.5.3 Chlorophyll a

The extraction and analysis of chlorophyll *a* were done according to the procedure of SHOAF and LIUM (1976). Five milliliters of microalgal culture were filtered through Millipore cellulose acetate membrane filters (pore size 0.45 µm). The membrane filters were then dissolved in a vial with 3 mL of dimethyl sulfoxide (DMSO) at room temperature. The vial was kept in the dark for 30 min. Then it was removed and shaken to properly dissolve the filter paper and

returned to the dark for another 15 min. The absorbance was read at the appropriate wavelengths using a HACH DR5000 spectrophotometer (Loveland, Co., USA) and the chlorophyll *a* concentration calculated through equation 3, as described in JEFFREY and HUMPHREY (1975).

$$Chl\ a\ (mgL^{-1}) = (11.47 * OD_{664}) - (0.4 * OD_{630})\ x/y \qquad (3)$$

Where x is the total volume of extraction solvent used and y represents the volume of culture filtered.

Note: OD is optical density at 664 nm and 630 nm wavelengths.

2.5.4 *Density (cells ml^{-1})*

Direct microscopic counts using a Leica microscope were used to determine cell density with the aid of an improved Neubauer haemocytometer.

2.5.5 Productivity

Biomolecule productivity was obtained from the product of biomass productivity at the moment of sampling and the biomolecule content according to the equation 4 below (GRIFFITHS and HARRISON, 2009; LV et al., 2010):

$$Qv = \mu X * b \qquad (4)$$

Where µX is the biomass productivity per day and b is biomolecule content. Biomolecule productivities (Qv) are reported in grams per liter per day (g L^{-1} d^{-1}).

2.6 Biochemical composition analyses

2.6.1 Total intracellular carbohydrates

Analysis for intracellular carbohydrates was performed according to the modified phenol-sulfuric acid technique (LIU et al., 1973) using glucose as standard. Five milliliters aliquots were taken from the culture and centrifuged at 1,500 rpm for 10 min using an Eppendorf Centrifuge 5702 R (Eppendorf; AG, Hamburg) to pellet the cells. The pellets were stored at -20°C until the time of analysis. The pellet obtained was re-suspended in 1 mL of distilled water and 1 mL phenol solution was added to it. After thoroughly mixing, 5ml of concentrated H_2SO_4 was quickly added, directing the flow at the liquid surface to obtain a good mixing. The mixture was left to stand at room temperature for 10 min and centrifuged at 3000 g for 10 min. The supernatant was read at 485 nm against a reagent blank. Carbohydrate concentrations were obtained from a calibration curve of glucose with concentrations from 10 µg mL^{-1} to 150 µg mL^{-1} (FIGURE 2.1).

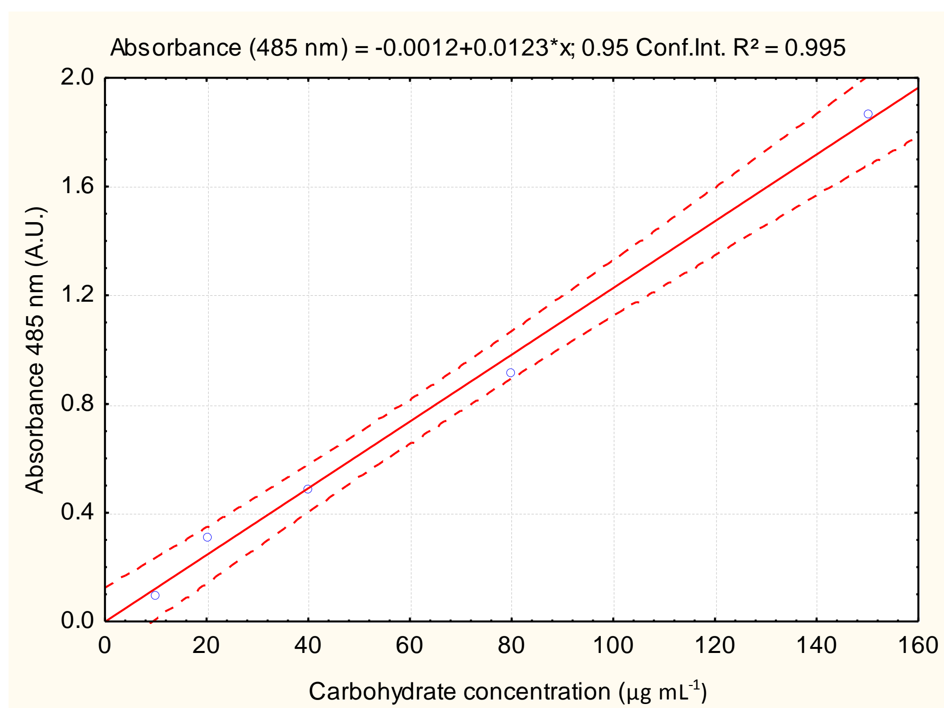

FIGURE 2.1: Carbohydrate calibration curve used in the present study.

2.6.2 Total proteins

To determine the total intracellular protein content of the microagal cells, the procedure of BRADFORD (1976) with bovine serum albumin (BSA) as protein standard was used.

The extraction procedure used was performed according to RAUSCH (1981). Five milliliters of algal culture was centrifuged at 1500 rpm and the pellet formed resuspended in 1.5 mL of 0.5 N NaOH. This sample was then extracted for 120 min at 100 °C in an oven. The extracted proteins were obtained by centrifuging at 4,400 rpm for 10 min and collecting the supernatant.

To every mililiter of the supernatant, 4 mL of Bradford reagent (0.01% Coomassie blue, 4.7% ethanol, and 8.5% phosphoric acid) prepared just prior to the assay, were added and allowed to stand for 5 min at room temperature. The absorbance of the solution was then read at 595 nm. The BSA used as standard was prepared in 0.5 N NaOH solution with concentrations ranging from 10 to 150 µg mL^{-1}. These values were used to plot the total protein standard curve (FIGURE 2.2).

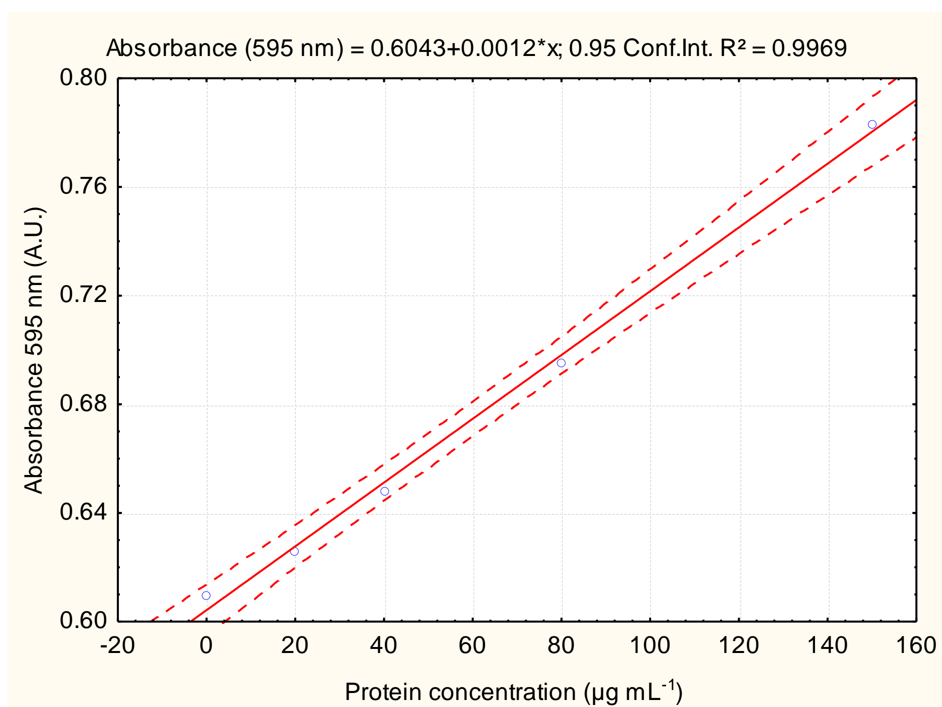

FIGURE 2.2: The protein calibration curve used in this study.

2.6.3 Calorific value

Calorific values of *C. vulgaris* under each phosphorus treatment was determined using an IKA C200 bomb calorimeter (IKA, Heitersheim, Germany). Algal samples (150 mL) were filtered through cellulose acetate filters (Sartorius) and dried at 60°C for 24 h prior to the analysis. These samples were then weighed before combustion in the calorimeter. The calorific values of blank filters were obtained by combusting several filters without the microalga. This value was subtracted from filters with algae (ILLMAN et al., 2000). The instrument was calibrated according to the manufacturer's instructions using pre-weighed tablets provided.

2.6.4 Lipids

Extraction: lipid extraction was carried out using the modified Folch method as described in PARISH (1987, 1999). Biomass was obtained by filtering 50 mL of culture through a glass fiber filter (BOECO, Germany) previously baked overnight at 400°C. Immediately after filtration of culture samples and prior to extraction, the filters were placed in screw capped Teflon centrifuge tubes (Nalgene) containing 6 mL dichloromethane (CH_2Cl_2):methanol (MeOH) (2:1) and spiked with 10 µg of internal standard (hexadecane-3-one). This was then sonicated using a UNIQUE sonicator (Unique Group, Indaiatuba, Brazil) for 3 min set at 60 for power using 3 cycles of 1 min each. Afterwards 1.5 mL ultrapure water (Barnstead EASYpure II; Thermo Scientific, Dubuque, IA, USA) was added to back wash the methanol and all non-lipid materials by centrifugation at 3000 rpm. This formed two phases: an upper aqueous layer containing methanol and a lower organic layer containing dichloromethane and lipids. All subsequent extractions were done with 100% CH_2Cl_2 to minimize the amount of MeOH in the final concentrate. MeOH should be removed to reduce its interference with the quantification of ME/WE content.

Analytical equipment and conditions of operation: Lipid class analysis was performed using an Iatroscan MK-6 (Mitsubishi Kagaku Iatron, Inc. Tokyo, Japan) according to the procedure described in PARRISH (1999). The Iatroscan was connected to a computer using a Model 333 Single Channel USB Chromatography A/D alongside an Auto Start Controller TU-600. On the computer, a PeakSimple version 6.78 for windows was used to record the chromatograms resulting from the scans.

Standards and Calibrations: A composite lipid stock solution containing nine different lipid standards was made in dichloromethane (table 2.5) and stored in a freezer (-20°C) under N_2. Serial dilutions were made from this composite standard solution and stored in 5 mL glass reaction vials fitted with Teflon caps. The concentrations of standards used on a daily basis ranged from 0.2 to 20 µg μL^{-1} and were regularly renewed from new dilutions of the stock solution. Each lipid class standard was individually chromatographed through the entire development procedure and scanned in the Iatroscan to determine its purity before the final composite standard solution was made. When a large number of samples were to be analyzed, calibration curves (FIGURES 2.3 to 2.10) were compiled throughout the whole period of sample analysis. One rod was chosen at random and used for analysis of the standards on each day of analysis. This was used to confirm the retention values, which depending on environmental conditions may vary within the development procedure in thin layer chromatography.

Standards and Sample application: Standards were routinely spotted onto the Chromarods II using Hamilton syringes. The Rod Holder SD-6 was placed on a warm hot-plate (35 ^{o}C), when application of samples to the rods was done. Solvent focusing was done three times when volumes greater than 10 µL were spotted on a rod (PARRISH and ACKMAN, 1983a, 1983b). Focusing was achieved by developing in acetone to just above the point of application to refocus any solute that may have spread away from the point of application.

Chromarod Conditioning: After spotting and before each development, the rods were conditioned for 5 min in a constant humidity chamber (~30%), which was a desiccator containing a saturated water solution of $CaCl_2$.

Developments and Partial Scans: Four different solvent systems were used to obtain three chromatograms per rod. The first solvent system, hexane:diethyl ether:formic acid (99:1:05), was used for a double development of 25 min, followed by a 20 min development. In-between the two developments, the rods were kept in the constant humidity chamber for 5 min. Each rod was then scanned to the lowest point behind the KET peak to obtain the first chromatogram (PPS 32). The second chromatogram was obtained after a 40 min development in the solvent system constituted by hexane:diethyl ether:formic acid (80:20:0.1); this time the scan continued to the lowest point behind the ST peak (PPS 15). For the last chromatogram, a complete scan was obtained after two double developments: the first was done in 100% acetone (15 min for each development) and the second in CH_2Cl_2:MeOH:water (5:4:1). The last development lasted for 10 min each. In-between the developments, the rods were always kept in the constant humidity chamber for 5 min.

TABLE 2.5: Lipid standards that were resolved in a three-step separation for lipid class determination in this study.

Class	Abbreviation	Principal representative Compound	Source
Aliphatic Hydrocarbon	HC	n-Nonadecane	Sigma
Wax Ester	WE	Octadecyl hexadecanoate	Sigma
Ketone	KET	Hexadecan-3one	K+KLABS
Triacylglycerol	TG	Glyceryl trihexadecanoate	Sigma
Free Fatty Acids	FFA	Hexadecanoic acid	Supelco
Free Aliphatic Alcohol	ALC	Hexadecan-1-ol	Sigma
Free Sterol	ST	Cholesterol	Sigma
Acetone Mobile Polar Lipids	AMPL	Glyceryl-1-monohexadecanoate	Sigma
Phospholipids	Pl	Phosphatidyl choline	Sigma

Operational Conditions of Scans: For all the thin layer chromatography analyses in this study, the Iatroscan system operation conditions were set at 173 ml min^{-1} H_2 flow rate, 4 mm s^{-1} scan velocity, and air flow of 2 L min^{-1}.

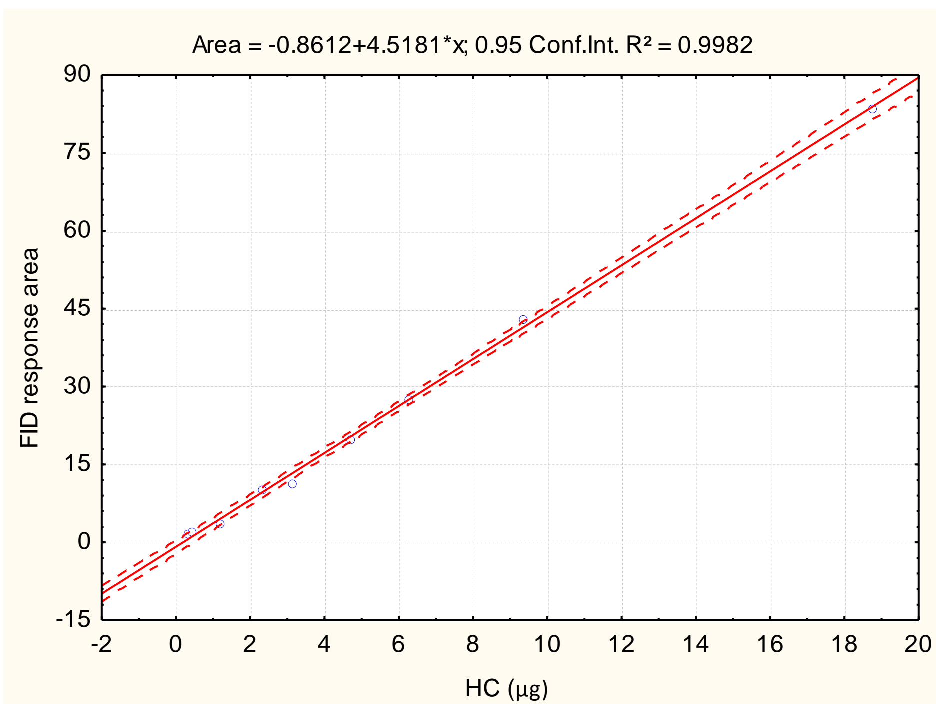

FIGURE 2.3: Aliphatic hydrocarbon (HC) calibration curve for lipid class determination.

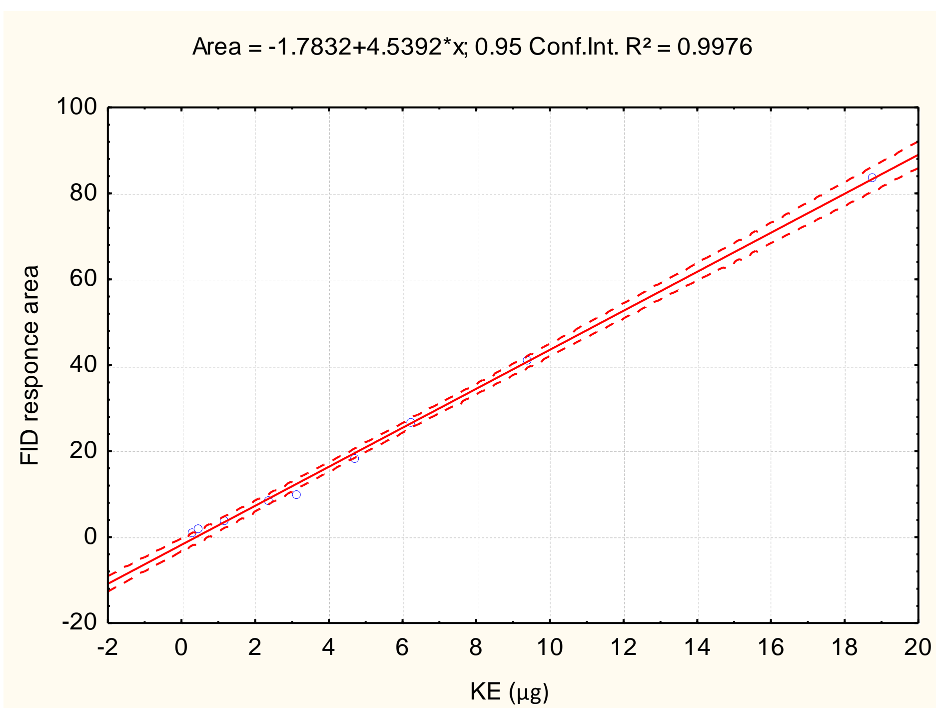

FIGURE 2.4: Internal standard (Hexadecan-3-one, KE) calibration curve for lipid class determination.

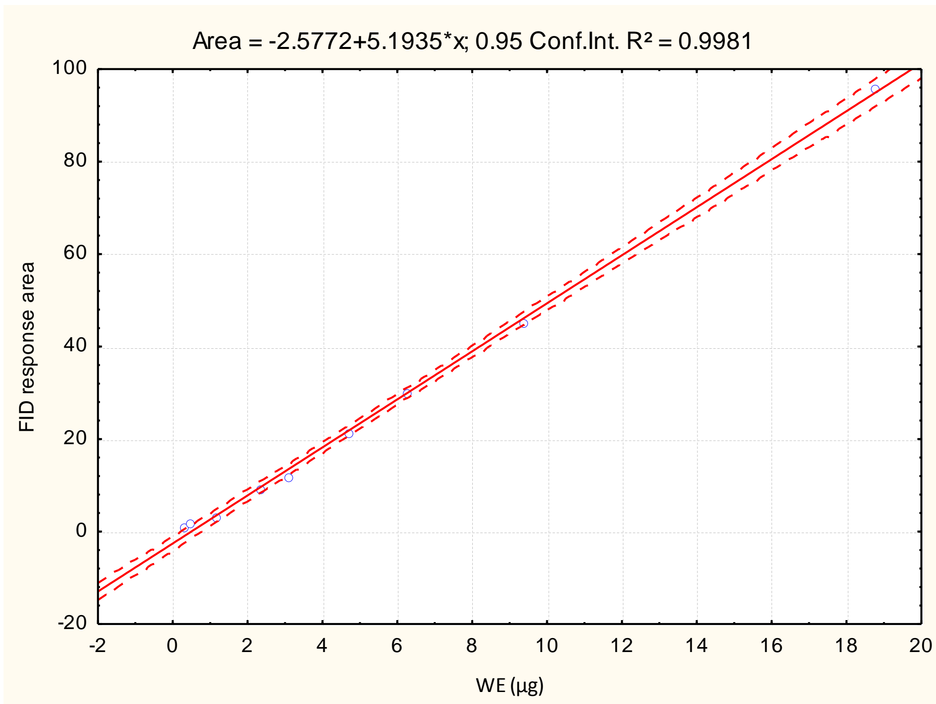

FIGURE 2.5: Wax ester (WE) calibration curve for lipid class determination.

