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Combined nitrogen limitation and cadmium stress stimulate total carbohydrates, lipids, protein and amino acid accumulation in *Chlorella vulgaris* (Trebouxiophyceae)

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ABSTRACT

Metals have interactive effects on the uptake and metabolism of nutrients in microalgae. However, the effect of trace metal toxicity on amino acid composition of *Chlorella vulgaris* as a function of varying nitrogen concentrations is not known. In this research, *C. vulgaris* was used to investigate the influence of cadmium (10^{-7} and 2.0×10^{-8} mol L^{-1} Cd) under varying nitrogen (2.9×10^{-6}, 1.1×10^{-5} and 1.1×10^{-3} mol L^{-1} N) concentrations on its growth rate, biomass and biochemical composition. Total carbohydrates, total proteins, total lipids, as well as individual amino acid proportions were determined. The combination of Cd stress and N limitation significantly inhibited growth rate and cell density of *C. vulgaris*. However, increasing N limitation and Cd stress stimulated higher dry weight and chlorophyll *a* production per cell. Furthermore, biomolecules like total proteins, carbohydrates and lipids increased with increasing N limitation and Cd stress. Ketogenic and glucogenic amino acids were accumulated under the stress conditions investigated in the present study. Amino acids involved in metal chelation like proline, histidine and glutamine were significantly increased after exposure to combined Cd stress and N limitation. We conclude that N limitation and Cd stress affects the physiology of *C. vulgaris* by not only decreasing its growth but also stimulating biomolecule production.

1. Introduction

Some aquatic ecosystems naturally have high metal contents due to the nature of their bedrock (e.g., volcanic lakes: Lohr et al., 2006) or sediment material (e.g., through mining activities: Johnson, 1998; Chia et al., 2011), while others are exposed to metal pollution from point and non-point sources that threatens the biota (Bonet et al., 2014). Cadmium (Cd) is a toxic metal that negatively influences major metabolic processes in phytoplankton, such as photosynthesis, respiration, protein synthesis, nitrogen and carbohydrate metabolisms (Duong et al., 2010; Suárez et al., 2010; Monteiro et al., 2011; Chia et al., 2013a,b). As a metal, Cd is not biodegradable; accumulates in the environment, and is transferred through food webs (Chan et al., 2003). It has low affinity with naturally occurring dissolved organic materials, which increases its availability to microalgae as free Cd^{2+} ions or labile species (Gouvêa et al., 2005; Tonietto et al., 2014). Therefore, investigations that consider the interactions of Cd ions with other dissolved nutrients are of ecological significance for understanding the physiological responses of microalgae to multiple stress factors (Chia et al., 2013a).

Incidences of eutrophication in freshwater ecosystems worldwide justify the need to study metal-nitrogen interactions because of the influence they have on microalgal physiology. Nitrogen is one of the major nutrients needed for algal growth, and variations in its availability controls phytoplankton community structures in aquatic ecosystems (Reynolds 2006; Chia et al., 2011). In response to nitrogen variability in the aquatic environment, microalgal cells undergo a series of metabolic acclimations, which often result in changes in cellular biomolecules composition (Illman et al., 2000; Gushina and Harwood, 2006; Widjaja et al., 2009; Bhola et al., 2011; Chia et al., 2013a). These changes are required by the microalgae to

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correctly adapt and survive the effect of nitrogen limitation, depletion or starvation at the expense of cell division (Bono et al., 2013).

The presence of metals like Cd can affect nitrogen availability and uptake by microalgae in water (Awasthi and Rai, 2005; Shun-Xing et al., 2007; Sivakumar et al., 2010). Cd can interact with enzymes like RuBisCo, nitrogenase and nitrate reductase (Devriese et al., 2001) to replace essential nutrients that are needed for their normal functioning (Suárez et al., 2010). Available evidence show that microalgal sensitivity to different metals including Cd is partly controlled by the amount of nutrient available in the aquatic environment, which may have serious ecological implications (Farias et al., 2003; Serra et al., 2010; Li et al., 2012). The interaction of Cd and N qualitatively and quantitatively alters lipid class and fatty acid composition of microalgae (Chia et al., 2013a), which renders them less prone to oxidative damage caused by stress conditions (Mock and Kroon 2002; Gushina and Harwood, 2006; Solovchenko et al., 2009; Pinto et al., 2011; Bonet et al., 2014).