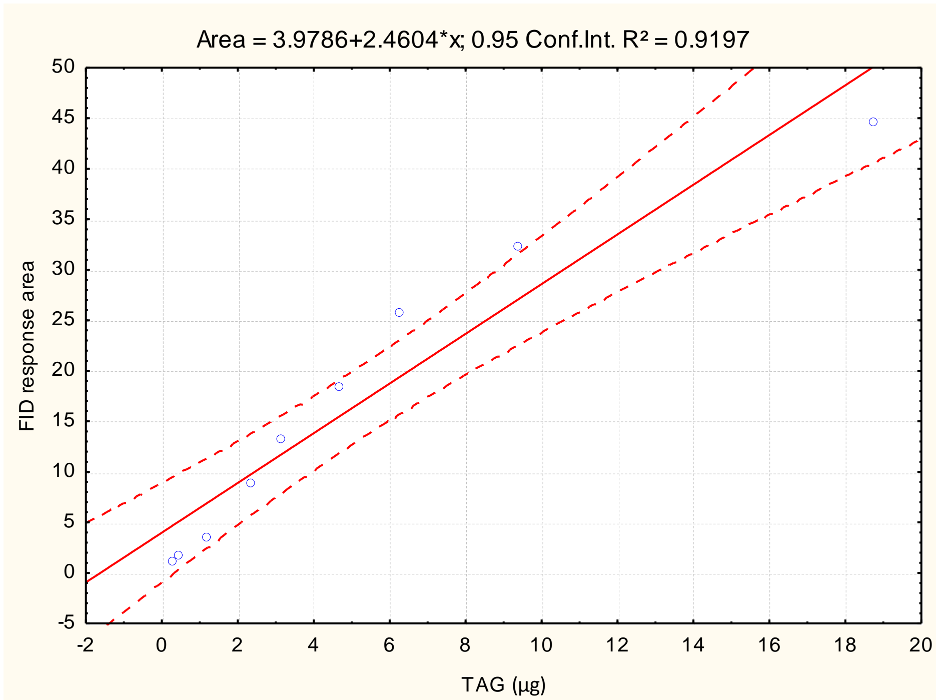

FIGURE 2.6: Triacylglycerol (TAG) calibration curve for lipid class determination.

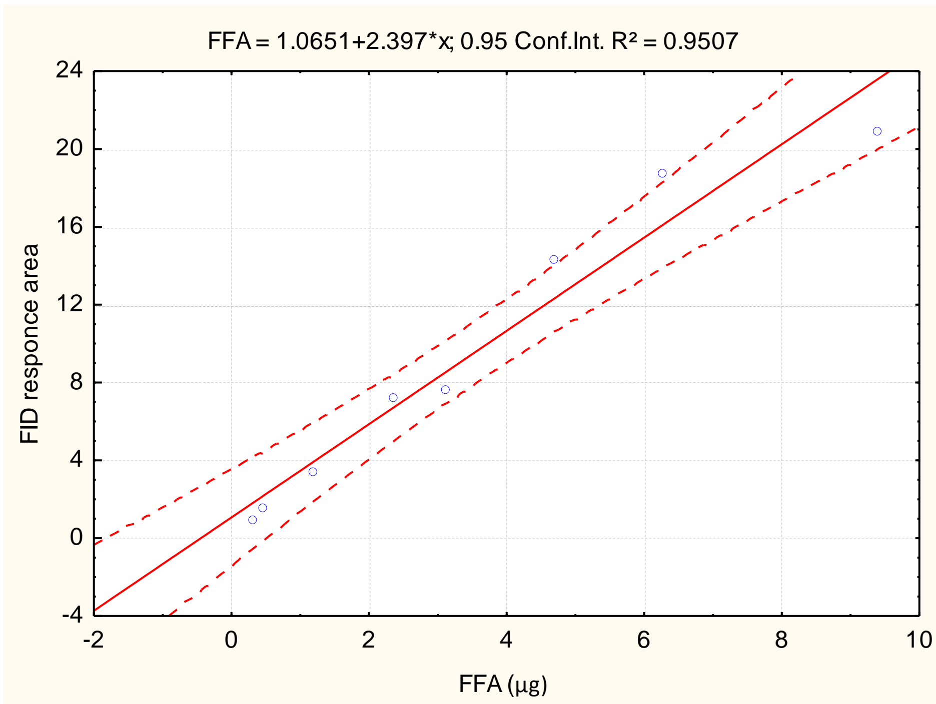

FIGURE 2.7: Free fatty acid (FFA) calibration curve for lipid class determination.

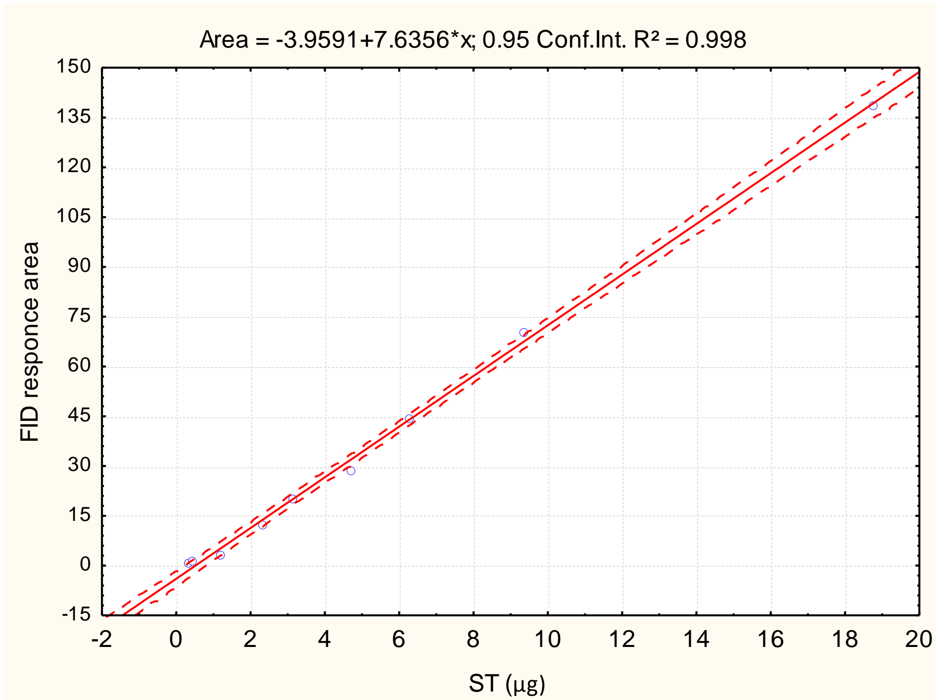

FIGURE 2.8: Free sterol (ST) calibration curve for lipid class determination.

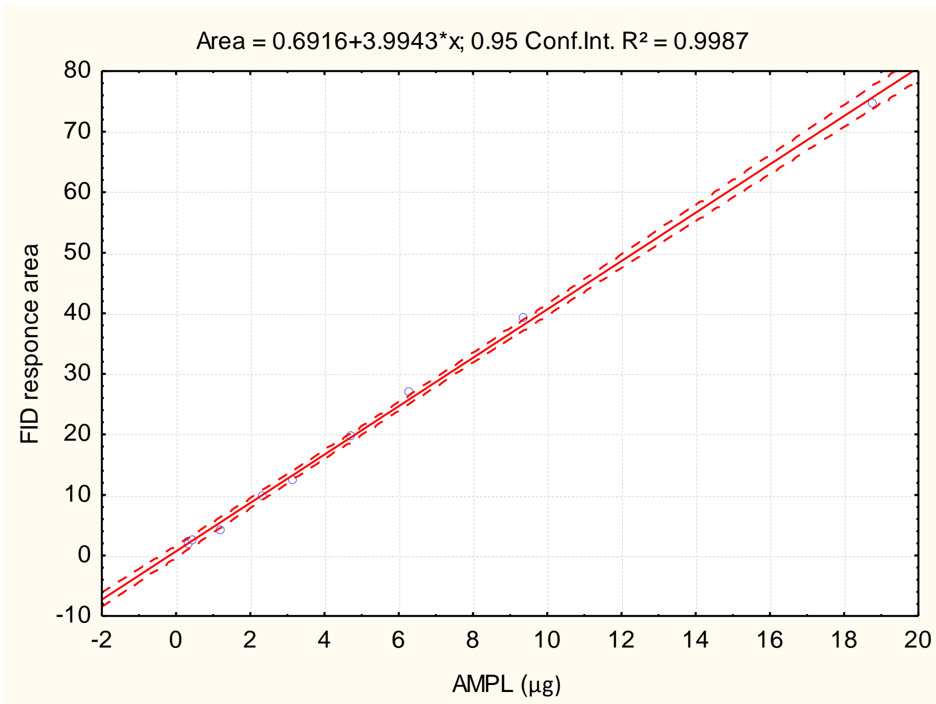

FIGURE 2.9: Acetone mobile polar lipid (AMPL) calibration curve for lipid class determination.

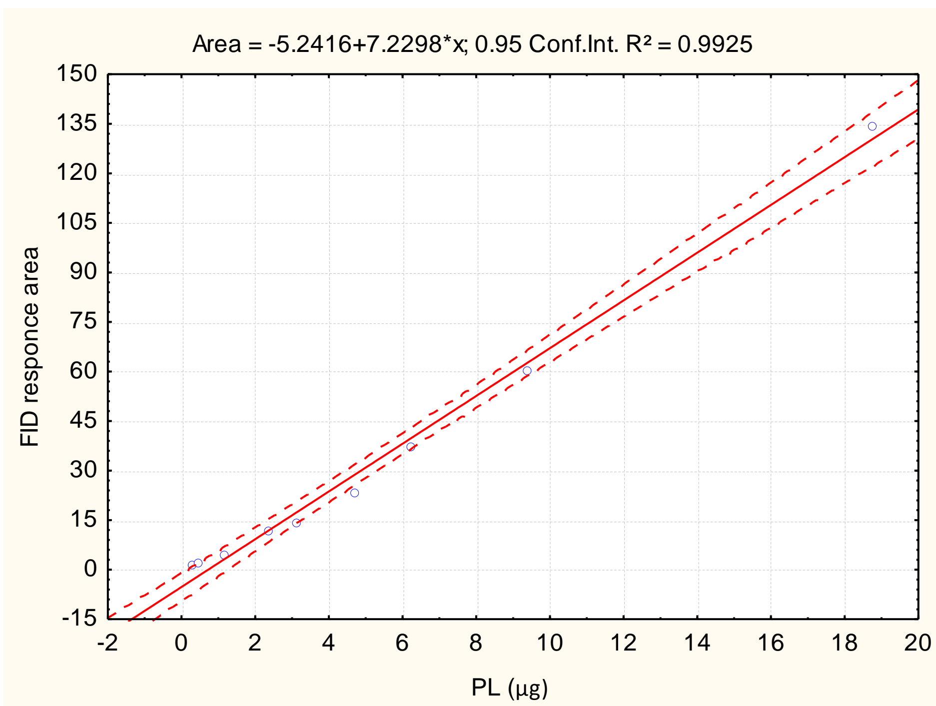

FIGURE 2.10: Phospholipid (PL) calibration curve for lipid class determination.

Fatty acid analysis: Samples for fatty acid analysis were dried under ultrapure N_2, transesterified to fatty acid methyl esters with 14% boron trifluoride at 85°C for 1.5 h. Analyses of the fatty acid methyl esters were carried out with a HP Agilent 6890 gas chromatograph (GC) equipped with 7683 autosampler and flexible fused-silica column (30 m x 0.32 mm internal diameter x 0.25 μm film thickness) coated with polyethylene glycol (ZBwax+, Phenomenex). Column temperature began at 65°C for 0.5 min, which was then raised to 195 °C at a rate of 40°C/min and held for 15 min. Temperature was then increased to 220°C at a rate of 2°C/min and maintained for 0.75 min. Injector temperature started at 150°C and was ramped at a rate of 120°C min^{-1} to a final temperature of 250°C, while the FID detector remained constant at 260°C. Fatty acid retention times were determined with Supelco's 37 component FAME mix (product number 47885-U; Sigma Aldrich), bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033) and PUFA 3 (product number 47085-U).

2.7 Chemical Analyses

2.7.1 Nitrogen

Nitrogen in the form of nitrate was determined following the method described in APHA (1998). Amalgamated Cd at 0.6 g was added to a flask and then 10 mL of each sample added to it. Three milliliter of ammonium chloride (2.6%) was added to the solution, which was followed by the addition of 1 mL of 2% borax. The flask was closed and kept shaking for 20 min, after which 7 mL was removed and transferred to a test tube and 1 mL sulfanilamide added. The total solution was mixed for 4 to 6 sec using a vortex and 1 mL n-1-naphthyl ethylenediamine added. The optical density of the total solution was determined at 543 nm using a UV-VIS Scanning Spectrophotometer equipped with an ASC-5 auto sampler change (Shimadzu, Japan).

Nitrate calibration curves were made using previously dried KNO_3 for 1 h at 105°C. The KNO_3 (0.36 g) was dissolved in deionized water and diluted to 500 mL. Nitrate concentrations used to obtain the calibration curve ranged from 10 to 400 μg L^{-1} (FIGURE 2.11).

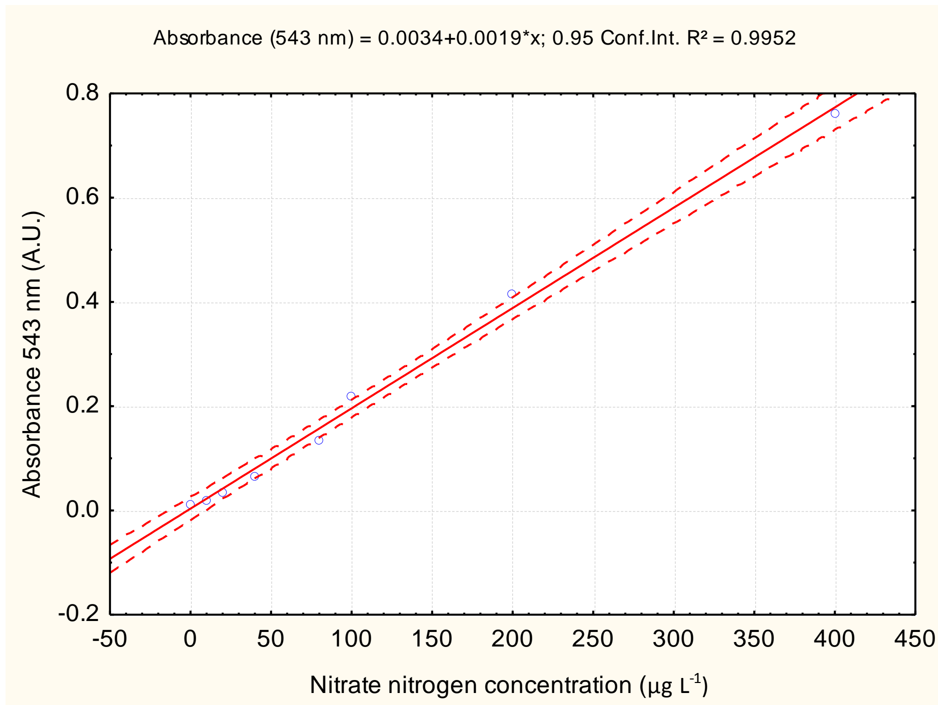

FIGURE 2.11: Nitrate calibration curve for nitrate determination.

2.7.2 Phosphorus

Phosphorus in the form of phosphate was determined according to APHA (1998) using the ascorbic acid method. Previously filtered culture samples (5 mL) were pipetted into a clean and dry test tube. One drop of phenolphthalein indicator solution was added to the sample. Where a red color developed, 5 N H_2SO_4 solution was added drop-wise to just remove the color. Then 0.8 mL of the combined reagent {5N H_2SO_4, $K(SbO)C_4H_4O_6.{}^{1}/_{2}H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $C_6H_8O_6$} was added and mixed thoroughly. After at least 10 min but not more

than 30 min, absorbance of each sample was determined at 878 nm, using reagent blank as the reference solution.

Calibration curves were made using K_2HPO_4 ranging from 0.06 to 4 mg L^{-1}. To make sure that all treatments were within the range of the calibration curve, where P concentrations were higher, dilutions were made. Ultrapure water was used as blank with the combined reagent to make photometric readings for the calibration curve (FIGURE 2.12).

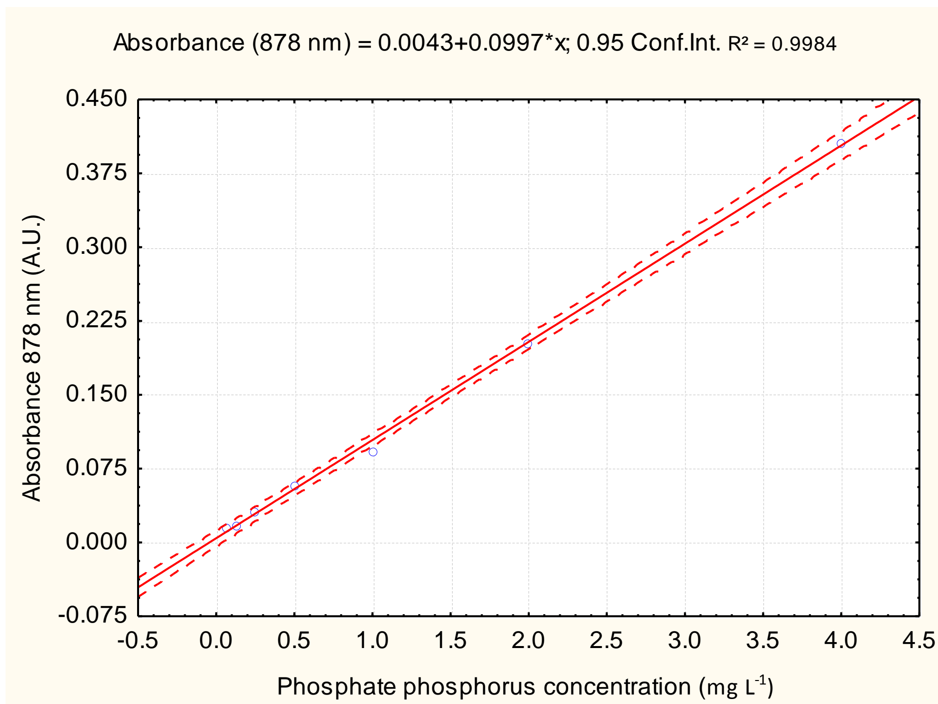

FIGURE 2.12: Phosphate calibration curve for phosphate determination.

2.8 Data analyses

The data were subjected to the Levene's test for homogeneity of variances. Factorial analysis of variance (ANOVA) and Tukey's HSD multiple range comparison tests were used to determine significant differences between means of analyzed parameters. A correlation matrix based principal components analysis (PCA) was used to detect any relationship among the analyzed parameters. PCA represents a statistical procedure that employs an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the first principal component has the largest possible variance (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to (i.e., uncorrelated with) the preceding components (COOPER et al., 1999; WIKIPEDIA, 2012). A combination of the first two axes gives the best plane of the relationship between the factors as exhibited by their distribution from the barycentre. PCA scores were grouped by cluster analysis using single linkage. All analyses were done at 95% confidence interval. ANOVA and post-hoc analyses were done using Statistica 8.0 (Stat Soft. Inc) software, PCA was done using the CANOCO 4.5 and PAST computer program for windows and cluster analysis with Minitab version 16.0.

3. RESULTS AND DISCUSSION

3.1 Growth, biomass production and biochemical composition of *Chlorella vulgaris* grown in different culture media

Results: The MINEQL$^+$ chemical equilibrium calculations for Cu, Fe and Zn showed that the lowest free and labile Cu concentrations were present in the WC medium, which can be expected due to the presence of the synthetic organic ligand, EDTA. In general, our results showed that for the speciation of Cu, Zn and Fe, LC Oligo had the highest values and Chu 10 medium presented intermediate concentrations of both labile and free metal ions (TABLE 3.1).

TABLE 3.1: MINEQL$^+$ metal speciation results for selected elements in the growth media. Values are in pCu, pFe and pZn, where p = negative base $\log_{10}$ of the molar concentrations.

		Media		
Element	Speciation	Chu 10	LC Oligo	WC
Copper	Free	9.56	7.76	12.84
	Labile	9.91	8.03	8.72
Iron	Free	5.62	5.52	5.89
	Labile	7.09	6.05	6.96
Zinc	Free	9.11	7.03	10.03
	Labile	10.80	8.53	11.49

Biomass results are shown in FIGURE 3.1. The highest growth (FIGURE 3.1a) and cell density (FIGURE 3.1b) were obtained in the LC Oligo medium. *C. vulgaris* growth rates obtained for the culture media tested were: LC Oligo: 0.84 d^{-1}, Chu: 0.79 d^{-1} and WC: 0.62 d^{-1} media.

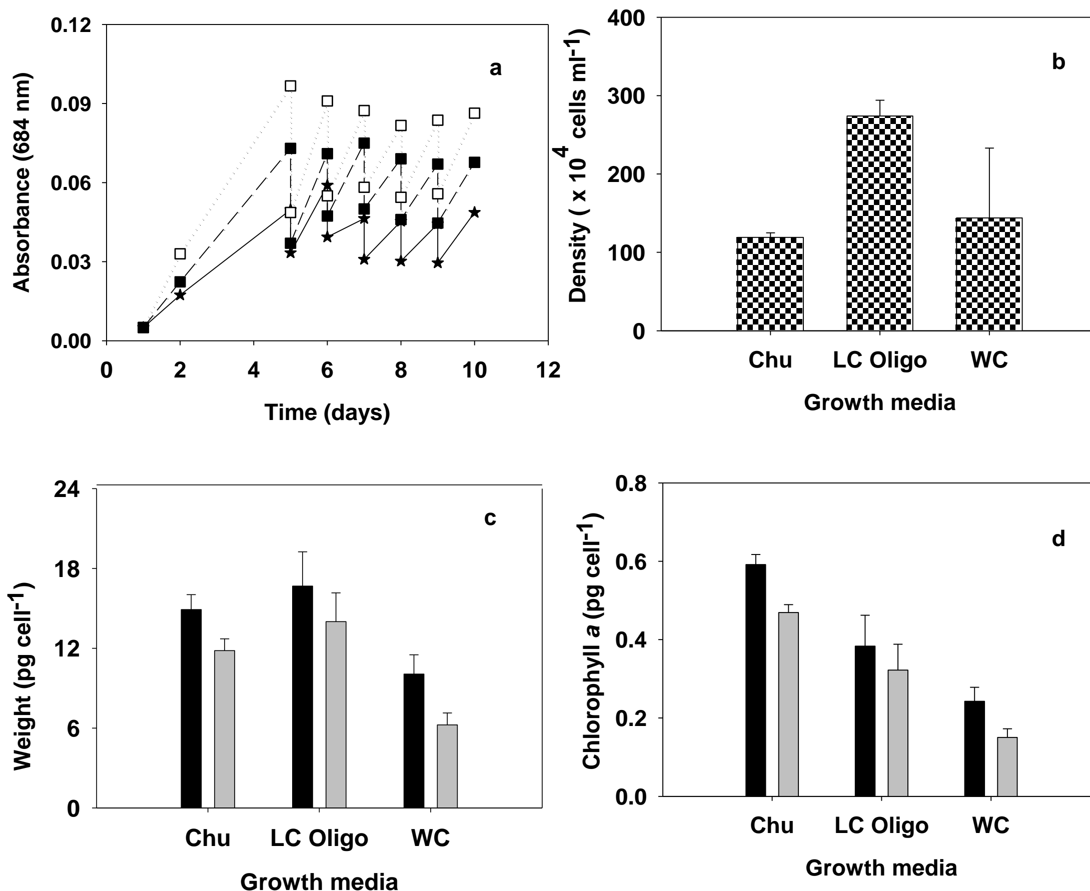

FIGURE 3.1: (a) Growth curve (star: WC; open square: LC Oligo; filled square: Chu) and specific growth rates (d^{-1}) for Chu (0.79), LC Oligo (0.84) and WC (0.62 d^{-1}); (b) density (cells mL^{-1}); (c) dry weight (pg $cell^{-1}$), and (d) chlorophyll a production (black bars) and yield (gray bars) (pg $cell^{-1}$) for *Chlorella vulgaris* grown in Chu (Chu medium), WC (WC medium), LC Oligo (LC Oligo medium). Error bars represent standard deviation for $n = 3$.

Dry weight production and yield (pg $cell^{-1}$) were highest in the LC Oligo medium and lowest in the WC medium (FIGURE 3.1c). Chlorophyll *a* production and yield were highest in the Chu medium and lowest in the WC medium (FIGURE 3.1d).

Total intracellular proteins, carbohydrates and lipids results are shown in FIGURE 3.2. The lowest protein and carbohydrate concentrations and yields were obtained in the LC Oligo medium, while the highest was found in the Chu medium. Lipid class composition analysis showed that HC, SE, FFA, ALC, AMPL and PL concentrations and yields were highest in the Chu medium, while the lowest value was recorded in the WC medium (FIGURE 3.3). Lipid class composition and total lipid production did not significantly ($P < 0.05$) differ between the LC Oligo medium and the Chu 10 medium.

Principal component analysis showed that the first two components accounted for 68% of the total variation observed in the PCA (FIGURE 3.4). Phosphorus and N concentrations were positively correlated with SE, TAG, FFA, ALC, and ST concentrations. In addition, P was significantly ($p < 0.05$) associated with cell density, dry weight, chlorophyll *a,* AMPL, total lipids and carbohydrate concentration. Chlorophyll a concentrations were positively correlated with AMPL levels. The concentration of the storage lipid class (TAG) was negatively correlated with the structural lipid class concentrations (PL).

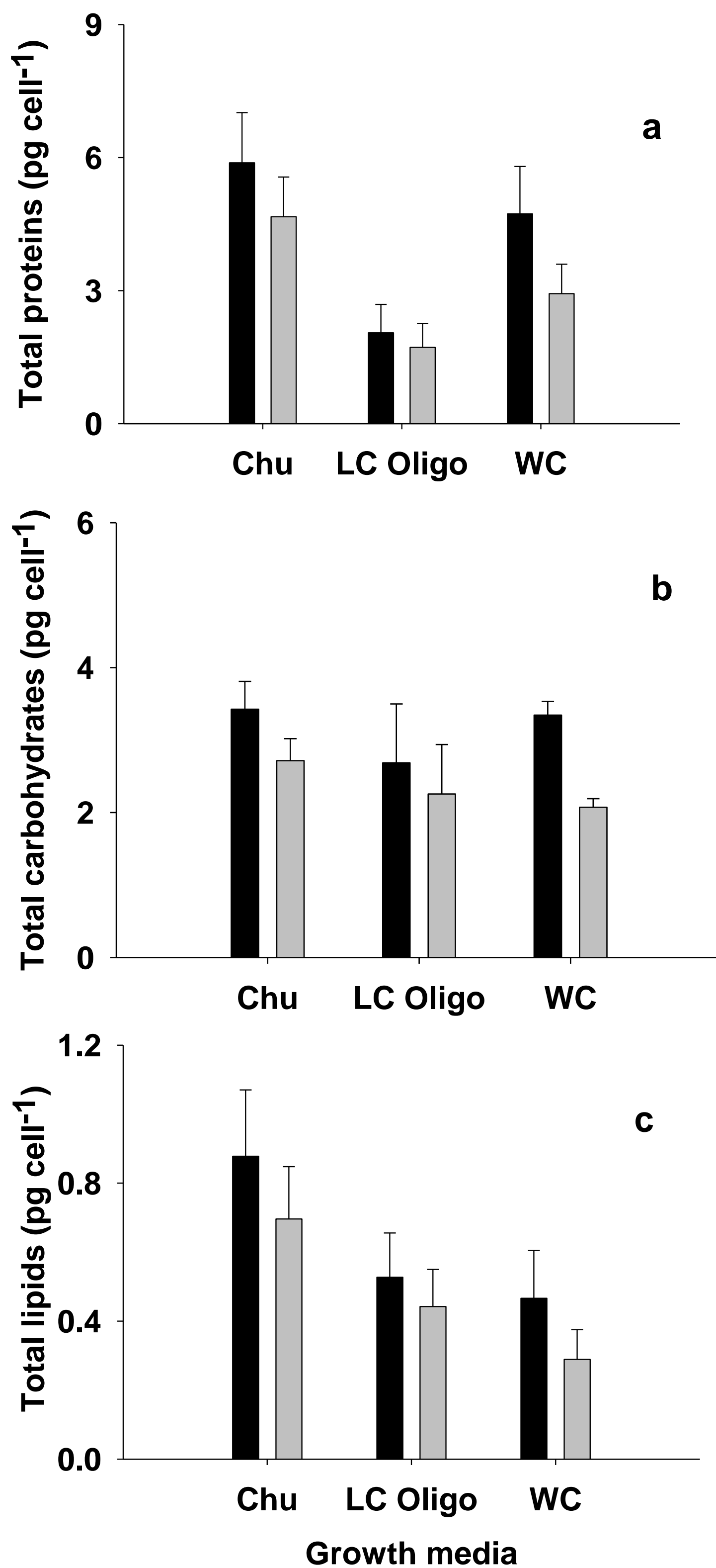

FIGURE 3.2: Total carbohydrate (a), total protein (b) and total lipid (c) production (black bars) and yield (gray bars) (pg cell^{-1}) of *Chlorella vulgaris* as a function of media type using semi continuous culture system. Error bars represent standard deviation for *n* = 3. Chu = Chu medium, WC = WC medium, LC Oligo = LC Oligo medium.

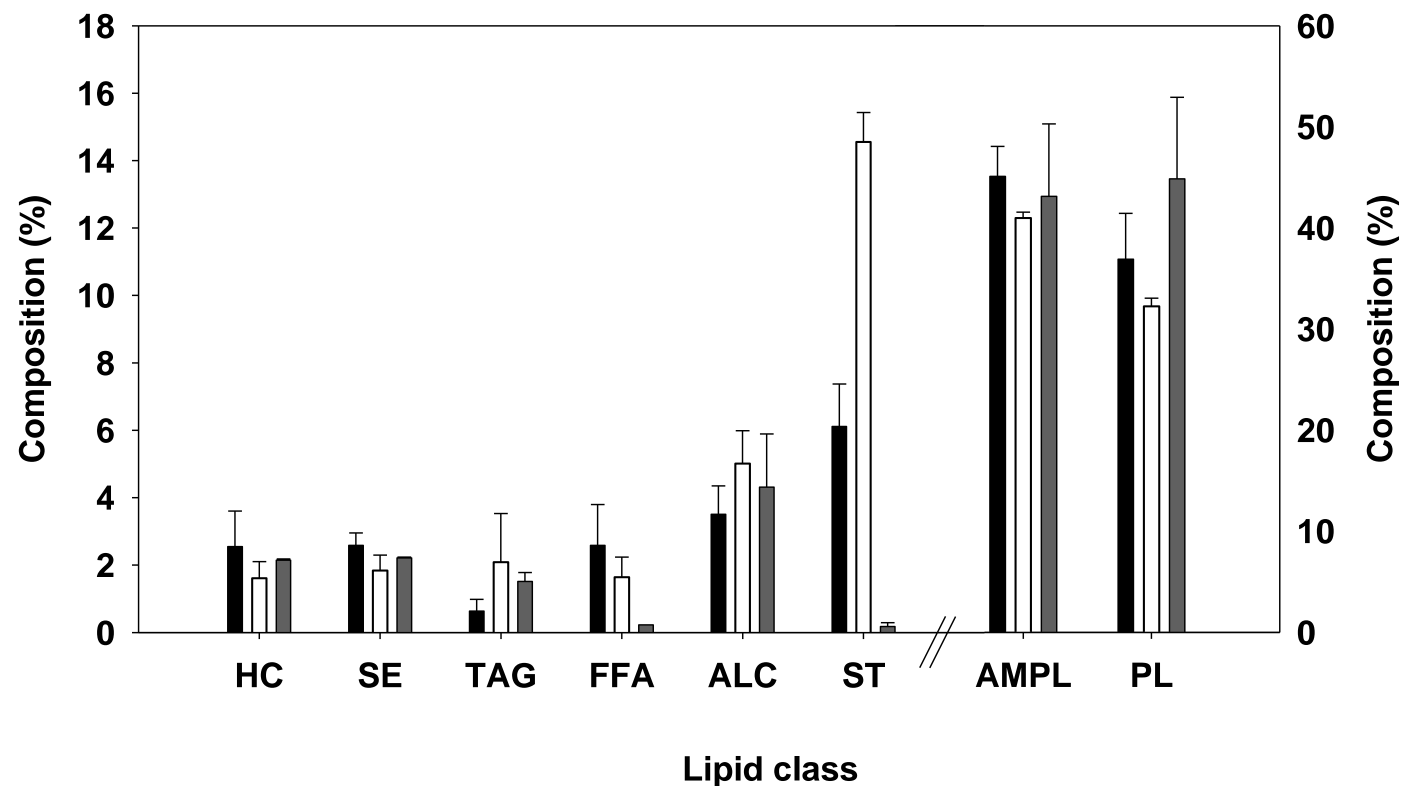

FIGURE 3.3: Lipid class composition (%) of *Chlorella vulgaris* grown in different growth media (black bars: Chu; white bars: LC Oligo; gray bars: WC). Neutral lipids are represented by HC, SE/WE, TAG, FFA, ALC and ST, while polar lipids, AMPL and PL. Error bars represent standard deviation for $n = 3$. Chu = Chu medium, WC = WC medium, LC Oligo = LC Oligo medium. Right axis applies to AMPL and PL classes.

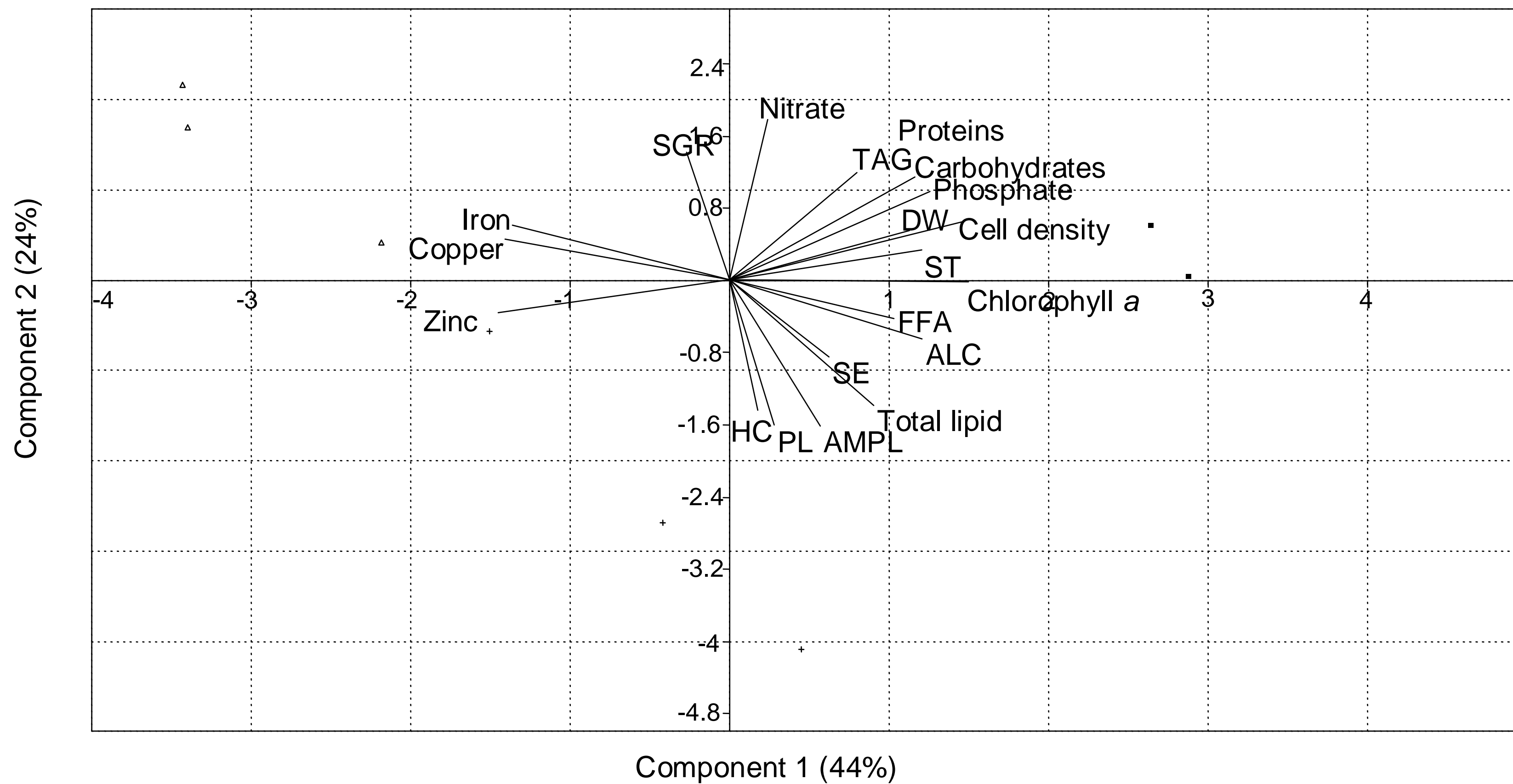

FIGURE 3.4: PCA biplot for the various parameters measured for *Chlorella vulgaris* cultured with different growth media using semi continuous culture. SGR = specific growth rate and DW = dry weight

Discussion: Growth performance was influenced by phosphate, copper, nitrate and iron concentrations in the media, supporting the significantly higher growth rate (0.84 d^{-1}) for *Chlorella vulgaris* in the LC Oligo medium in comparison with the other two culture media tested. This growth rate value is within those reported in the literature for *Chlorella.* ONG et al. (2010) obtained specific growth rates of 0.40-0.85 d^{-1} for *C. vulgaris* grown in f/2 medium, which are higher than that reported in OBATA et al. (2009) for *C. vulgaris* in enriched C medium under semi-continuous conditions (0.40 d^{-1}).

Cell density, an important biomass parameter varied significantly from medium to medium with LC Oligo having almost twice (2.74 x 10^6 cells mL^{-1}) the value found in the other growth media we investigated. These results are lower than those of OHSE et al (2008), where the authors showed a maximum cell density for *C. vulgaris* cultures of 5.7x10^6 cells mL^{-1} in tubular bioreactors, a value that is about 2.5 times higher than the present ones. However, our results are similar to those of SCRAGG et al. (2002) and OBATA et al. (2009) that also investigated the growth of *C. vulgaris* under controlled conditions.

Chlorophyll *a* production by phytoplankton cells is known to vary with growth conditions; in our experiments, the maximum production of chlorophyll *a* by *C. vulgaris* was 1 µg mL^{-1} in the LC Oligo cultures. These results are lower than those reported by CHINNASAMY et al. (2009), which showed that batch cultures of *C. vulgaris* grown in BG 11 medium, reached a maximum chlorophyll *a* concentration of approximately 5 µg mL^{-1} within the first 10 days. However, if the results are reported as chlorophyll *a* concentration per cell unit (not per volume), the present results show a high production for the Chu media cultures (5.9 x 10^{-13} g $cell^{-1}$), which are higher than those obtained by BERTOLDI et al. (2008). These authors obtained a maximum chlorophyll *a* production for *C. vulgaris* grown in the BBM medium of 2.8 x 10^{-13} g $cell^{-1}$.

The highest dry weight (16.82 pg $cell^{-1}$) concentration achieved using LC Oligo medium can be due to its composition, as this medium had the highest N and P concentrations. Similar to our study, CHINNASAMY et al. (2009) obtained a dry weight production of ~50 µg mL^{-1} during the exponential phase of *C. vulgaris* grown at 0.036%

CO_2 and 30°C, which falls within the values (13-54 μg mL^{-1}) obtained in this study in the media tested.

Protein and carbohydrate concentrations were closely related to the media components nitrate, phosphate and free copper ion concentrations. The protein production of 50% (7.0 mg L^{-1}) in Chu and (6.8 mg L^{-1}) in WC media are higher than those obtained in other studies, but similar to the results of BERTOLDI et al. (2008). BERTOLDI et al. (2008) reported that *C. vulgaris* cultivated in BBM had a protein content of approximately 52.4% (dry weight). Our results for protein content in Chu and WC media are about twice higher than those reported by ILLMAN et al. (2000), who showed 29% (dry weight) protein production by *C. vulgaris* in Watanabe medium.

The highest carbohydrate production by *C. vulgaris* in the present conditions was obtained in the LC Oligo cultures (7.36 μg mL^{-1}). This value is lower than that reported in CHINNASAMY et al. (2009), who obtained 40 μg mL^{-1} carbohydrate production for *C. vulgaris* grown in BG 11 medium. However, our results are similar to those reported in HABIB et al. (2003). These authors showed a carbohydrate production of 30.8 g per 100 g of *C. vulgaris* grown in an NPK fertilizer enriched culture medium, while we obtained an equivalent of 33.74 g per 100 g for *C. vulgaris* in Chu medium. ILLMAN et al. (2000) reported carbohydrate concentrations of up to 50% for *C. vulgaris* in Watanabe medium, which is higher than the highest value of 37% we obtained for *C. vulgaris* grown in WC medium. In addition, LIANG et al. (2009) showed that growing *C. vulgaris* in media with glucose, acetate and glycerol, carbohydrate production values up to 44% may be obtained.

In relation to lipid production, not all lipid classes were affected by the culture medium used. Aliphatic hydrocarbons (HC), free fatty acids (FFA), free aliphatic alcohols (ALC) and sterol esters (SE) were within 5% of the total lipid production. This percentage value is within the normal range expected for most microalgae under healthy growth conditions independent of the culture medium (HU, 2004; DOE, 2010; SCARSELLA et al., 2010). In the present experiments, structural lipids, such as the AMPL and PL classes dominated the percent lipid composition in *C. vulgaris*

independent of the culture medium used. Polar lipids (AMPL and PL) production of *C. vulgaris* under autotrophic and healthy growth are usually up to 90% of the total lipids, while under heterotrophic and also healthy growth the value is around 37% (HU et al., 2008; SCARSELLA et al., 2010), which agrees with the findings of this study. This is important for aquaculture once polar lipids contribute to the production of PUFAs (MEIRELES et al., 2003); PUFAs are known as effective growth promoters in aquaculture (FEIRREIRA et al., 2009; HILL et al., 2011).

Triacylglycerol lipid class in the present research was kept relatively low, within 2% of the total lipids; denoting cultures were healthy despite the differences in media composition. According to LOMBARDI and WANGERSKY (1991), TAG content can be a better guide to the physiological state of phytoplankton cells than is the C:N:P ratio and any TAG content higher than 15% of the total lipids imply nutrient stress. The negative relationship between TAG and PL reflects the physiological route towards structural lipid synthesis that occurs under nutrient replete conditions, confirmed by the increase in biomass in these situations. This is confirmed by the results of RODOLFI et al. (2008) and GRIFFITH and HARRISON (2009) which showed that under nutrient replete conditions, the precursors involved in TAG and PL synthesis are channeled towards the production of PL, needed for cell membranes. Acetone mobile polar lipids are a polar lipid class that may contain 35 - 50% chlorophyll *a* (PARRISH and WANGERSKY, 1987; LOMBARDI and WANGERSKY, 1991; 1995; ILLIJA et al., 2009), which explains the significant association between them.