The survival and/or dominance of particular algal groups or species in Cd polluted aquatic ecosystems having different nitrogen concentrations is dependent on their adaptive capabilities, their ecophysiological specialization, which is controlled by their biochemical composition and the ecological advantages that may exist (Nixdorf et al., 1998; Cid et al., 2010). While studies on Cd and N are important, current knowledge of Cd tolerance, adaptability or physiological response have been restricted to single stress (Cd) factor studies (Suárez et al., 2010; Carfagna et al., 2013). In addition, most studies have only focused on the role of the amino acid proline in combating Cd and metal toxicity in general (Tripathi et al., 2006; Choudhary et al., 2007; Zhang et al., 2008; Çelekli et al., 2013), and lipid composition changes (Chia et al., 2013a,b), while the interactive effect of Cd and N on total carbohydrates, total proteins and amino acid composition of *Chlorella vulgaris* or any other freshwater microalgae to the best of our knowledge, is not known. Proline detoxifies free radicals by forming a stable complex with them, thereby maintaining NAD(P)+/NAD(P)H ratios during stress at values similar to normal conditions (Hare and Cress, 1997; Backor et al., 2004; Sharma and Dietz, 2006; Kovacik et al., 2010). However, in higher plants, the accumulation of some amino acids besides proline contributes to amelioration of the damaging effects of metal stress (Chaffei et al., 2004; Konotop et al., 2012). Furthermore, qualitative and quantitative variations in total lipids and carbohydrates are necessary as food storage materials, alternative energy sources and bulk raw materials required for structural modifications that are needed for successful adaptation to metal and other stress conditions (Hu et al., 2008; Chia et al., 2013c; Yilancioglu et al., 2014).

In order to contribute to the limited knowledge of the effect of nitrogen–Cd stress on the physiology of algae in natural metal enriched and polluted aquatic ecosystems, the objective of the present study was to investigate the effects of Cd at different N concentrations on growth, biomass production, total carbohydrate, total lipid, total protein and amino acid composition of *C. vulgaris*. This is important because microalgae encounter complex conditions with nutrient and toxic metal mixtures varying simultaneously, which can have different effects on their cell physiology and adaptation (Serra et al., 2010; Chia et al., 2011). Hence, we exposed *C. vulgaris* to Cd stress under varying nitrogen concentrations to observe how the combined conditions affect physiological endpoints like growth, biomass production and biochemical composition.

2. Materials and methods

2.1. Stock cultures

The *C. vulgaris* (012) strain used in the present study was obtained from the Culture Collection of the Botany Department, Federal University of São Carlos, Brazil. It is registered at the World Data Center for Microorganisms (WDCM) with the identification number 835. Cultures of the microalga were maintained in LC Oligo medium (AFNOR, 1980) under controlled conditions (light intensity, 150 μmol m^{-2} s^{-1}; light/dark cycle, 16:8 h; and temperature, 20 ± 2 °C). Prior to autoclaving, the growth medium pH was adjusted to 7.0 ± 0.2. Culture medium sterilization was performed 24 h before use by autoclaving at 121 °C for 20 min.

2.2. Cadmium, phosphorus and nitrogen

Nitrogen (N) in the form of KNO_3 was provided at 2.9×10^{-6}, 1.1×10^{-5} and 1.1×10^{-3} mol L^{-1} (control) concentrations. The concentrations represent limiting and replete environmentally relevant N concentrations recorded in aquatic ecosystems (Larned 1998; Reynolds 2006). The effects of N were investigated one at a time, and then in combination with Cd as $Cd(NO_3)_2$ at 2.0×10^{-8} and 1.0×10^{-7} mol L^{-1}. These Cd concentrations fall within the levels reported for aquatic ecosystems in different parts of the world (Simon et al., 2011; Mutia et al., 2012). The control had N at 1.1×10^{-3} mol L^{-1} without Cd addition. Approximately 98% of the added Cd remained as free Cd^{2+} ions in the cultures (Chia et al., 2013a), as obtained from calculations through the chemical equilibrium software MINEQL+ v4.62.3 (Environmental Research Software, Hallowell, ME, USA). In the determination of Cd speciation was done taking into consideration the culture conditions of pH, temperature, ionic strength and open system for gas exchange.