Total lipid production varied among the culture media tested in this research, being lowest in WC grown *C. vulgaris*, suggesting that cells were under healthy growth conditions. Literature data show that total lipid production can be affected by N:P ratios in culture media, which for the ones used here were Chu 10:1; LC Oligo 10:1; WC 100:1. This phenomenon is well demonstrated in Wikfors (1986) who showed an increase in total lipid production in *Dunaliella tertiolecta* at N:P ratios of ~15:1, but not at 30:1. This confirms the importance of media characteristics on the biochemical composition of

phytoplankton, having implications for their nutritional value (GRIFFITH and HARRISON, 2009; PITTMAN et al., 2011).

Thus, from these results we conclude that the biochemical composition of *C. vulgaris* varies according to the growth media in which they are cultivated. However, this is to a limited extent as there is a similar behavior that can define healthy and stressed cells independently of the growth media. High growth rate and biomass production were obtained in the LC Oligo medium in relation to the other growth media. Therefore, the LC Oligo medium was selected as the preferred medium due to its cost effectiveness.

3.2 Calorific values of *Chlorella vulgaris* cultured under varying phosphate concentrations using semi-continuous culture.

Results: Specific growth rate ranged from 0.46 to 0.85 d^{-1} in all treatments with the lowest P concentration having the lowest growth rate. Dry weight per cell increased with decreasing P (TABLE 3.2). As expected, the highest chlorophyll *a* production was recorded in the control, while the lowest was recorded at the lowest P concentration. Carbohydrate and lipid concentrations increased with decreasing P, while protein concentration was highest (12.12 pg $cell^{-1}$) at $2.3x10^{-6}$ mol L^{-1} P and lowest (1.84 pg $cell^{-1}$) at $6.0x10^{-7}$ mol L^{-1} P. Calorific values of *C. vulgaris* increased with decreasing P concentration.

Concentrations of lipid classes showed that storage lipids like HC, SE and TAG were highest at P concentrations lower than the control. The TAG component was the most accumulated lipid class among all lipids when P was limiting growth. Aliphatic hydrocarbons and ST content did not differ significantly between the different treatment conditions (TABLE 3.3). The polar lipid composition showed that the lowest concentrations per cell of AMPL and PL were recorded in the control, while the highest concentration was detected at $2.3x10^{-6}$ mol L^{-1} P (TABLE 3.3).

Principal components analysis showed that P was significantly and positively associated ($p<0.05$) with PL, ST, AMPL, cell density, chlorophyll *a,* and growth rates (FIGURE 3.5). However, a significant negative relationship ($p<0.05$) was observed between P concentration and TAG, dry weight, total lipids, carbohydrates and calorific values. The PCA showed that 77.3% of all the accounted variations observed were within the first two components.

TABLE 3.2: A summary of biomass production, biochemical composition and calorific value of *C. vulgaris* at different phosphorus concentrations. Values represent the mean of $n = 3 \pm$ standard deviation. Rows with the same superscript letters are not significantly different at 95% significance level ($P < 0.05$).

	Phosphorus (mol L^{-1})		
Parameter	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}
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}	1.07 ± 0.10^{b}	0.75 ± 0.10^{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})	13.78 ± 1.66^{a}	30.47 ± 5.48^{b}	33.07 ± 3.98^{b}

TABLE 3.3: Lipid class composition (pg $cell^{-1}$) of *C. vulgaris* at different phosphate regimes (mol L^{-1}) using semicontinuous cultures. Values are mean $\pm$ standard deviation for n = 3. Rows with the same superscript letters are not significantly different at 95% significance level ($P < 0.05$).

	Phosphorus concentration (mol L^{-1})		
Lipid class	Control	$2.3x10^{-6}$	$6.0x10^{-7}$
HC	0.02 ± 0.01^{a}	0.43 ± 0.14^{b}	$0. 19 \pm 0.01^{b}$
SE	0.03 ± 0.01^{a}	0.11 ± 0.03^{b}	-
TAG	0.03 ± 0.02^{a}	1.00 ± 0.49^{b}	5.41 ± 1.36^{c}
FFA	0.02 ± 0.01	-	-
ALC	0.07 ± 0.01	0.11 ± 0.05	0.03 ± 0.01
ST	0.21 ± 0.12	0.22 ± 0.04	0.08 ± 0.04
AMPL	0.59 ± 0.01^{a}	3.06 ± 0.42^{b}	$1.77 \pm 0. 50^{a}$
PL	0.47 ± 0.01^{a}	2.23 ± 0.20^{c}	1.62 ± 0.21^{b}

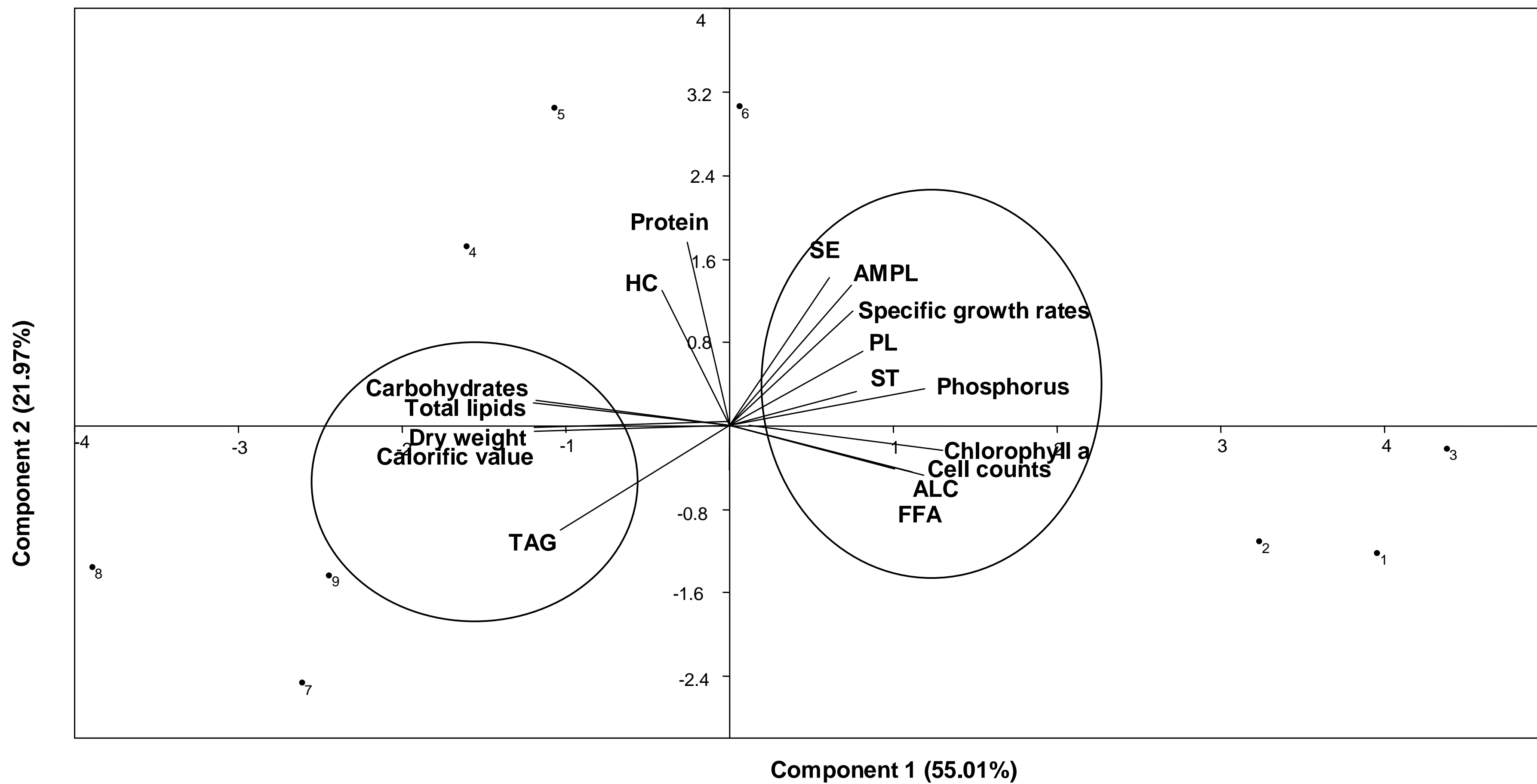

FIGURE 3.5: Principal components analysis showing the relationship between phosphate levels and physiological parameters analyzed for *C. vulgaris*.

Discussion: Phosphorus is needed for microalgal growth as it plays important regulatory roles in their physiology and growth (BHOLA et al., 2011). This explains the higher specific growth rates obtained in the control. Other authors have also shown that sufficient phosphorus concentrations increase growth rates and biomass production (KHOZIN-GOLDBERG and COHEN, 2006; CHISTI, 2008; WIDJAJA et al., 2009; BHOLA et al., 2011). Similar to the results of KHOZIN-GOLDBERG and COHEN (2006) that showed a reduction in cell division and chlorophyll synthesis of *Monodus subterraneus* under phosphorus limitation, we recorded a decrease in chlorophyll *a* production per cell under P limitation.

Biochemical parameters like total lipid and carbohydrate that increased under P stress supported the high calorific value. This agrees with other studies that have shown that microalgae tend to store carbohydrates and total lipids under stress conditions, thereby causing their calorific values to increase (ILLMAN et al., 2000; SCRAGG et al., 2002). Carbohydrates and lipids are high energy yielding molecules. The calorific value found in the control (13.78 kJ g^{-1}) is lower than those reported in other studies (18-21 kJ g^{-1}) when *Chlorella* spp. were grown under healthy growth conditions (ILLMAN et al., 2000; SCRAGG et al., 2002; BHOLA et al., 2011). The authors reported that under normal growth conditions microalgal calorific values fall between 18 and 21 kJ g^{-1} (SCRAGG et al., 2002; CHANGDONG and AZEVEDO, 2005; BHOLA et al., 2011). However, the values we recorded under P limitation are higher than that reported by these authors (28 kJ g^{-1}). The still high growth rate (0.67 d^{-1}) at 2.3×10^{-6} mol L^{-1} P and high calorific value may be a promising combination for biomass production directed towards biofuel extraction. ILLMAN et al. (2000), SCRAGG et al. (2002) and BHOLA et al. (2011) 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. This is because it will permit higher calorific yields per unit time compared to cultures with lower growth rates.

When P is deficient in an environment, microalgae tend to accumulate more of triacylglycerols in place of other storage lipid classes (HU et al., 2008; GOUTX et al.,

2009). Under limited P condition, *C. vulgaris* showed an increased TAG/PL ratio, which is indicative of nutrient stress condition (GUSCHINA and HARWOOD, 2006b). This is further supported by the fact that under stress conditions, the production of TAG progresses with precedence over that of PL.

The significant association we obtained between phosphorus and phospholipids is explained by the important role phosphorus plays in the PL molecule.

The percentage increase of triacylgycerols and decrease of polar lipids to total lipids we obtained under phosphorus stress is in agreement with the results presented in 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 relationship between AMPL and chlorophyll *a* we obtained is in accordance with other literature results and it is related to the extraction procedure that for this class includes pigments such as chlorophyll (LOMBARDI and WANGERSKY, 1995; HU et al., 2008).

In conclusion, higher production of total lipids rich in TAGs, and carbohydrates supported higher calorific values under P limitation compared to the control. The 2.3×10^{-6} mol L^{-1} P treatment produced high calorific values with minimal growth retardation, which means it has the potential to be applied for renewable energy purposes.

3.3 Cadmium, nitrogen and phosphorus induced changes in growth, biomass production and biochemical composition of *Chlorella vulgaris*

Results: In general, *Chlorella vulgaris* growth and biomass production (cell density, chlorophyll *a* and dry weight) were lowest in the most extreme growth conditions, i.e., highest Cd/lowest P, and highest Cd/lowest N concentrations (FIGURES 3.6 and 3.7). The highest growth rate, cell density, chlorophyll *a* and dry weight were recorded in the control, which had the highest P and N concentrations, and no Cd addition. Significant differences were detected for specific growth rates and biomass production among treatments ($p<0.05$) (TABLE 3.4). Specific growth rate, chlorophyll *a*, dry weight, and cell densities were positively and significantly correlated with N and P concentrations ($p<0.05$). For all P and N concentrations, the presence of Cd at 10^{-7} mol L^{-1} caused a significant reduction in biomass productivity ($p<0.05$; table 3.4) and growth rate ($p<0.05$; TABLE 3.5).

The biochemical composition of *C. vulgaris* was affected by both N and P limitation. In addition, when N limitation was combined with Cd, lower organic production was generally obtained (FIGURE 3.8). Under P limitation, Cd at 2 x 10^{-8} mol L^{-1} resulted in higher biomolecule production. The lowest carbohydrate and protein productivity were obtained at the lowest N and P concentrations with 10^{-7} mol L^{-1} Cd. Total carbohydrates were highest in intermediate N limitation. The highest protein production was recorded in the control (FIGURE 3.8). Total lipid productivity significantly increased under P and N limitation. It is notable that the lipid increase under P limitation was ~4.5 times higher than the highest value found under N limitation (FIGURE 3.8).

Principal components analysis of the data showed that growth and biomass production of *C. vulgaris* were closely related to P and N concentrations (FIGURES 3.9 and 3.10). Cadmium was positively correlated with total lipids and carbohydrates

($p<0.05$). Total protein was positively associated with N concentrations ($p<0.05$). The PCAs for both N and P experiments showed that the first two components were responsible for over 70% of the total variation recorded.

The percent contribution considering each biomolecule (carbohydrates:proteins:lipids) for the total biochemical composition is shown in TABLE 3.5. These results showed that *C. vulgaris* favored the synthesis of storage molecules, namely carbohydrates and lipids to the detriment of proteins under conditions of N and P limitation.

TABLE 3.4: Analysis of variance results for the effect of phosphorus, nitrogen and cadmium on the growth and biomass of *Chlorella vulgaris*. Values represent *F* values and those in parenthesis represent *P* value. Note: values with $P < 0.05$ are significant.

	Experiment					
	P	Cd	P/Cd	N	Cd	N/Cd
Specific growth rate	25.66(0.00)	59.83(0.00)	14.59(0.00)	226.41(0.00)	351.06(0.00)	38.54(0.00)
Density	79.63(0.00)	17.20(0.00)	7.15(0.0)	152.03(0.00)	65.73(0.00)	5.74(0.00)
Dry weight	21.91(0.00)	13.02(0.00)	8.83(0.00)	231.51(0.00)	110.23(0.00)	24.44(0.00)
Chlorophyll a	68.24(0.00)	76.81(0.00)	13.72(0.00)	198.04(0.00)	39.38(0.00)	28.21(0.00)
Total carbohydrate	1.11(0.35)	2.67(0.10)	2.19(0.11)	1.80(0.19)	7.56(0.00)	0.94(0.46)
Total protein	57.80(0.00)	11.05(0.00)	13.41(0.00)	53.92(0.00)	46.18(0.00)	8.55(0.00)
Total lipids per cell	22.82(0.00)	0.95(0.40)	1.05(0.40)	26.19(0.00)	12.22(0.00)	4.70(0.01)

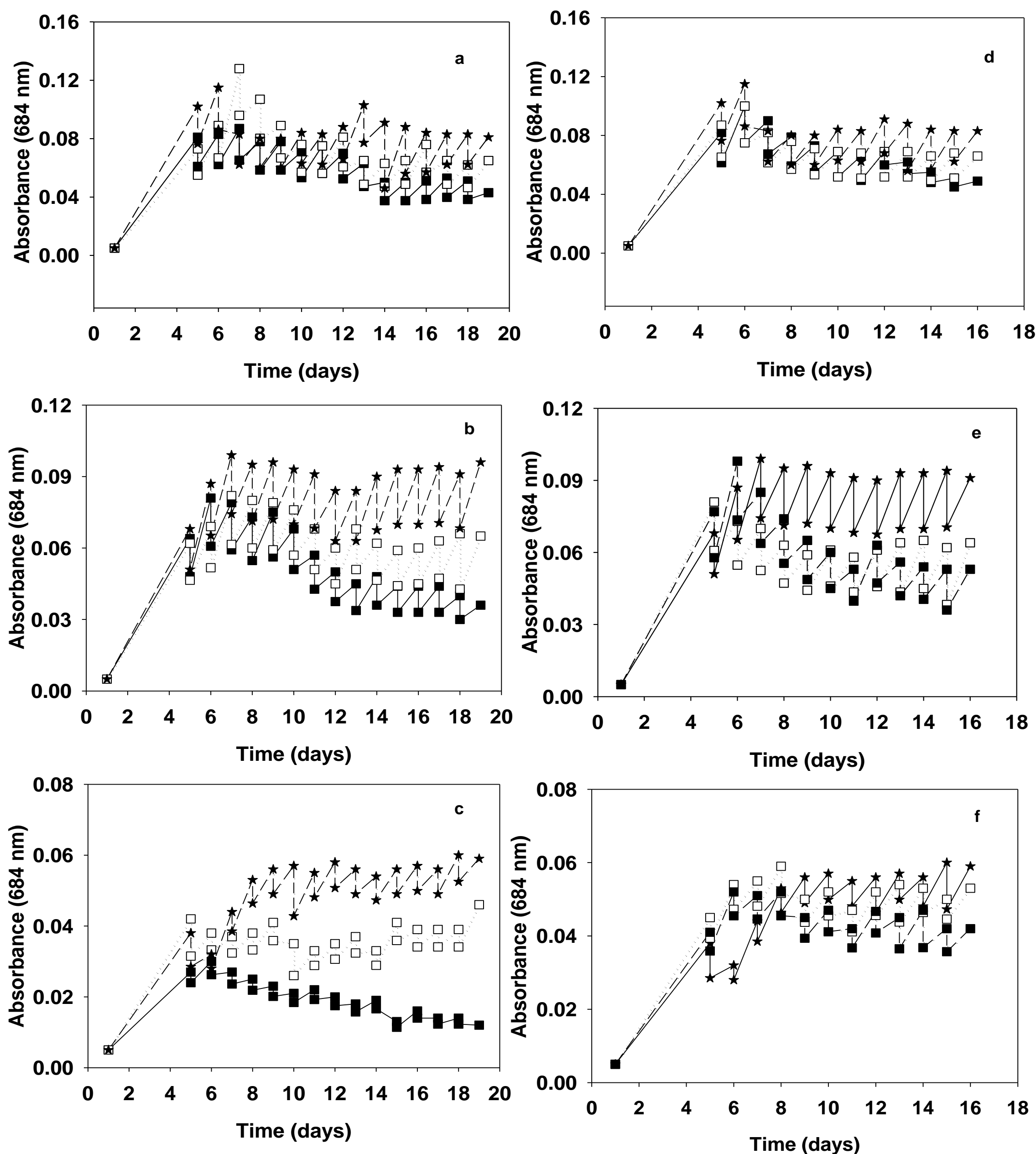

FIGURE 3.6: Growth curve for *Chlorella vulgaris* at different Cd, N and P concentrations reported as absorbance (684 nm) *vs* time (days) in semi-continuous cultures. Stars represent 2.3×10^{-4} mol L^{-1} P or 1.1×10^{-3} mol L^{-1} N without Cd, empty squares represent 2.3×10^{-6} mol L^{-1} P or 1.1×10^{-5} mol L^{-1} N, and filled squares 6.0×10^{-7} mol L^{-1} P or 2.9×10^{-6} mol L^{-1} N. **a.** P without Cd; **b.** P with 10^{-8} mol L^{-1} Cd; **c.** P with 10^{-7} mol L^{-1} Cd; **d.** N without Cd; **e.** N with 10^{-8} mol L^{-1} Cd; **f.** N with 10^{-7} mol L^{-1} Cd.