2.3. Experimental cultures

Semi-continuous cultures were performed using 1 L polycarbonate bottles (Nalgene, U.S.A.) containing 250 mL of culture medium. Before the beginning of the experiments, *C. vulgaris* was acclimated to specific N concentrations through consecutive culture transfers of exponentially growing cells from batch cultures. This ensured that *C. vulgaris* metabolism reflected the culture medium condition. Growth rates were measured for each new culture until 3 consecutive statistically similar growth rates were obtained. The semi-continuous cultures were inoculated with acclimated cells (~10^6 cells mL^{-1}), and culture medium removal and replacement were performed according to growth rates of the microalga under each nitrogen and cadmium treatment combination. This maintained the cell density within a known range throughout the experiments which lasted 16 days, after which, culture samples were taken for biochemical analyses and the experiment ended. Each treatment was carried out with three experimental replicates.

2.4. Growth and biomass determinations

Prior to daily dilutions of the semi-continuous cultures, aliquots were taken for biomass determinations through the measurement of optical density at 684 nm (HACH DR5000 spectrophotometer, USA). With these values, the specific growth rates (μ) were calculated using the equation proposed by Lombardi and Maldonado (2011).

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

Where

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

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

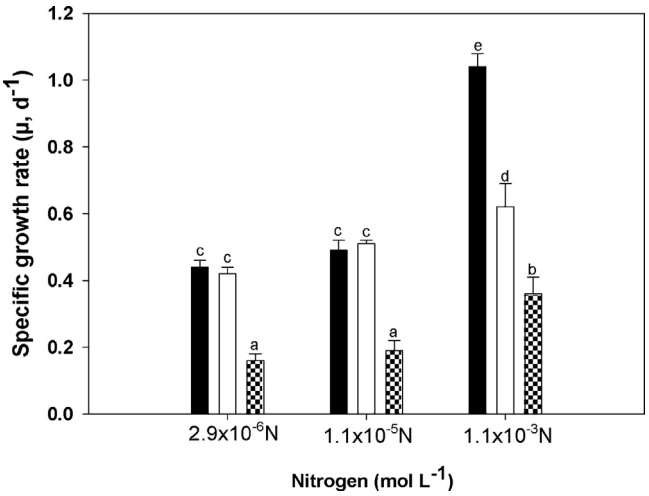

Fig. 1. Specific growth rate (μ, d^{-1}) of *Chlorella vulgaris* exposed to different Cd and N concentrations. Black bars represent treatments without Cd, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Bars with the same alphabets are not significantly different at $p < 0.05$. Error bars represent standard deviation for $n = 3$.

Cell densities were determined microscopically using an improved bright lined Neubauer haemocytometer. Algal dry weight was determined gravimetrically with a Sartorius MC21S analytical balance (Precision Weighing Balances, Bradford, MA), using previously dried (60 °C for 24 h) 0.45 μm pore size cellulose acetate membrane filters (Millipore, Brazil).

2.5. Chemical analyses

Chlorophyll *a* extraction and quantification were performed according to Shoaf and Lium (1976). Five milliliters of microalgal culture were filtered through Millipore cellulose acetate membrane filters (pore size 0.45 μm). The filters were subsequently dissolved in 3 mL of dimethyl sulfoxide (DMSO) and left in the dark at room temperature for 45 min with periodic shaking to ensure proper extraction. The absorbance was read at 664 and 630 nm using a HACH DR5000 spectrophotometer (Loveland, Co., USA). Chlorophyll *a* concentrations were computed using the equation described in Jeffrey and Humphrey (1975).

$$\text{Chl a } (\text{mg} \times \text{L}^{-1}) = (11.47 \times \text{OD}_{664}) - (0.4 \times \text{OD}_{630})\ x/y \quad (3)$$

Where *x* is the total volume of extraction solvent used and y represents the volume of culture filtered. OD is the optical density at 664 nm and 630 nm wavelengths.