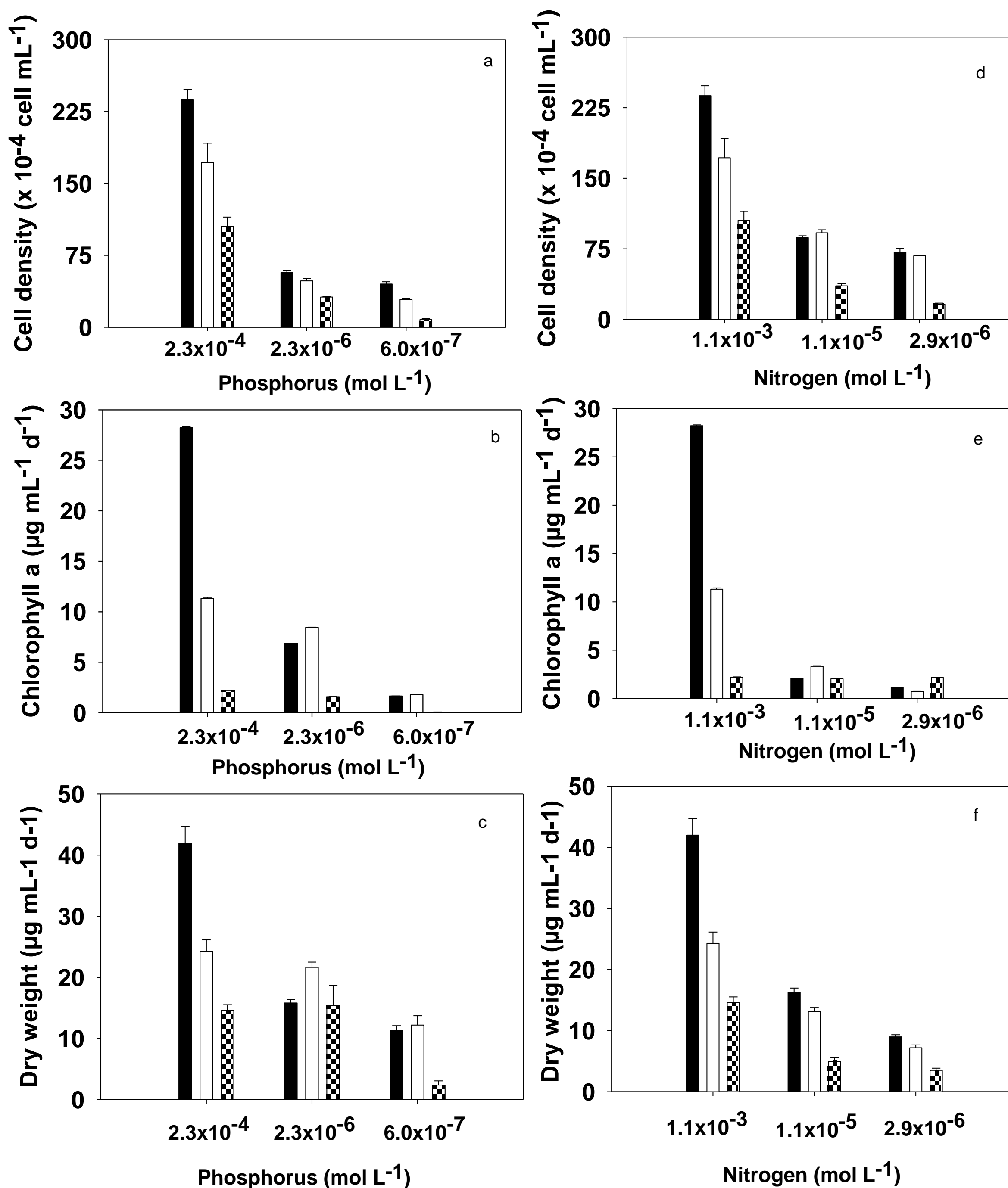

FIGURE 3.7: Effect of Cd on cell density, chlorophyll *a* and dry weight at different P and N concentrations (mol L^{-1}). Black bars represent treatments without Cd, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

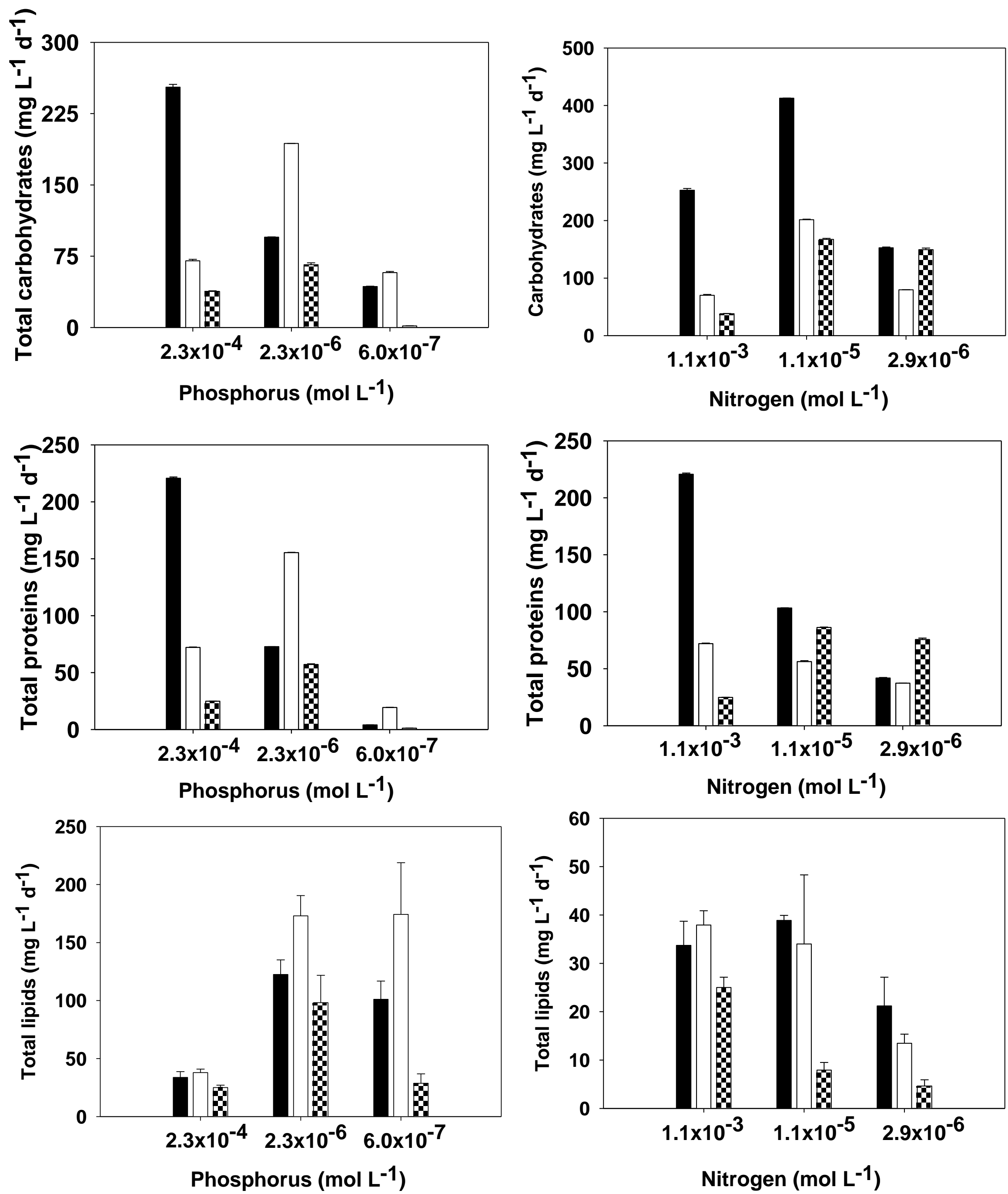

FIGURE 3.8: Total carbohydrate, proteins and lipids production as a function of different Cd, for the P and N concentrations (mol L^{-1}). Black bars represent treatments without Cd, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for n = 3.

TABLE 3.5: Percent organic contribution of total carbohydrates, proteins and lipids (Carb:Pr:L) and specific growth rate (d^{-1}) in parenthesis of *Chlorella vulgaris* acclimated to different Cd, P and N concentrations in mol L^{-1}.

Treatment	Without Cd	Cd – 2.0×10^{-8}	Cd – 1.0×10^{-7}
	Carb:Pr:L	Carb:Pr:L	Carb:Pr:L
Control	47:39:14 (1.05)	38:40:22 (0.62)	53:34:13 (0.36)
P – 2.3×10^{-6}	44:34:22 (0.67)	47:38:15 (0.76)	48:43:9 (0.39)
P – 6.0×10^{-7}	63:6:31 (0.46)	57:19:24 (0.51)	51:35:15 (0.22)
N – 1.1×10^{-5}	71:21:8 (0.49)	72:20:7 (0.51)	64:34:2 (0.19)
N – 2.9×10^{-6}	71:19:10 (0.44)	58:27:15 (0.42)	64:33:2 (0.16)

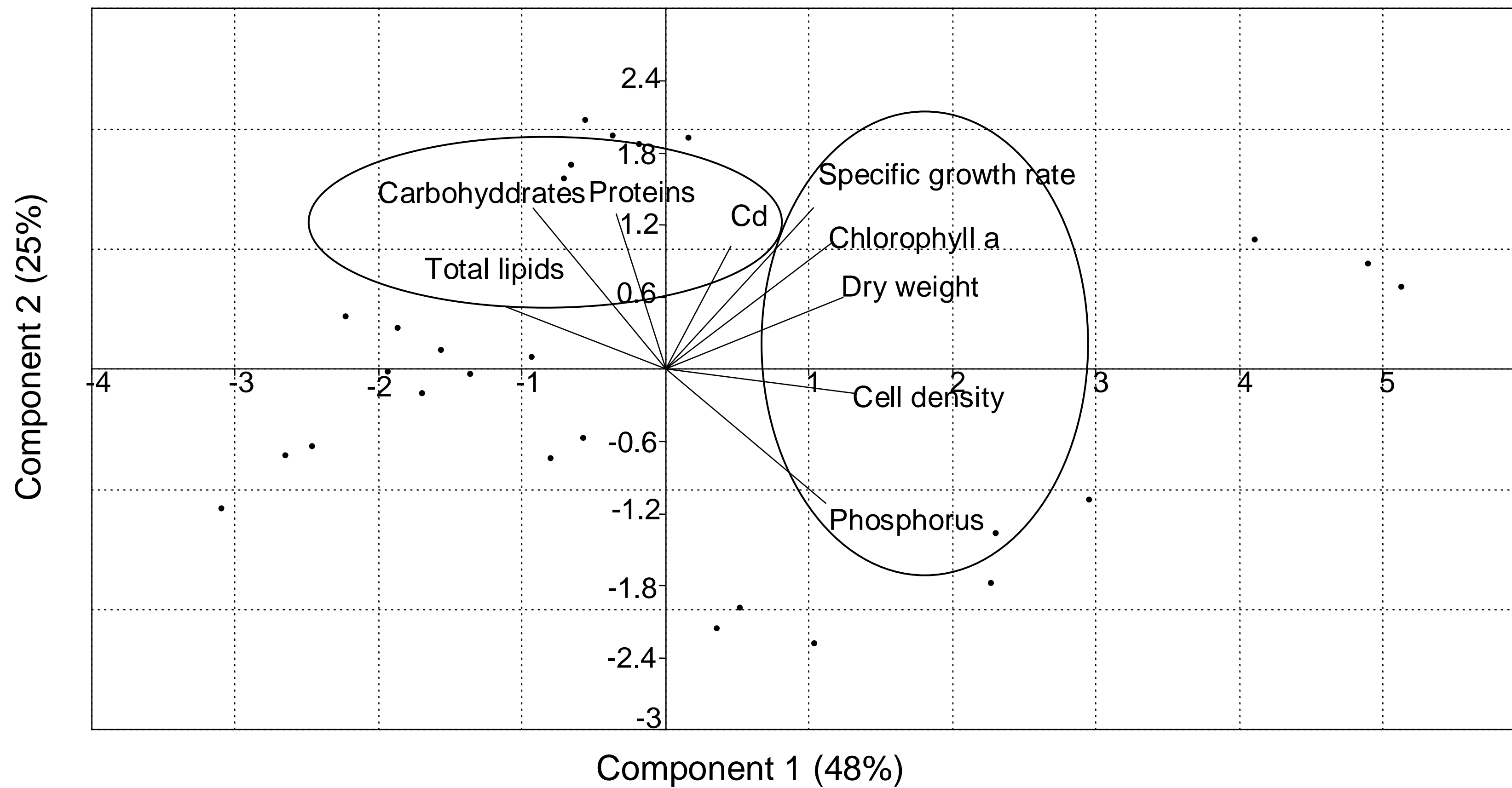

FIGURE 3.9: PCA biplot of different parameters analyzed for *Chlorella vulgaris* at different Cd and P concentrations (mol L^{-1}).

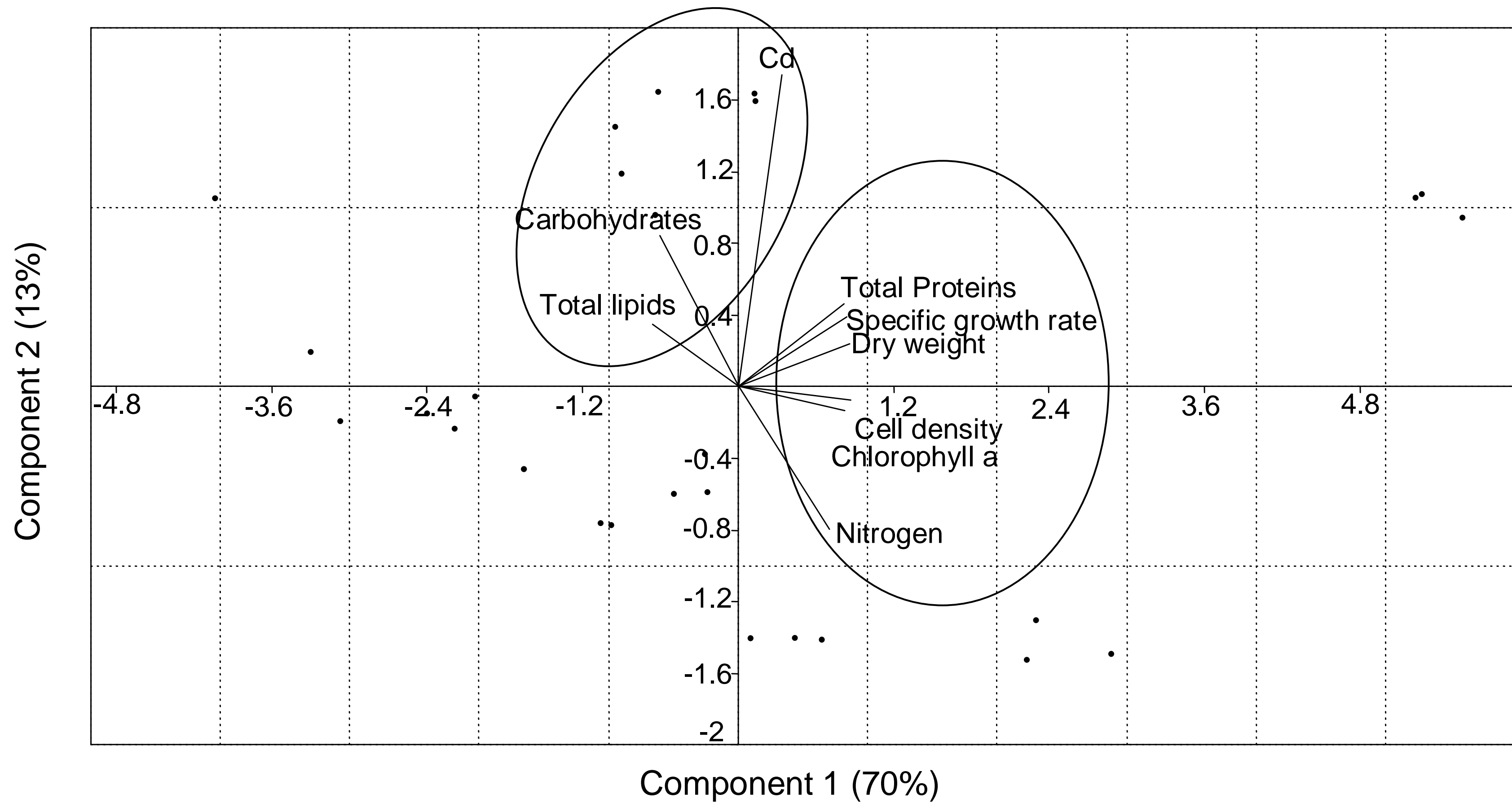

FIGURE 3.10: PCA analysis biplot of different parameters analyzed for *Chlorella vulgaris* as a function of Cd and N concentrations to which the alga was exposed.

Discussion: The positive correlation of growth rates and biomass with the concentrations of N and P without Cd addition supports the importance of these nutrients for cell replication. These results are in accordance with other literature data (HU and ZHOU, 2010; BHOLA et al., 2011) that also reported decreased algae growth and biomass with decrease in N and P. The 50% reduction of *C. vulgaris* growth rates and biomass when exposed to Cd also agrees with other literature results that show a decrease in cell division and nutrient uptake by microalgae in the presence of this metal (SERRA et al., 2010; MONTEIRO et al., 2011). However, healthy concentrations of P and N significantly reduced the inhibitory effect of Cd. Similar to our results, EL-NAGGAR et al. (1998) and BAJGUZ (2011) showed that growth rate and biomass of *C. vulgaris* decreased up to 50% in relation to the control when exposed to Cd concentrations of $5x10^{-6}$ mol L^{-1} and higher.

The negative effect of Cd on the production of chlorophyll *a* obtained in the present study agrees with the results of QIAN et al. (2009), who showed a decrease in chlorophyll *a* concentration in cultures of *C. vulgaris* exposed to a range of Cd concentrations (0 to $2x10^{-6}$ mol L^{-1}). According to QIAN et al. (2009) and BAJGUZ (2011), this decrease in chlorophyll *a* concentrations can lead to a decrease of photosynthesis, consequently affecting growth rate and biomass concentrations in the cultures.

The accumulation of carbohydrates by *C. vulgaris* under conditions of N or P stress agrees with the results of URBANI et al. (2005), who showed that P limited ($6x10^{-6}$ mol L^{-1}) marine diatoms accumulated carbohydrates. The authors propose that this is due to a decreased rate of carbohydrate catabolism. The addition of Cd to P stressed cells further stimulated the accumulation of carbohydrates, as demonstrated in the present research. This is in agreement with the results of PISTOCCHI et al. (2000) who obtained carbohydrates accumulation by microalgae as a general response to Cd exposure. According to BEARDALL et al. (2005), carbohydrate accumulation under nutrient limiting conditions can be due to down regulation of the carbon concentrating mechanism and decreased growth rate.

The positive correlation observed between protein productivity and N concentration can be explained from the fundamental role N plays in protein metabolism, as it is a key element for amino acid synthesis (SCRAGG et al*.,* 2002). According to BRANCO et al. (2010) and BAJGUZ (2011), Cd stimulates the production of reactive oxygen species scavenging enzymes, and affects protein synthesis. In agreement with BAJGUZ (2011), who reported decrease in protein accumulation by a factor of 2 for *C. vulgaris* exposed to 10^{-4} - 10^{-6} mol L^{-1} Cd, we recorded 2.5 fold decrease in protein content in the presence of 10^{-7} mol L^{-1} Cd in relation to the control. However, under sub-lethal Cd concentration ($2.0x10^{-8}$ mol L^{-1}), higher total protein concentration was obtained, which is in agreement with the results of EINICKER-LAMAS et al. (2002), BRANCO et al. (2010) and HARISH et al. (2011). These authors also reported increased microalgal protein content in the presence of sublethal concentrations of Cd and Cu.

Total lipid productivity of *C. vulgaris* under N and P replete concentrations we detected agrees with those reported for *Chlorella* sp. in MATA et al. (2010). The authors reported a total lipid productivity of 40 mg L^{-1} d^{-1} and our results showed 42.1 mg L^{-1} d^{-1}. In the present research, total lipids were most accumulated when *C. vulgaris* was grown under conditions of P limitation together with $2.0x10^{-8}$ mol L^{-1} Cd. This lipid accumulation at trace metal induced stressing conditions is in accordance with the results of EINICKER-LAMAS et al. (1996), and GUSHINA and HARWOOD (2006) that reported total lipid increase in Cd exposed microalgae. The inverse correlation between lipid accumulation and N and P concentrations we obtained are in general agreement to the results of WIDJAJA et al. (2009) and BHOLA et al. (2011), who showed that when P and N were limiting, total lipids content in *C. vulgaris* doubled. Our findings further showed that without Cd, P limitation resulted in higher lipid content than did N limitation. Nitrogen limitation resulted in growth rate decrease and consequently lipid productivity decrease, which agrees with the results of WIDJAJA et al. (2009).

The percent contribution of each biomolecule to the biochemical composition was significantly affected under the conditions we tested. Higher protein in relation to carbohydrates and lipids means the cell is healthy and growing, whereas lower protein in

relation to the other two biomolecules means that the synthesis of storage products instead of structural molecules is taking place (WILSON, 2002). Usually, storage products are synthesized in excess when algae face a stressing situation (LOMBARDI and WANGERSKY, 1995; GRIFFITH et al., 2011).

From the results above, it can be concluded that N and P limitation reduced the growth, biomass production and protein productivity of *C. vulgaris.* The negative effects of N and P limitation were further enhanced in the presence and increasing concentrations of Cd. However, higher lipid and carbohydrate productivities were obtained under nutrient limitation and Cd stress. Replete concentrations of N and P significantly reduced the inhibitory effects of Cd on growth and biomass of the microalga.

3.4 Changes in *Chlorella vulgaris* lipid composition as a function of different cadmium and phosphorus concentrations

Results: In general, the presence of cadmium induced metabolic alterations in *C. vulgaris*; it stimulated the synthesis of chlorophyll *a*, while the percent total lipids decreased (TABLE 3.6).