Dissolved nitrogen (as nitrate) concentrations in culture media were determined according to APHA (1998). Amalgamated Cd at 0.6 g was added to a flask and then 10 mL of each sample added to it. Three milliliters 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 shaken for 20 min, after which 7 mL was removed and transferred to a test tube and 1 mL sulfanilamide added. The whole solution was mixed for 6 s by vortexing and 1 mL *n*-1-naphthyl ethylenediamine added. The optical density of the solution was determined at 543 nm using a UV–vis scanning spectrophotometer equipped with an ASC-5 Autosampler change (Shimadzu, Japan). Nitrogen calibration curves were made using previously dried KNO_3 for 1 h at 105 °C.

The phenol-sulfuric acid technique was used for total carbohydrate concentration determination with glucose as the standard (Liu et al., 1973). Five milliliters aliquots were taken from the cultures and centrifuged at 1500 rpm for 10 min using an Eppendorf centrifuge 5702 R (Eppendorf, AG, Hamburg) to pellet the cells. The pellets were re-suspended in 1 mL of distilled water and 1 mL phenol solution (10% w/v) was added to it. After thorough mixing, 5 mL

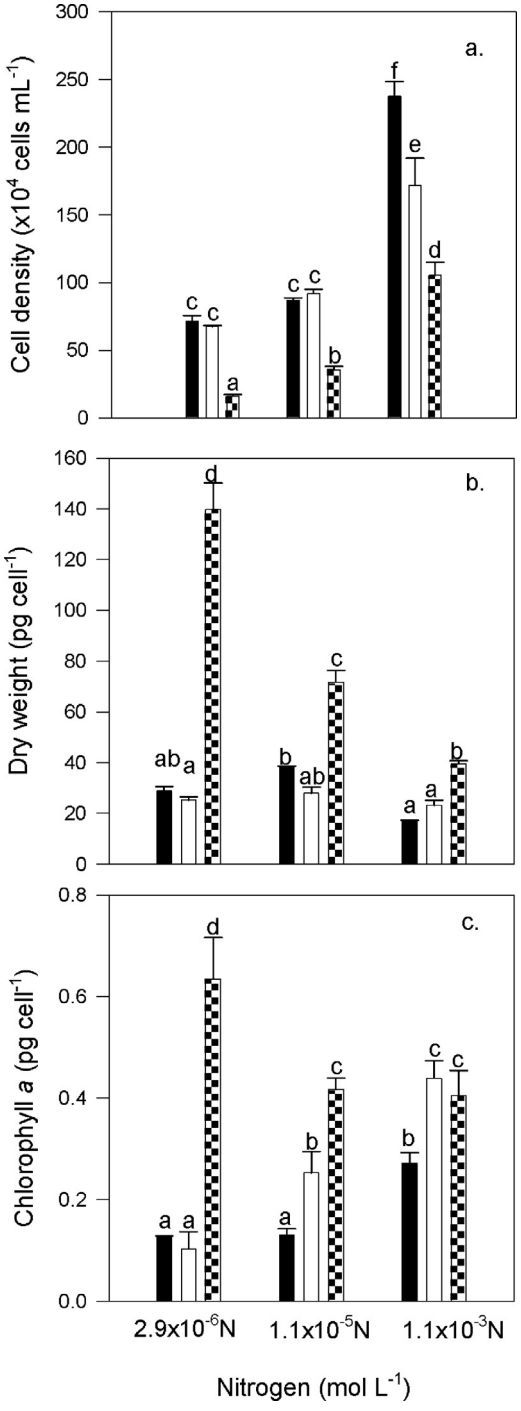

Fig. 2. Effect of Cd on cell density (a), dry weight (pg $cell^{-1}$) (b) and chlorophyll *a* (pg $cell^{-1}$) (c) at different N concentrations (mol L^{-1}). Black bars represent treatments without Cd, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Bars with the same alphabets are not significantly different at $p < 0.05$. Error bars represent standard deviation for $n = 3$.

of concentrated H_2SO_4 was quickly added, directing the flow to the liquid surface to obtain good mixing. The mixture was left to stand for 10 min at room temperature 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 to 150 μg mL^{-1}.

Total protein extraction was performed according to Rausch (1981). Five milliliters of algal culture was centrifuged at 1500 rpm and the pellet formed was re-suspended in 1.5 mL 0.5 M NaOH.