In relation to lipid classes, aliphatic hydrocarbon (HC) showed no specific concentration pattern, being kept mostly at <6% in relation to total lipids either with or without Cd, P limited or P replete concentration (FIGURE 3.11a). Triacylglycerol (TAG) concentrations increased with decreasing P, its synthesis being specially stimulated whenever Cd was present (FIGURE 3.11b). Free aliphatic alcohol (ALC) and free sterol (ST) concentrations were lowest in the control, but were not significantly affected in the different treatments and were kept within <5% (ALC) and <7% (ST) of the total lipids. Acetone mobile polar lipids (AMPL) and phospholipids (PL) concentrations were lowest in the $2.3x10^{-4}$ mol L^{-1} P treatments, while the highest concentrations of both lipid classes were detected at decreased P concentrations, $2.3x10^{-6}$ for AMPL and $6.0x10^{-7}$ mol L^{-1} P for PL (FIGURE 3.11e and f). In terms of percent contribution to total lipids, both AMPL and PL decreased as P became limited. About 57% of AMPL was present in the control, followed by 46% at $2.3x10^{-6}$ mol L^{-1} P and 19% at $6.0x10^{-7}$ mol L^{-1} P; PL was present at 35% in the control, 30% at $2.3x10^{-6}$ mol L^{-1} P and 20% at $6.0x10^{-7}$ mol L^{-1} P. The levels of AMPL and PL were significantly ($p<0.05$) different among the P treatments (TABLE 3.7).

Table 3.6: Chlorophyll *a* concentration (pg cell^{-1}), percentage contribution of chlorophyll *a* in relation to total lipids and total lipids (TL, pg cell^{-1}) for *Chlorella vulgaris* at the several Cd and P concentrations (mol L^{-1}) tested. Values are ± standard deviation for *n = 3*

Phosphorus	Cadmium								
	Without Cd			2.0×10^{-8}			1.0×10^{-7}		
	Chla	Chla %	TL	Chla	Chla %	TL	Chla	Chla %	TL
2.3×10^{-4}	0.27±0.02	35	0.80±0.08	0.44±0.03	28	1.57±0.09	0.40±0.05	24	1.70±0.04
2.3×10^{-6}	1.15±0.08	15	7.79±0.88	1.08±0.05	14	8.00±0.77	0.84±0.07	14	6.38±0.77
6.0×10^{-7}	0.73±0.09	9	9.14±1.85	1.02±0.07	8	13.85±1.97	1.67±0.32	13	12.39±1.21

Fatty acids analysis showed that total saturated fatty acids (SAFA) levels increased in P limited *C. vulgaris* (FIGURE 3.12a). Cadmium concentrations tested in this study did not cause any significant variations in the SAFA content (TABLE 3.7). Total mono-unsaturated fatty acids (MUFA) concentration increased with lowering P concentration (FIGURE 3.12b), and the presence of Cd caused a significant MUFA increase ($p \leq 0.05$). Total poly-unsaturated fatty acids (PUFA), ω3 PUFA and PUFA:SAFA ratios significantly ($p \leq 0.05$) decreased with increasing P limitation and Cd concentrations (FIGURE 3.12c to 3.12e). Significant ($p \leq 0.05$) interactions between P and Cd on total PUFA and ω3 PUFA, and PUFA/SAFA ratios were recorded (TABLE 3.7).

FIGURE 3.13 reports the effect of the environmental conditions we tested on SAFA and MUFA. It shows that 14:0, 16:0 and 18:0 concentrations increased as P limitation increased, with the levels of 16:0 and 18:0 increasing in the presence of Cd. The concentrations of 16:1*n* – 11 and 18:1*n* – 7 decreased with increasing P limitation, whereas 18:1*n* - 9 increased under the same conditions (FIGURE 3.13). The presence of Cd caused a significant ($p<0.05$) increase in the concentration of 18:1*n* – 7 and 18:1*n* - 9. Most members of the PUFA group recorded in this study had their concentration decreased under P limitation and in the presence of Cd, with some exceptions (FIGURE 3.14); the concentration of 18:2*n* – 6 increased under P limitation.

Principal components analysis confirmed that phosphorus had a significant and positive relationship with PL, AMPL, ST, and PUFA ($p<0.05$). Triacylglycerol concentrations were negatively correlated with P ($p<0.05$), as shown in FIGURE 3.15. Total SAFA, MUFA, 14:0, 18:0, 18:2n – 6, 18:1n – 9 and 16:0 were negatively related to P and total MUFA, 18:0 and 18:1*n* – 7 were negatively correlated with Cd. Principal components analysis showed that the first two components accounted for 70% of the total variation recorded in this study. With the addition of a third component to the first two, almost 80% of the total variation is accounted for.

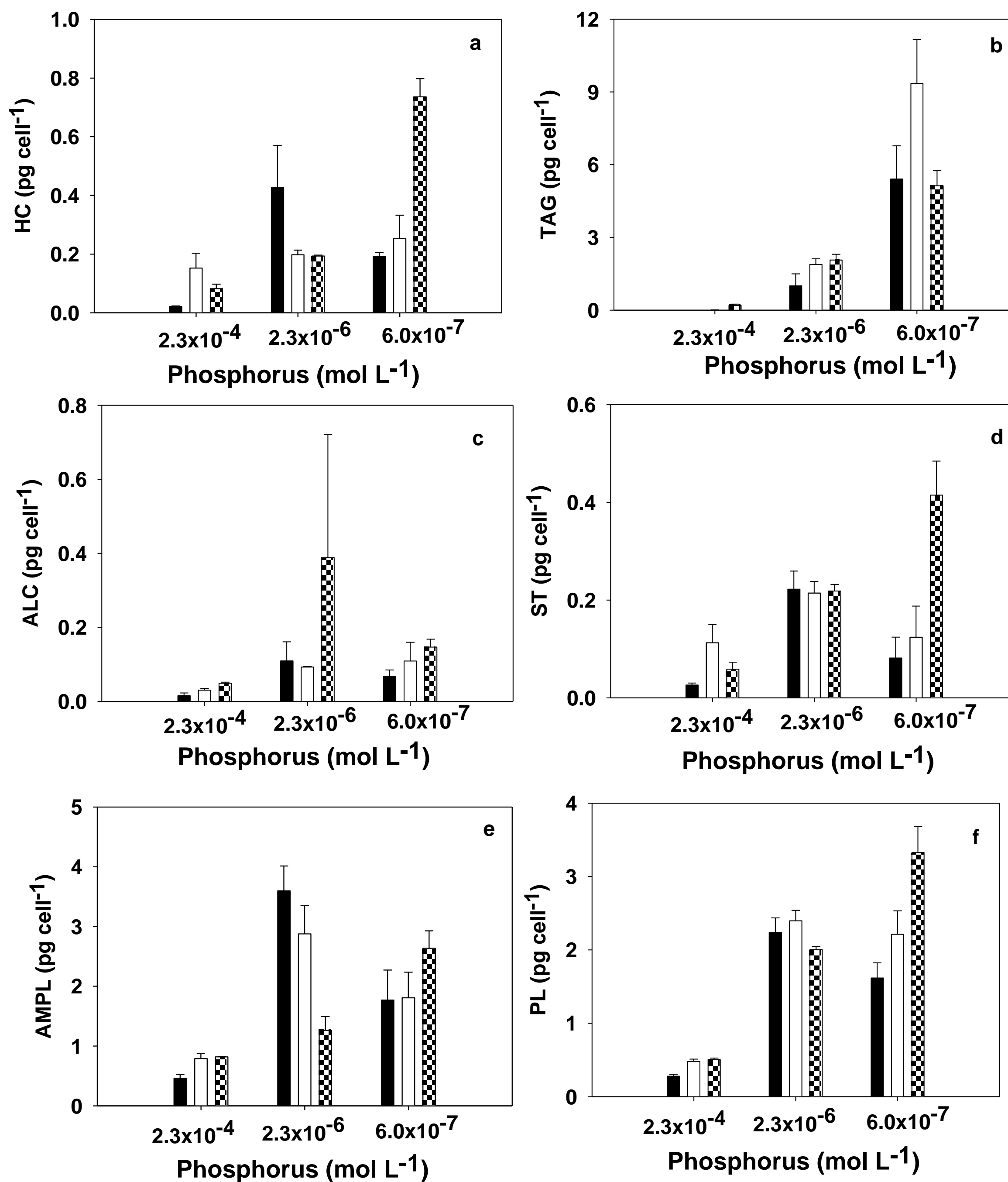

FIGURE 3.11: Lipid class composition of *Chlorella vulgaris* at different P and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

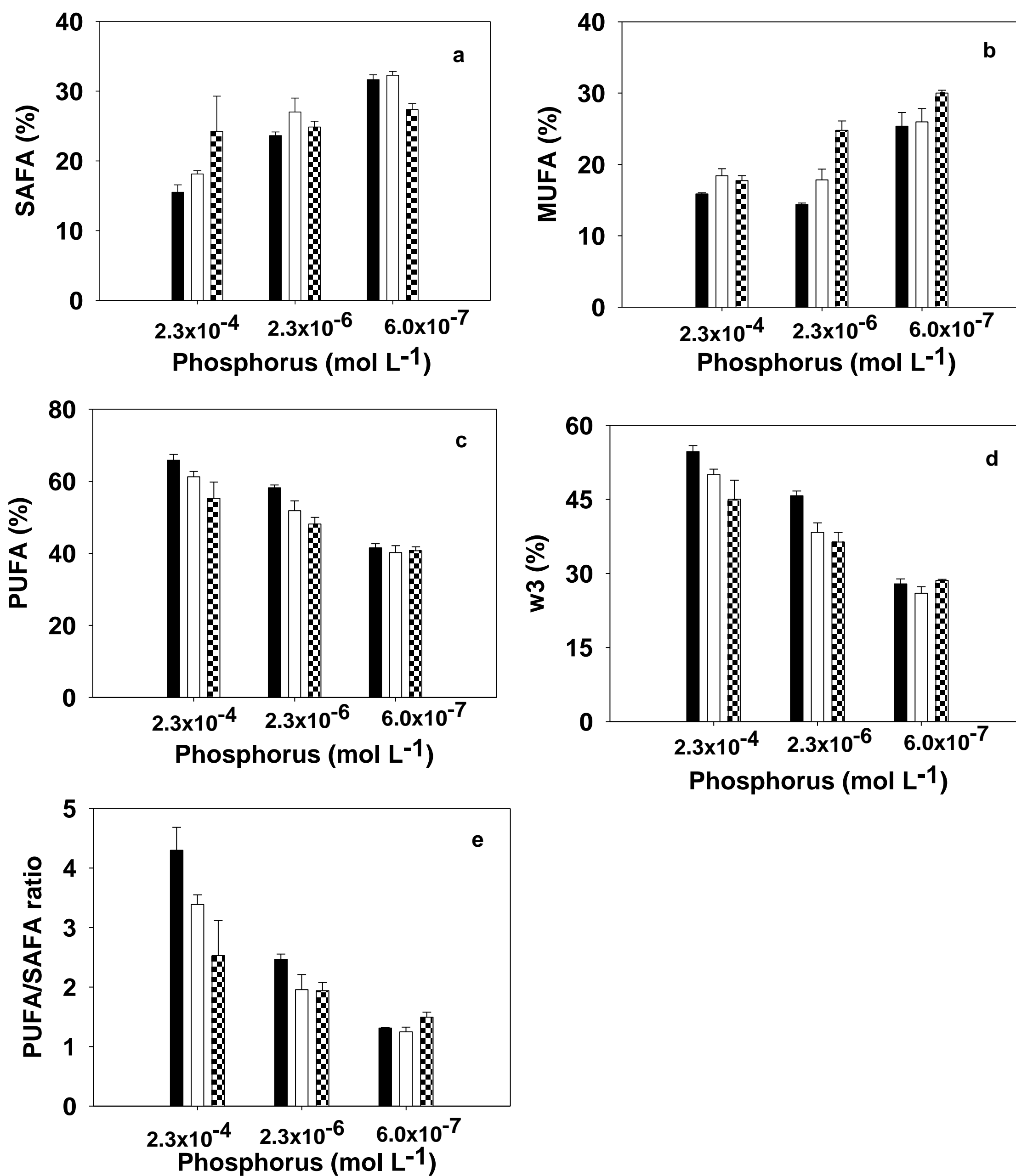

FIGURE 3.12: The production of total SAFA, MUFA, PUFA, ω3 and PUFA/SAFA ratios of *Chlorella vulgaris* as a function of different P and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

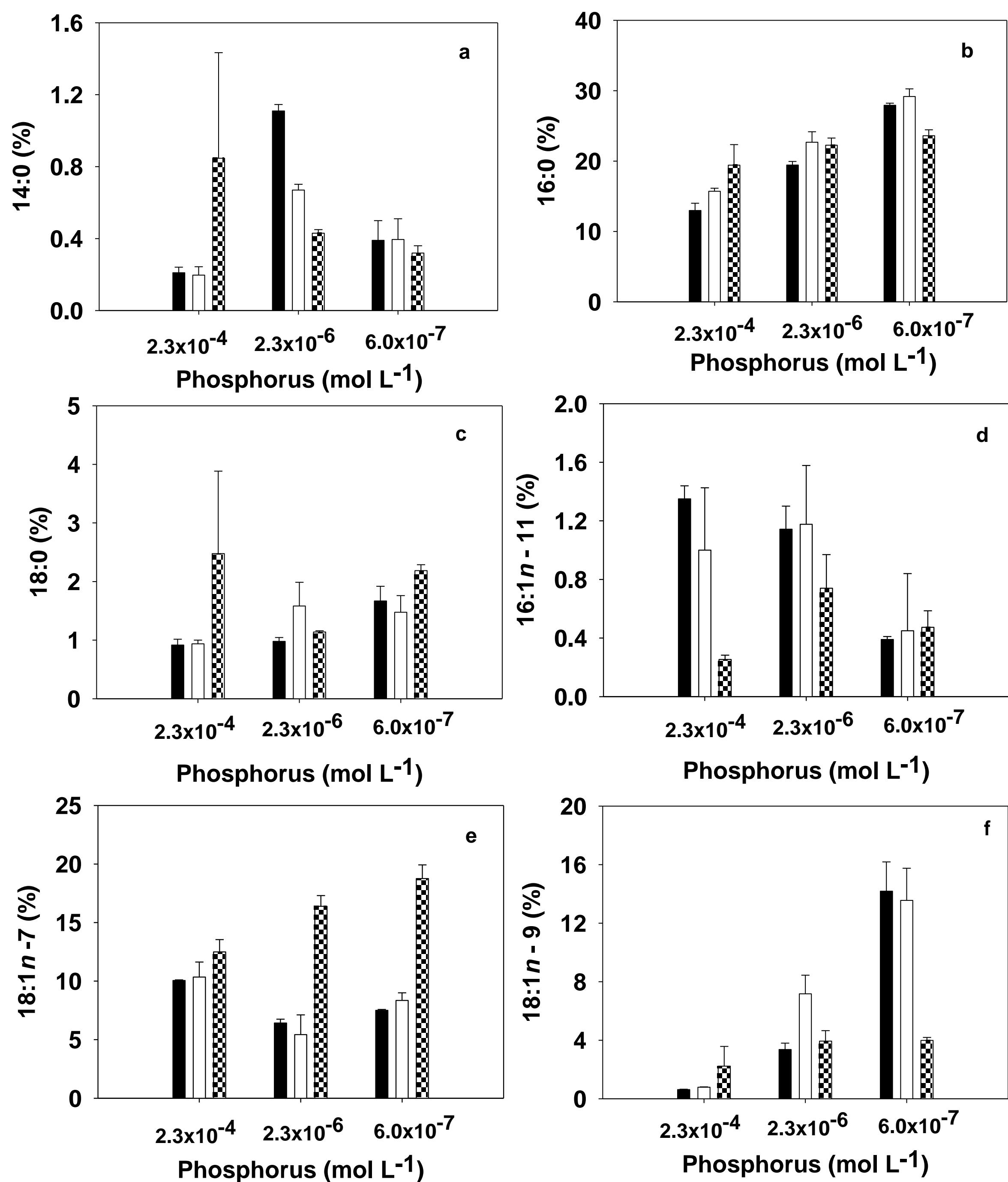

FIGURE 3.13: Saturated fatty acids (SAFA) and monounsaturated fatty acids (MUFA) for *Chlorella vulgaris* exposed to different P and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

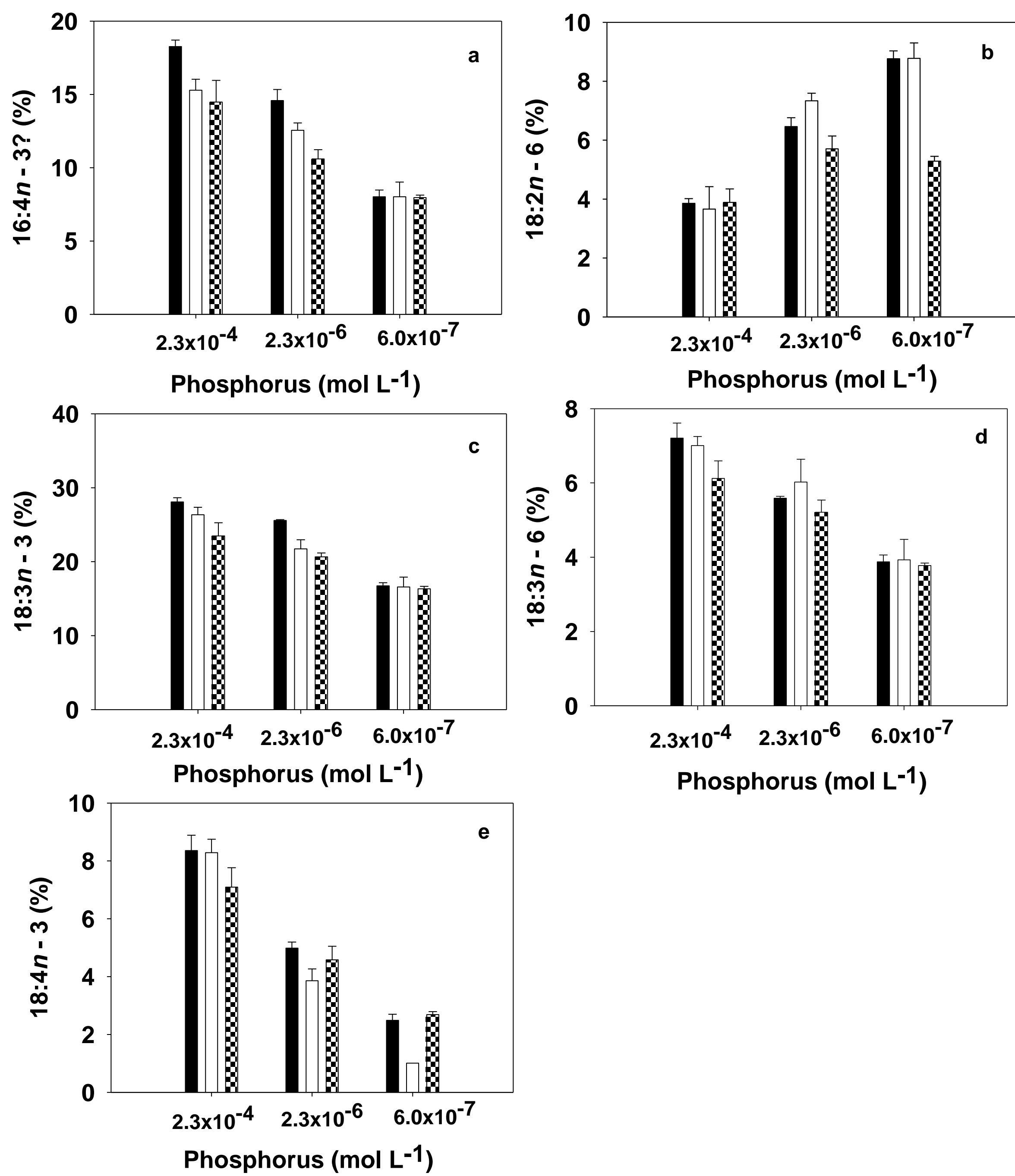

FIGURE 3.14: Polyunsaturated fatty acids (PUFA) composition of *Chlorella vulgaris* as a function of different P and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

TABLE 3.7: Analysis of variance results for the effect of phosphorus and cadmium on the lipid composition of *Chlorella vulgaris*. F values are reported and the values in parenthesis represent P values. Note: where $P \leq 0.05$, F value is significant.