Extraction was carried out for 120 min at 100 °C in an oven. The extracted proteins were obtained by collecting the supernatant after sample centrifugation at 4400 rpm for 10 min. Total protein concentration was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard. To every milliliter of supernatant, 4 mL of Bradford reagent (0.01% Coomassie blue, 4.7% ethanol and 8.5% phosphoric acid) prepared just prior to the assay, was added and allowed to stand for 5 min at room temperature. The absorbance of the solution was then read at 595 nm. Total protein concentration was obtained via calibration curves made with BSA from 10 to 150 $\mu g\,mL^{-1}$.

Lipid extraction was carried out according to Parrish (1999) with dichloromethane:methanol (2:1) as the extraction solvent. Cultures were filtered through previously dried (400 °C, 12 h) glass fiber filters (GF/C; BOECO, Germany). After filtration and prior to the extraction, the filters were spiked with hexadecan-3-one, used as internal standard. Extracted samples were concentrated under ultrapure N_2 gas, spotted onto silica gel-coated TLC rods (Chromarods-SIII) and analyzed using an Iatroscan FID (Iatron Laboratories Inc., Tokyo, Japan) according to Chia et al. (2013a,b). The analytical conditions for the Iatroscan runs were: hydrogen flow 173 mL min^{-1}, air flow 2 L min^{-1} and scan speed 4 mm s^{-1}. The composition of lipid classes was obtained using their peak areas recorded and processed by PeakSimple software version 6.78 (SRI Instruments, Menlo Park, California, USA) for windows. Total lipid concentration was obtained by adding the concentration of individual lipid classes.

For amino acid analysis, algal samples were homogenized using a Polytron homogenizer in 10 mL ultrapure (Milli-Q) water. An aliquot of the homogenate (0.5 mL) was hydrolyzed with 0.5 ml HCL/phenol (1% by weight) at 100 °C in micro-reaction vials for 24 h. Total amino acids were derivatized using an EZ:faast™ GC-FID amino acid analysis Kit. Following hydrolyzation and derivatization, samples were run on a Varian 3800 GC-FID with a column length of 10 m and a diameter of 0.25 mm (ZB–AAA Zebron amino acid, Phenomenex, USA). The injector maintained a constant temperature of 250 °C and used 2.0 μL of sample with a 1:15 split. The column temperature started at 110 °C, was raised to 320 °C at a rate of 32 °C/min, and maintained for 2 min to ensure elution of all amino acids. The flow rate of the carrier gas, helium, was kept constant at 1.5 mL/min. The detector temperature remained steady at 320 °C. Peaks integration was done using the Varian Galaxie Chromatography Data System, version 1.9.3.2 (Agilent Technologies, Colorado Springs, Colorado) to obtain quantitative amino acid profile. Peak areas were quantified in comparison with an internal standard and a four-level calibration curve (level 1:50 nmols/mL, level 2:100 nmols/mL, level 3: 150 nmol/mL, and level 4: 200 nmol/mL). Standard solutions were supplied with the EZ:faast™ kit. Taurine and arginine are not determined using the EX:faast™ method.

Table 1
Analysis of variance results for the effect of phosphorus, nitrogen and cadmium on the growth and biomass of *Chlorella vulgaris*. Values represent *F* value and those in parenthesis represent *p* value.

Parameters	Factors		
	N	Cd	N/Cd
Specific growth rate	226.41(**0.00**)	351.06(**0.00**)	38.54(**0.00**)
Density	152.03(**0.00**)	65.73(**0.00**)	5.74(**0.00**)
Dry weight	231.51(**0.00**)	110.23(**0.00**)	24.44(**0.00**)
Chlorophyll a	198.04(**0.00**)	39.38(**0.00**)	28.21(**0.00**)
Total carbohydrate	7.35(**0.01**)	16.86(**0.00**)	3.25(**0.03**)
Total protein	53.92(**0.00**)	46.18(**0.00**)	8.55(**0.00**)
Total lipids per cell	26.19(**0.00**)	12.22(**0.00**)	4.70(**0.01**)
Sarcosine	0.17(0.68)	1.28(0.31)	0.30(0.75)
Glycine	0.73(0.41)	1.02(0.39)	0.30(0.75)
Amino-n-butyric acid	5.88(**0.03**)	3.76(**0.05**)	1.58(0.25)
Valine	1.87(0.20)	0.48(0.63)	0.43(0.66)
Leucine	0.35(0.57)	0.15(0.86)	0.02(0.98)
Isoleucine	0.73(0.41)	1.86(0.20)	0.58(0.058)
Proline	2.06(0.18)	2.18(0.16)	0.58(0.58)
Asparagine	0.04(0.85)	1.01(0.39)	0.36(0.70)
Thioproline	0.95(0.35)	1.12(0.36)	1.23(0.33)
Aspartic acid	0.38(0.55)	0.94(0.42)	0.85(0.45)
Methionine	0.38(0.55)	0.86(0.45)	0.46(0.64)
Phenylalanine	0.99(0.34)	1.13(0.35)	0.75(0.49)
A-aminopimelic acid	0.88(0.37)	0.33(0.73)	0.87(0.44)
Glutamine	0.37(0.55)	0.47(0.64)	0.28(0.76)
Ornithine	0.36(0.56)	0.82(0.46)	0.99(0.40)
Lysine	0.34(0.57)	0.37(0.70)	0.47(0.64)
Histidine	0.44(0.52)	2.45(0.13)	0.47(0.64)
Tyrosine	0.03(0.86)	1.63(0.24)	0.36(0.70)

Note: values with $p \leq 0.05$ are significant.

2.6. 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

Table 2
Amino acid compositions (g per 16 g N) of *Chlorella vulgaris* in response to different nitrogen and cadmium concentrations. Abbreviations for the amino acids in parenthesis are those used in the principal components analysis.

Amino acids	1.1×10^{-3} M		1.1×10^{-5} M			2.9×10^{-6} M		
	No Cd	2×10^{-8} M Cd	No Cd	2×10^{-8} M Cd	1×10^{-7} M Cd	No Cd	2×10^{-8} M Cd	1×10^{-7} M Cd
Sarcosine (SAR)	14.56	13.59	15.41	15.94	13.15	13.69	17.42	19.23
Glycine (GLY)	4.69	3.76	4.47	4.02	3.39	4.36	4.85	5.78
Amino-n-butyric acid (ABA)	0.95	7.20	3.60	1.54	0.88	0.87	0.62	0.26
Proline (PRO)	2.59	1.88	1.78	2.32	2.47	2.50	3.95	2.66
Valine (VAL)	6.42	25.69	4.77	4.60	4.27	5.64	14.47	12.57
Leucine (LEU)	1.71	1.28	0.94	1.07	1.73	1.18	1.19	2.13
Isoleucine (ILE)	7.49	19.8	9.39	13.23	4.94	9.24	13.95	15.31
Asparagine (ASN)	6.77	3.05	7.05	4.19	7.57	6.54	5.62	4.83
Thioproline (TPR)	1.77	1.77	1.49	1.55	2.46	1.67	3.03	2.04
Aspartic acid (ASP)	7.08	2.35	5.10	4.37	5.81	7.49	5.11	6.05
Methionine (MET)	1.46	1.39	1.35	1.37	1.66	1.25	2.18	1.74
Phenylalanine (PHE)	3.19	1.42	2.50	2.07	3.14	2.39	2.87	3.15
α-aminopimelic acid (APA)	3.63	1.65	2.72	2.98	4.87	3.19	2.37	1.53
Glutamine (GLN)	5.14	4.70	6.62	3.54	7.30	3.79	3.09	7.51
Glutamic acid (GLU)	1.22	–	1.25	0.62	2.07	–	–	0.68
Ornithine (ORN)	1.55	0.00	1.27	0.37	2.84	0.90	0.93	1.29
Lysine (LYS)	2.35	0.87	1.87	1.35	2.91	1.87	1.44	1.50
Histidine (HIS)	15.69	9.98	14.57	13.89	14.88	16.54	11.00	7.24
Tyrosine (TYR)	1.68	0.00	0.81	0.53	3.42	0.07	1.29	2.77

differences between means of analyzed parameters. A correlation matrix based principal component analysis (PCA) was used to detect any relationship between the analyzed parameters. ANOVA and post hoc analyses were done using Statistica 8.0 (Stat Soft. Inc.) software, and PCA was done using PAST 2.17c computer program for Windows at 5% significance level.