	ANOVA		
Parameter	P	Cd	P/Cd
HC	4.03(0.04)	0.73(0.49)	4.16(0.01)
TAG	88.77(0.00)	2.18(0.14)	8.12(0.00)
ALC	2.93(0.08)	3.37(0.06)	2.37(0.09)
ST	7.56(0.00)	1.28(0.30)	2.95(0.05)
AMPL	50.57(0.00)	7.97(0.00)	2.91(0.05)
PL	6.18(0.01)	0.73(0.50)	1.49(0.25)
14:0	2.77(0.09)	0.44(0.65)	2.09(0.13)
16:0	44.11(0.00)	2.26(0.13)	6.60(0.00)
16:1n – 11?	7.50(0.00)	0.91(0.41)	0.49(0.74)
16:4n – 3?	45.65(0.00)	7.06(0.01)	1.78(0.18)
18:0	0.60(0.60)	1.76(0.20)	1.00(0.44)
18:1n – 9	71.46(0.00)	7.93(0.00)	11.70(0.00)
18:1n – 7	1.71(0.21)	16.91(0.00)	2.97(0.05)
18:2n – 6	30.85(0.00)	1.90(0.18)	1.68(0.20)
18:3n – 6	28.23(0.00)	1.72(0.21)	1.83(0.17)
18:3n – 3	18.90(0.00)	0.53(0.60)	2.54(0.08)
18:4n – 3	42.03(0.00)	1.02(0.38)	2.80(0.06)
SAFA	23.51(0.00)	1.21(0.32)	4.59(0.01)
MUFA	19.21(0.00)	6.94(0.01)	1.21(0.34)
PUFA	32.72(0.00)	5.01(0.02)	2.85(0.05)
ω3	40.97(0.00)	1.97(0.17)	3.31(0.03)
PUFA/SAFA	34.57(0.00)	5.00(0.02)	4.67(0.01)

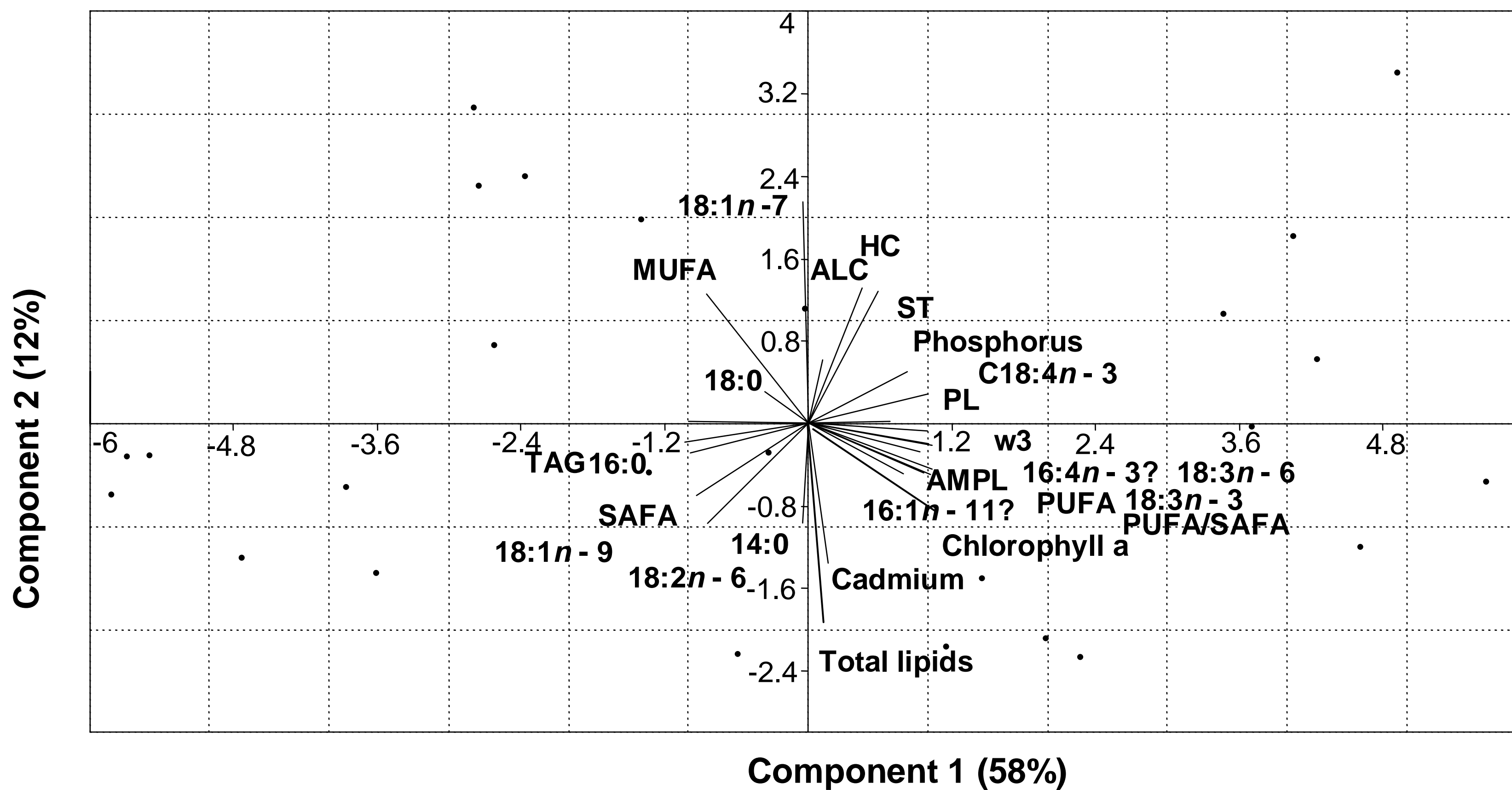

FIGURE 3.15: PCA biplot for lipid composition of *Chlorella vulgaris* at different P and Cd concentrations.

Discussion: The accumulation of total lipids in *C. vulgaris* under the stress conditions we studied can be traced to the increased proportion of neutral lipids, which corresponded to ~ 62% of total lipids in the newly assimilated carbon. This agrees with the results of LOMBARDI and WANGERSKY (1991), HARWOOD and GUSHINA (2009), BHOLA et al. (2011) for microalgae under P stress. The addition of Cd further stimulated lipid accumulation, which confirms literature results that with a combination of stress factors acting simultaneously, it is possible to stimulate higher total lipid production per cell than a single factor alone (GARDNER et al., 2011; PAL et al., 2011).

Aliphatic hydrocarbon, FFA, ALC and ST are neutral lipids that can be found in algae in quantities generally <5% even under environmental stress conditions (LOMBARDI and WANGERSKY, 1991; GUSHINA and HARWOOD, 2006b). The increase in TAG content under P limitation and Cd stress can be interpreted in the context of significant interactions between P and Cd, which agrees with the results of GUSHINA and HARWOOD (2006b) for *Coccomyxa* spp. under P limitation, Cu and Pb stress. The strong negative relationship between TAG and PL implies a mechanism that would lead to the accumulation of TAGs in P limited cells, in which PL synthesis is reduced. As PL and TAG have common precursors in their biosynthetic pathway, the common intermediates are likely to be channeled towards TAG synthesis if PL synthesis is hindered (PARRISH and WANGERSKY, 1987; HU et al., 2008). HU et al. (2008) report that TAGs may account for as much as 80% of total lipids in cells under conditions of nutrient stress. We obtained TAG values of up to 66% for *C. vulgaris* at the lowest P concentration tested. Acetone mobile polar lipids constitute a principal lipid component of *C. vulgaris* under normal unstressed growth condition, making up more than 35% of total lipids. The positive correlation observed between chlorophyll *a* and AMPL supports the fact that between 35 to 50% of this lipid class may contain chlorophylls and other pigments (PARRISH and WANGERSKY, 1987; LOMBARDI and WANGERSKY, 1991; HU, et al. 2008).

The dominance of C16 and C18 fatty acids *C. vulgaris* is in accordance with other literature results for green algae (BRETT et al., 2006; PETKOV and GARCIA, 2007; VILLAR-ARGAIZ et al., 2009; GRIFFITH et al., 2011). In agreement with our study, KHOZIN-GOLDBERG and COHEN (2006) showed that in *Monodus subterraneus,* the proportion of 16:0, 18:0 and 18:1 increased significantly under P limited growth. According to GUSHINA and HARWOOD (2009), and SPIJKERMAN and WACKER (2011) even though photosynthetic rates may reduce during P limitation, carbon accumulation takes place and is stored in the form of TAGs that are rich in SAFA and MUFA. Consistent with the results in Pinto et al. (2011), Cd, like other trace metals, caused significant increases in SAFA and MUFA content in the microalgae, which can be related to oxidative stress and the production of reactive oxygen species (PINTO et al., 2003; ROCCHETTA et al., 2006; PINTO et al., 2011). MCLARNON-RICHES et al. (1998) and DE SCHAMPHELAERE et al. (2007) suggested that this increase in the relative proportions of oleate may arise from a decrease in acyl-ACP transacylase activity that would slow fatty acid transfer into complex lipids, thus enhancing chain elongation of palmitate to stearate, and subsequent desaturation to oleate in the presence of trace metals. The significant association between TAG and most MUFAs and SAFAs can be related to the presence of these fatty acid groups in TAG composition, except for some species that have TAG with relevant proportions of PUFA (SPIJKERMAN and WACKER, 2011; REZANKA et al., 2011).

In this research, we showed that sufficient P concentration supported higher ω3 PUFA and total PUFA content in *C. vulgaris*, which means that the degree of saturation can be used to monitor the effect of P limitation and Cd stress on this microalga. Our findings are in agreement with those of VILLAR-ARGAIZ et al. (2009), and SPIJKERMAN and WACKER (2011) that showed ω3-PUFA increase in green algae under P sufficient conditions. The increased PUFA content can be explained by the synthesis of C_{18} PUFAs. A description by LEU et al. (2006) highlights that the pathway for the biosynthesis of C_{18} PUFAs starts at C18:0. Healthy P concentration triggers the

activation of this biosynthetic pathway towards the formation of 18C PUFAs (after the insertion of double bonds) at the expense of the precursor 18:0 (LEU et al., 2006; VILLAR-ARGAIZ et al., 2009). An exception to the above variations in PUFA composition as a function of the stress conditions tested was the 18:2n – 6, which increased with increasing P limitation and Cd stress. Its significant positive association with Cd and negative relationship with P means that it can serve as important stress indicator or fatty acid biomarker in this microalga. A similar behavior has been recorded in *Nannochloropsis salina* grown under high nickel concentration, where the proportion of this fatty acid was doubled from 6 to 12% (MOHAMMADY and FATHY, 2007). Similar behavior was also obtained for *Acer pseudoplatanus* grown under sub-lethal copper exposure (PÁDUA et al., 2003).

Conclusions can be drawn that P limitation in combination with Cd stress significantly affects the lipid composition of *C. vulgaris*. TAG concentrations were further increased by the combination of P limitation and Cd stress. MUFA and SAFA content increased under P limitation and the presence of Cd, whereas PUFA content decreased under these stress conditions.

3.5 Effect of cadmium and nitrogen on the lipid composition of *Chlorella vulgaris*

Results: Chlorophyll a concentrations decreased with decreasing N in the cultures, and Cd has a less significant effect than N starvation for the synthesis of chlorophyll a. However, Cd had a more significant effect on lipid synthesis than did N, which made the percent contribution of chlorophyll a in relation to total lipids lower than in the control. These results are presented in TABLE 3.8.

Lipid class composition is shown in FIGURE 3.16. Triacylglycerols, ALC, ST and AMPL were the lipid classes related either to N depletion or Cd stress, or both in combination. For the other classes (FFA, HC and PL), no clear tendency was obtained. Triacylglycerol concentrations increased with increasing N limitation, but within this trend it decreased with Cd increase in the cultures. A contrary behavior was obtained for free aliphatic alcohol concentrations, it increased with increasing N and Cd concentration in the cultures. Acetone mobile polar lipids concentrations decreased with N decrease (FIGURE 3.16e), which is possibly related to the effect of N in the chlorophyll *a* synthesis.

Table 3.8: Chlorophyll *a* concentration (pg cell^{-1}), percentage chlorophyll *a* in relation to total lipids and total lipids (pg cell^{-1}) of *Chlorella vulgaris* as a function of different Cd and N concentrations (mol L^{-1}). Values are plus or minus standard deviation for *n = 3*

Nitrogen	Cadmium								
	Without Cd			2.0×10^{-8}			1.0×10^{-7}		
	Chla	Chla %	TL	Chla	Chla %	TL	Chla	Chla %	TL
1.1×10^{-3}	0.27±0.02	35	0.80±0.08	0.44±0.03	28	1.57±0.09	0.40±0.05	24	1.70±0.04
1.1×10^{-5}	0.13±0.01	6	2.74±0.85	0.25±0.042	13	2.03±0.22	0.42±0.02	12	3.65±0.68
2.9×10^{-6}	0.13±0.00	4	3.41±0.36	0.10±0.03	3	4.01±1.86	0.63±0.08	6	9.85±0.64

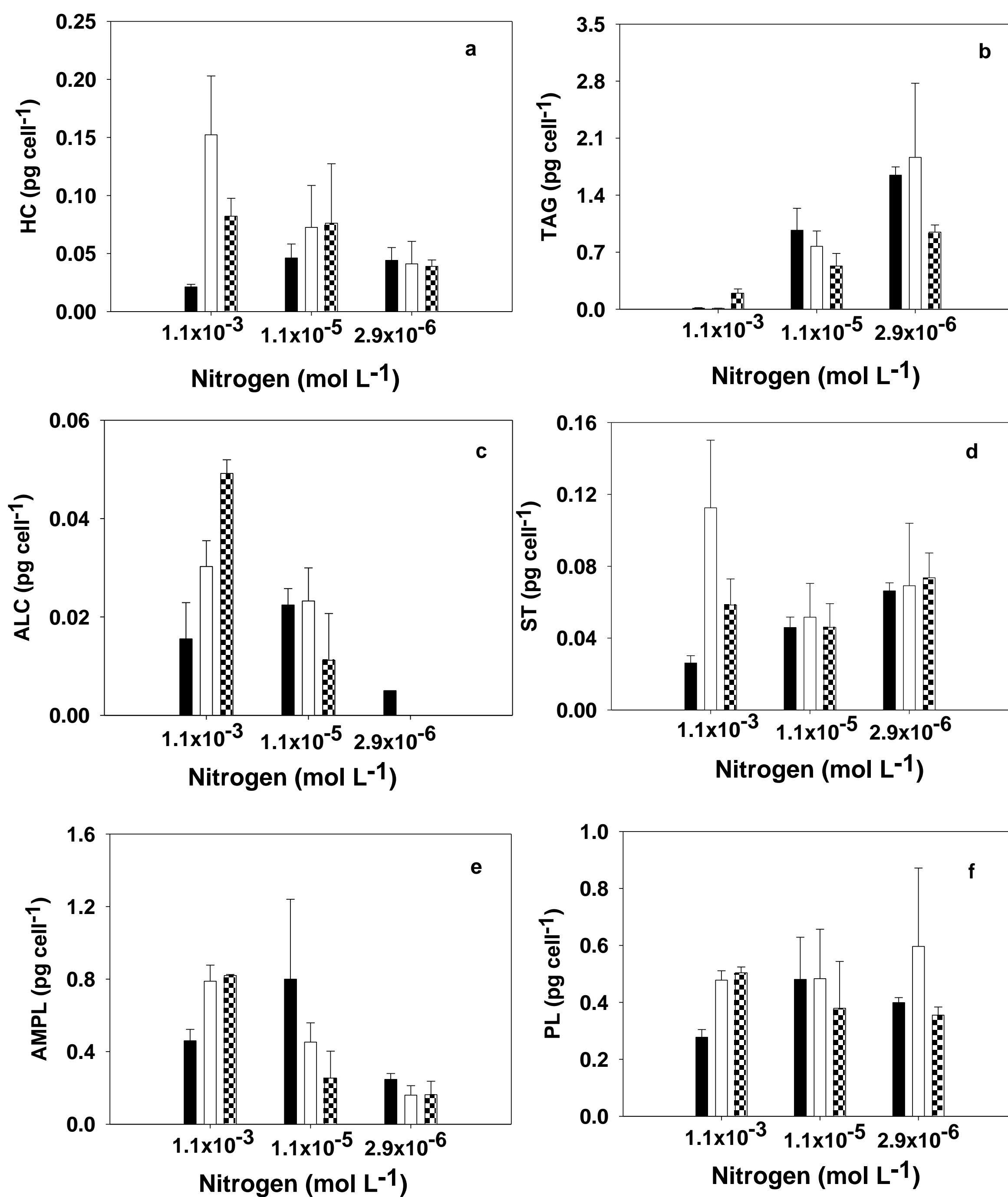

FIGURE 3.16: Lipid class composition of *Chlorella vulgaris* at different N and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

The accumulation of total saturated fatty acids (SAFA) and monounsaturated fatty acids (MUFA) are reported in FIGURE 3.17. It shows that both SAFA and MUFA increased under N limitation, but no difference within Cd concentrations were detected (ANOVA, $p>0.05$). Contrary to the results for SAFA and MUFA, total polyunsaturated fatty acids (PUFA), ω3 PUFA and PUFA/SAFA ratios decreased under N limitation and Cd stress (FIGURE 3.17 – ANOVA, $p<0.05$).

FIGURE 3.18 shows that Cd stimulated higher production of SAFA and MUFA. The 18:1n – 9 and 18:1n – 7 fatty acids concentrations were lower in the control than in N limited cultures. The combination of N limitation and Cd stress resulted in higher production of 14:0, 18:0 and 18:1n – 7 fatty acids, but not 16:1n – 11, which was higher under high N and sub-lethal Cd, an opposite behavior in relation to the production of the other MUFAs. FIGURE 3.19 shows that most of the PUFA members decreased under N limitation and Cd stress. However, the accumulation of 18:2n – 6 was stimulated under N limitation.

PCA analysis showed that the first two components were responsible for 72% of the total data variation (FIGURE 3.20). The concentrations of ST, HC, PL, ALC, AMPL and PUFA presented significant positive correlation with N, whereas TAG, MUFA, SAFA, 18:1n – 9, 16:0, 18:2n – 6 and 18:1n – 7 were negatively correlated with N. Cadmium was positively correlated with 18:2n – 6 and 18:1n – 7, while it showed negative correlations with PL, HC, ST, 14:0 and 18:0.

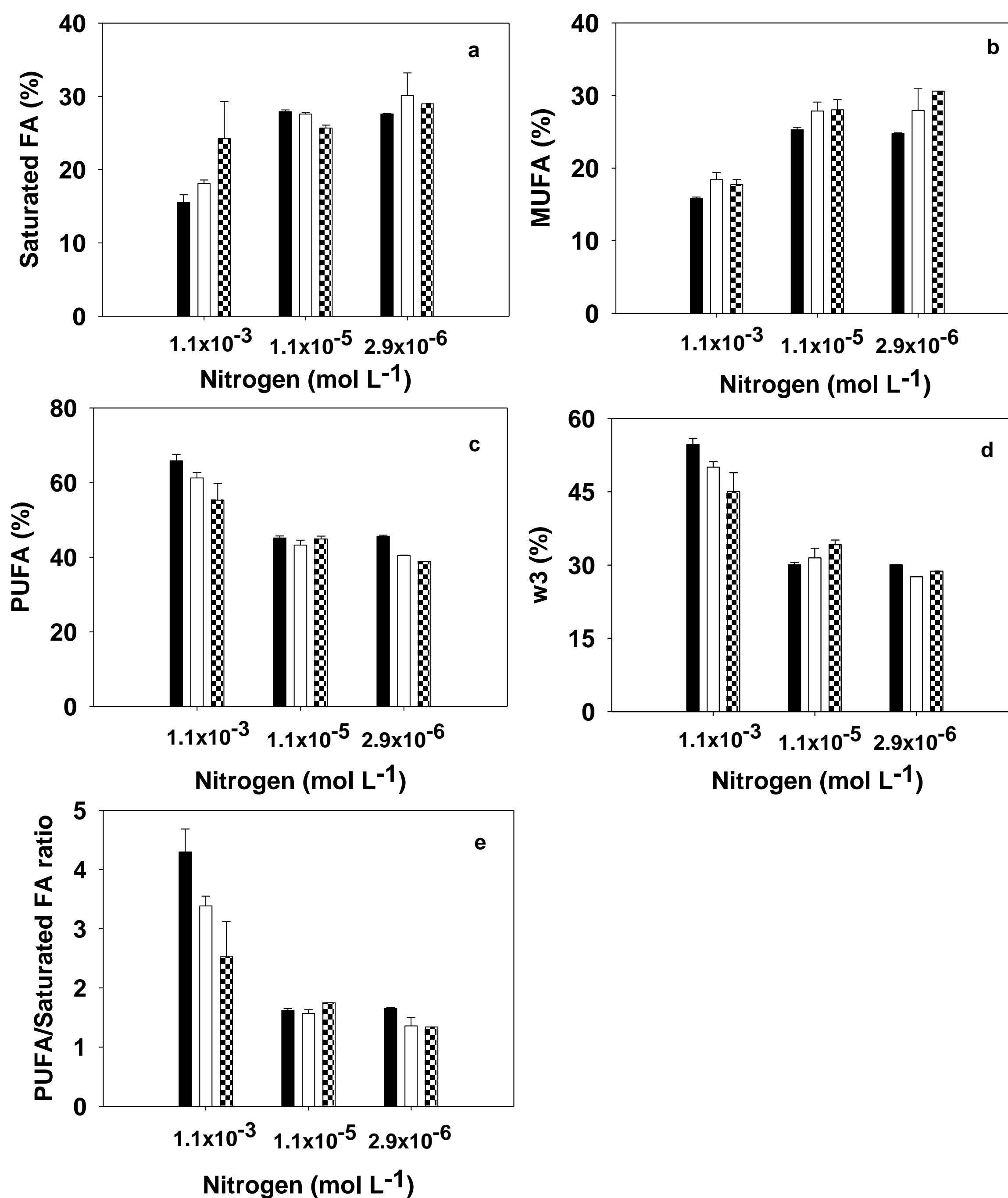

FIGURE 3.17: The production of total SAFA, MUFA, PUFA, ω3 and PUFA/SAFA ratios of *Chlorella vulgaris* as a function of different N and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