3. Results

The growth rate of *C. vulgaris* was highest in the control, while increasing Cd concentrations decreased the growth of the microalga (Fig. 1). The combination of N limitation and Cd stress further decreased the growth of the microalga than its exposure to Cd under replete N conditions. The highest cell density was found in the control, while at the highest Cd concentration significantly ($p < 0.05$) lower densities were recorded in all N concentrations (Fig. 2a). The changes in cell density and growth rates were significantly ($p < 0.05$) different between Cd and N treatments (Table 1). In addition, significant interactions were recorded between Cd and N treatments ($p < 0.05$) on cell density and specific growth rates. The combination of Cd stress and N limitation significantly ($p < 0.05$) increased dry weight and chlorophyll *a* concentration per cell (Fig. 2b and c).

High cellular levels of total carbohydrate, protein and lipid were obtained after exposure of *C. vulgaris* to the highest Cd in combination with all tested N concentrations (Fig. 3). Furthermore, the concentrations of total protein and total lipid per cell were usually lowest in the control. The effect of N, Cd, and Cd vs N interactions on total lipid, total carbohydrate and total protein content of *C. vulgaris* were statistically significant ($p < 0.05$, Table 1). Amino acid composition showed that proline, sarcosine, valine, phenylalanine, isoleucine and methionine concentrations increased with increasing Cd stresss and N limitation (Table 2). Nitrogen limitation resulted in a decrease in the synthesis of proline, leucine, thioproline, methionine, α-aminopimelic acid and tyrosine. However, most of these amino acids that had lower values under N limitation presented higher concentrations after exposure to Cd.

Principal component analysis of the data showed that growth rate and cell density of *C. vulgaris* were positively related to N concentrations (Fig. 4). Cadmium was positively correlated with total lipids, proteins and carbohydrates, chlorophyll *a* and dry weight production per cell ($p < 0.05$). Nitrogen concentrations were positively related to most of the amino acids, while proline, tyrosine, valine, isoleucine, methionine and leucine were positively correlated with Cd ($p < 0.05$). The PCA results showed that the first two components were responsible for about 50% of the total variation recorded.

4. Discussion

4.1. Growth and biomass

The positive association of growth rates and cell density with the concentrations of N without Cd addition demonstrates the importance of this nutrient for cell replication. These findings are in agreement with other literature data (Hu and Zhou, 2010; Bhola et al., 2011; Chia et al., 2014). The reduction of *C. vulgaris* growth rates and cell density when exposed to Cd can be related to cell division and nutrient uptake inhibition by the metal as demonstrated for other microalgae (Serra et al., 2010; Monteiro et al., 2011). Omar (2002) attributed the reduction in growth rate to an inhibition of normal cell division due to metal binding to sulfhydryl groups that are crucial in regulating plant cell division. In agreement with our study, Serra et al. (2010) showed that copper sensitivity in natural periphyton communities followed the gradient of nutrient concentrations found in the Fluvià River, Catalonia, Spain. This may explain the significant interactive effect observed of Cd and N on the growth and cell density of *C. vulgaris*, which means that the sensitivity to Cd increased as the microalga was grown in limiting N conditions. Furthermore, in agreement with our results, Liu et al. (2014) showed that ammonium reduces chromium toxicity in *C. vulgaris* because at high nitrogen concentration the microalga was able to withstand the negative effect of the metal.