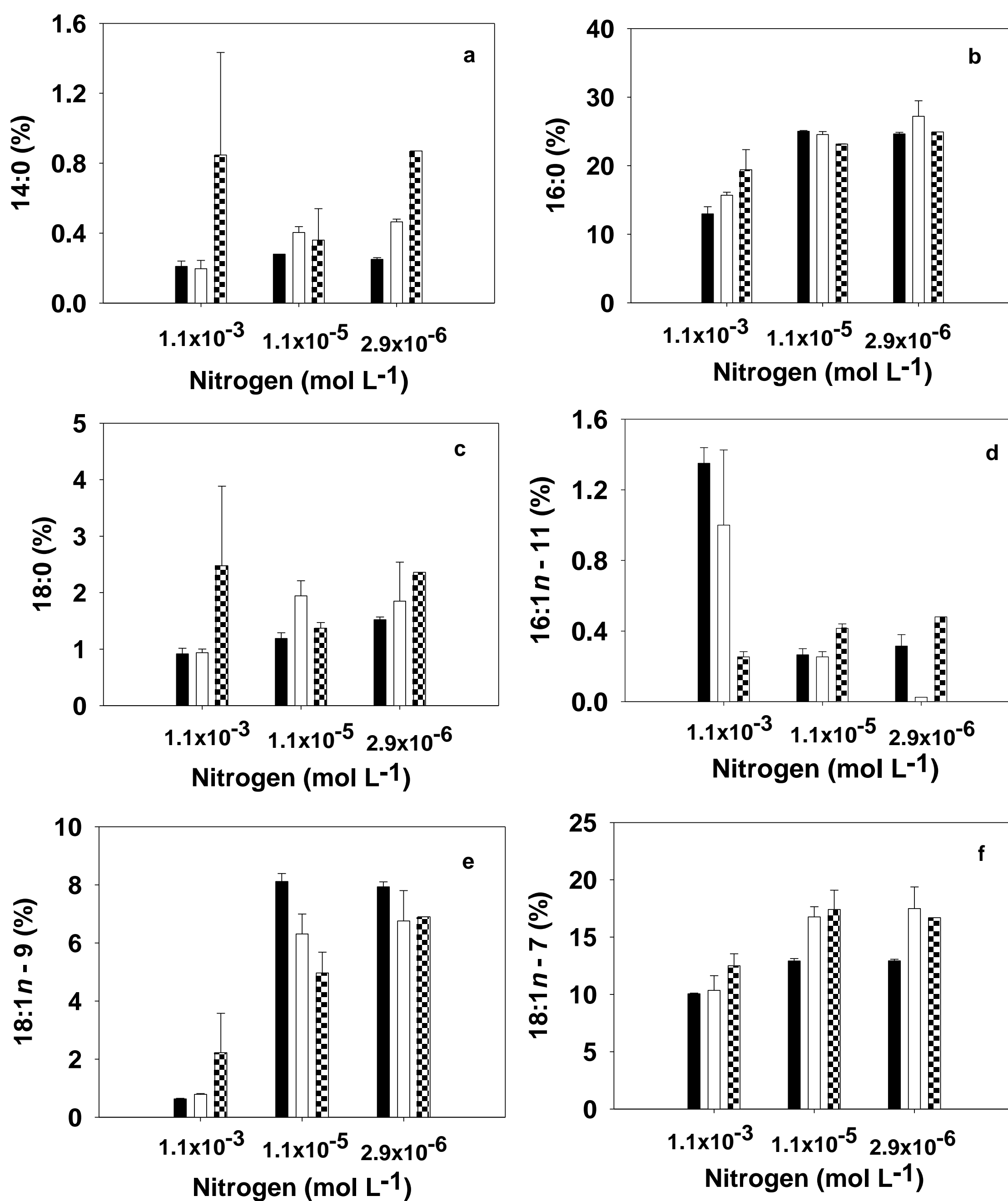

FIGURE 3.18: Saturated fatty acids (SAFA) and mono-unsaturated fatty acids (MUFA) composition of *Chlorella vulgaris* exposed to different N and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

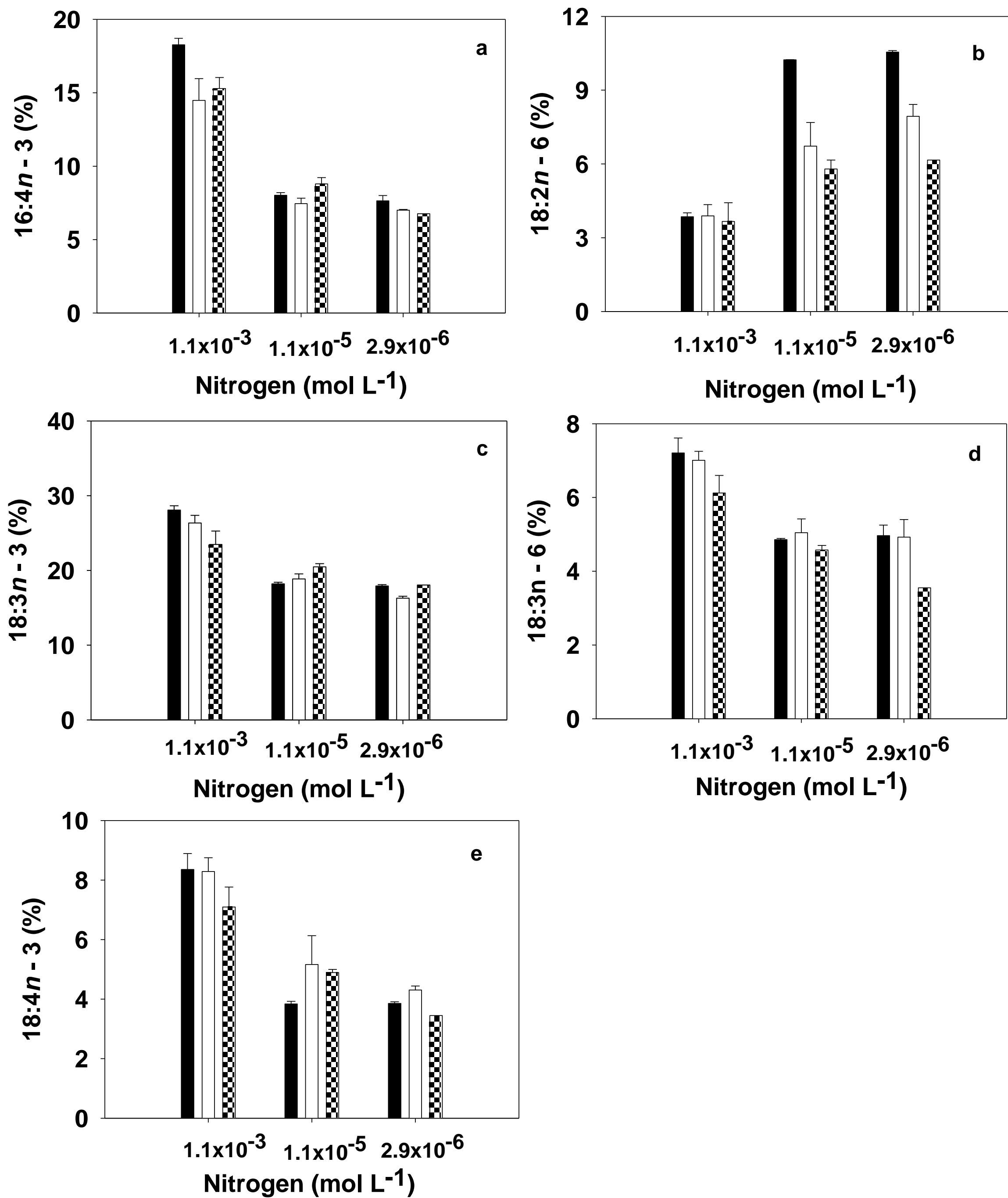

FIGURE 3.19: Poly-unsaturated fatty acids (PUFA) composition of *Chlorella vulgaris* as a function of different N and Cd concentrations. Black bars represent the controls, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Error bars represent standard deviation for $n = 3$.

TABLE 3.9: Analysis of variance results for the effect of nitrogen and cadmium on the lipid composition of *Chlorella vulgaris.* Values represent F values and those in parenthesis represent P values. Note: F values with $P \leq 0.05$ are significant.

	ANOVA		
Parameter	N	Cd	N/Cd
HC	4.32(0.03)	2.23(0.14)	1.87(0.16)
TAG	43.05(0.00)	0.08(0.93)	0.43(0.79)
ALC	29.60(0.00)	0.23(0.79)	1.63(0.21)
ST	2.58(0.10)	1.34(0.29)	2.35(0.09)
AMPL	66.84(0.00)	2.18(0.14)	0.62(0.65)
PL	6.36(0.01)	0.24(0.79)	0.76(0.57)
14:0	0.51(0.61)	4.29(0.03)	0.87(0.50)
16:0	56.41(0.00)	1.58(0.23)	3.80(0.02)
16:1n – 11?	20.42(0.00)	2.37(0.12)	0.89(0.49)
16:4n – 3?	192.55(0.00)	5.98(0.01)	3.11(0.04)
18:0	0.54(0.59)	2.17(0.14)	1.19(0.35)
18:1n – 9	16.63(0.00)	0.62(0.55)	0.37(0.82)
18:1n – 7	4.16(0.03)	2.76(0.09)	0.47(0.76)
18:2n – 6	83.26(0.00)	31.88(0.00)	8.37(0.00)
18:3n – 6	56.20(0.00)	11.14(0.00)	0.85(0.51)
18:3n – 3	13.64(0.00)	1.19(0.33)	0.62(0.65)
18:4n – 3	62.82(0.00)	2.68(0.10)	1.25(0.32)
Saturated FA	21.03(0.00)	1.32(0.29)	2.61(0.07)
MUFA	101.67(0.00)	9.67(0.00)	1.45(0.26)
PUFA	105.88(0.00)	8.64(0.00)	2.63(0.07)
ω3	49.64(0.00)	1.95(0.17)	0.79(0.55)
PUFA/Saturated FA	57.19(0.00)	5.23(0.02)	4.27(0.01)

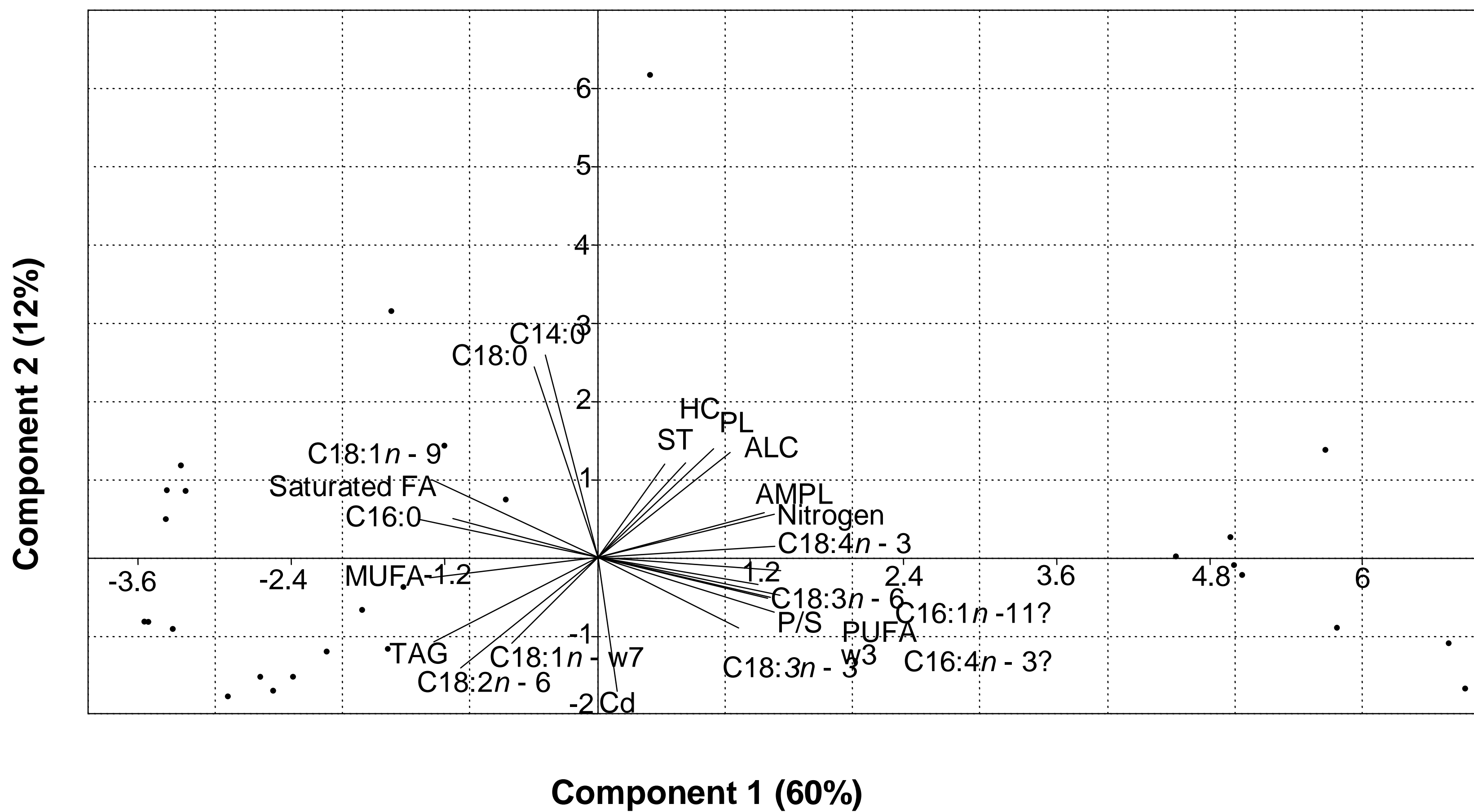

FIGURE 3.20: PCA biplot for lipid composition of *Chlorella vulgaris* at different Nand Cd concentrations.

Discussion: The increased total lipid production per cell we obtained for *Chlorella vulgaris* under N limitation and Cd stress is consistent with other investigations (EINICKER-LAMAS et al., 1996; GUSHINA and HARWOOD, 2006a; HARWOOD and GUSHINA, 2009; BHOLA et al., 2011). These authors showed increased lipid production under either nutrient limitation or Cd stress.

The 5% contribution of the HC, ALC and ST lipid classes to the total lipids we obtained agrees with other literature results for microalgae in general (LOMBARDI and WANGERSKY, 1991; GUSHINA and HARWOOD, 2006b). The accumulation of TAG in response to N limitation has also been observed in different algae species (PARRISH and WANGERSKY, 1987; LOMBARDI and WANGERSKY, 1991; BASOVA, 2005; MERZLYAK et al., 2007; GARDNER et al., 2011). However the neutral lipid class TAG decreased with increasing Cd. This is in agreement with the results of PAL et al. (2011), who demonstrated that combinations of stress factors can, under certain circumstances, be detrimental to neutral lipid production by *Nannochlropsis* sp. However, according to SOLOVCHENKO et al. (2010) the increase in neutral lipids under N limitation, predominantly TAGs, plays a protective role and renders microalgae less prone to oxidative damage. So, it would be expected that Cd stress would induce TAG synthesis, which in fact did not occur with *C. vulgaris*.

The inverse relationship we obtained between TAG and PL has been reported previously and suggested to be due to changes in *de novo* synthesis of both lipid classes, which results from modifications of their metabolic pathways towards the formation and accumulation of neutral lipids via the sequestering of *de novo*-produced fatty acid moieties in the form of TAG (PAL et al., 2011).

The significant positive relationship between AMPL and chlorophyll *a* is associated with the fact that a significant percentile (~50%) of this lipid class is usually constituted of microalgae pigments (PARRISH and WANGERSKY, 1987; LOMBARDI and WANGERSKY, 1991; HU et al., 2008).

Fatty acid production by *C. vulgaris* exhibited a high degree of plasticity with changing culture conditions. Most fatty acids were constituted by medium chain length groups of 16 and 18 carbon atoms, which is in accordance to literature data (BRETT et al., 2006; PETKOV and GARCIA, 2007; VILLAR-ARGAIZ et al., 2009). The increased SAFA and MUFA production by *C. vulgaris* in the presence of Cd we detected agrees with the results of PINTO et al. (2011), who showed that SAFA and MUFA content of *Gracilaria tenuistipitata* increased with increasing Cd concentration. The accumulation of these fatty acids groups is related to oxidative stress and the production of reactive oxygen species (PINTO et al., 2003; ROCCHETTA et al., 2006; PINTO et al., 2011). In our experiments the fatty acid tentatively identified as $16{:}1n - 11$ did not increase with N depletion and/or Cd stress, instead it decreased with increasing either stress condition. So, we suggest that the $16{:}1n - 11$ fatty acid can be considered a stress biomarker. The significant association of most monounsaturated and saturated fatty acids with TAGs is in accordance with literature data, which show that these fatty acids groups are the main contributors to TAG in chlorophytes (SPIJKERMAN and WACKER, 2011; REZANKA et al., 2011).

The PUFA/SAFA ratios are an important physiological index of stress as they indicate the degree of fatty acid saturation (MENDOZA GUZMÁN et al., 2011). The higher ω3 and PUFA content in *C. vulgaris* we obtained in N replete than in N limited cultures agrees with the results of GRIFFITH et al. (2011). These authors reported 50% higher PUFA in N replete cultures which is in agreement with the 40% we obtained for N replete conditions. MENDOZA GUZMÁN et al. (2011) reported that variations in MUFA and PUFA content especially the increase of 18:1 can be a response to N starvation and activation strategies. According to these authors, the thylakoid membranes play a major role in the ω3 PUFA synthesis using 18:1 as its pathway starting point. In addition, CHOI et al. (2011) showed a 2.6 fold increase in the expression of the stearoyl-AC desaturase gene (*sad*) encoding a stearoly-ACP desaturase involved in the synthesis of oleic acid by *Botryococcus braunii,* under N limitation than under N-sufficient conditions. Their findings can be used to explain the MUFA increase

and PUFA decrease in cultures under N limitation in our study. Also in accordance with the results of other studies (ROCCHETTA et al., 2006; PINTO et al., 2011), PUFA contents are significantly affected and their levels reduced in the presence of trace metals. We suggest that the fatty acid 18:2n – 6 can be used as a nutrient stress indicator in *C. vulgaris* since its concentration increased under N limitation and Cd stress unlike what is observed for other PUFAs. The significant association of individual PUFAs and total PUFA with PL and AMPL is because these lipid classes are principally made up of this fatty acid group (HILL et al., 2011).

We conclude that N limitation and Cd stress significantly affected the lipid composition of *C. vulgaris*. TAG concentrations were accumulated under N limitation, while increasing Cd concentration decreased the TAG content. MUFA and SAFA content increased under N limitation and the presence of Cd, whereas the trend of PUFA content under these conditions was reversed.

4. GENERAL CONCLUSION

The overall yield of *C. vulgaris* grown in WC medium for most parameters measured was the lowest among the treatments; the highest cell density supported by the LC Oligo medium in relation to Chu 10 and WC resulted in overall higher system productivity in LC Oligo cultures. This confirms that LC Oligo can be a cost effective alternative to the other media due to the absence of vitamins and EDTA.

Using semi-continuous cultures improves the calorific values of *C. vulgaris* under varying phosphorus concentrations. High concentrations of biochemical parameters such as carbohydrate and total lipid, and biomass (dry weight) concentrations significantly positively correlate with high calorific values of this microalga. Increasing the severity of phosphorus limitation retards growth and biomass production of *C. vulgaris* and it accumulates high concentrations of storage lipids (TAGs) over structural lipids. The best calorific values with less growth retardation were obtained at the 10^{-6} mol L^{-1} phosphorus concentration.

Significant effects on *C. vulgaris* growth, biomass production and biochemical composition were observed with varying N, P and Cd in culture media. Decrease in growth rate and biomass were a general trend with decreasing N or P concentrations, which were further decreased with Cd addition. Healthy cells, grown at N and P replete concentrations turned less severe the toxic effects of Cd. Total intracellular carbohydrates and lipids accumulated under low N or P concentrations. With Cd addition at limiting P or N, higher accumulation of such molecules was obtained. Phosphorus limitation leads to higher total lipid concentration than N limitation. Protein productivity decreased with decreasing N and P concentration.

The results of this study showed that the combination of Cd stress and P limitation affect lipid composition in *Chlorella vulgaris.* Under such conditions, a higher production of TAG compared to the other lipid classes was obtained. Saturated fatty acids and MUFA concentrations increased by lowering P and increasing Cd concentrations in culture media. Polyunsaturated fatty acids levels

and PUFA/SAFA ratios increased with increasing P concentrations without Cd, but at increasing P limitation and Cd stress, the polyunsaturated lipid 18:2n – 6 increased.

Nitrogen had a more significant effect on the total lipid production, lipid class and fatty acid composition of *C. vulgaris* than did Cd. Under N limitation, Cd did not have any significant effect on fatty acid composition. Triacylglycerols were most accumulated under N stress, but the presence and increasing concentrations of Cd caused the TAG levels to decrease. Acetone mobile polar lipids and PL percentages in relation to total lipids decreased with increasing N limitation. The degree of fatty acid unsaturation increased with increasing N concentration in the medium, which was demonstrated by the individual PUFAs, ω3 and PUFA/SAFA levels in the different treatments. However, the behavior of C16:1n – 11 and 18:2n – 6 exhibited tendencies that were different to general trends shown by the MUFAs and PUFAs, respectively. Hence, they have the potential to be used as indices or biomarkers of nutrient levels with respect to the physiology of *C. vulgaris*. Our study has shown that *C. vulgaris* lipid composition is more controlled by the N than Cd under N limitation.

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