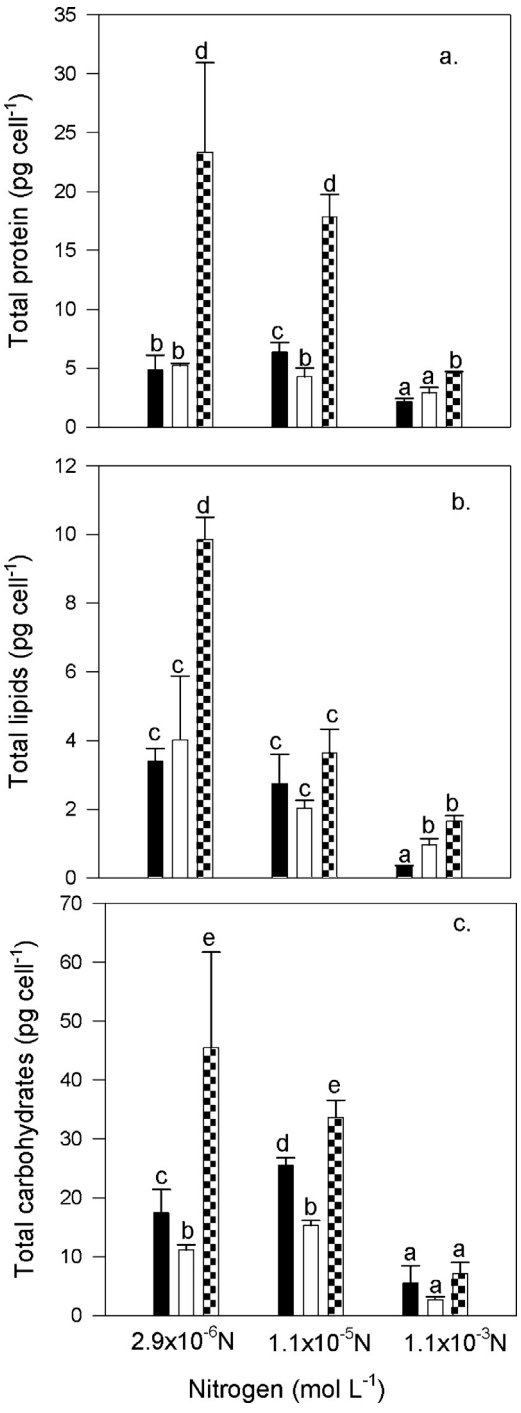

Fig. 3. Total proteins (a), lipids (b) and carbohydrates (c) production as a function of different Cd and N concentrations (mol L^{-1}). Black bars represent treatments without Cd, white bars 10^{-8} mol L^{-1} Cd and checked bars 10^{-7} mol L^{-1} Cd. Bars with the same alphabets are not significantly different at $p < 0.05$.Error bars represent standard deviation for $n = 3$.

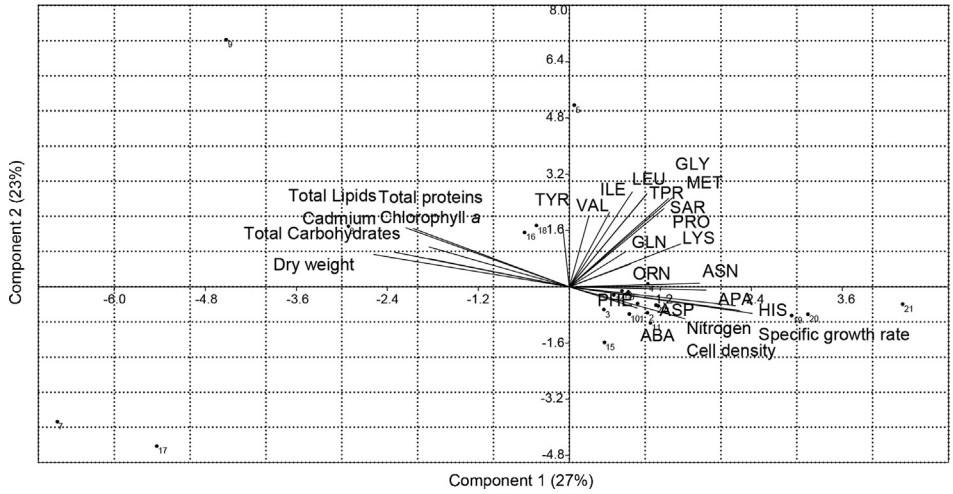

Fig. 4. PCA biplot of different parameters analyzed for *Chlorella vulgaris* after exposure to different Cd and nitrogen concentrations.

An additive effect was observed on the dry weight and chlorophyll *a* production of the microalga with increasing Cd stress and N limitation. This is because the combination of both conditions stimulated higher dry weight and chlorophyll *a* production per cell at the expense of reduced growth rate. The combination of nitrogen limitation with Cd stress affects photosynthesis due to the increase in chlorophyll *a* concentration. The increase in chlorophyll *a* content will support higher photosynthetic rate, which in turn will increase carbon dioxide fixation and allocation of carbon into sugars and carbohydrates (Chia et al., 2013c). The sugars and carbohydrates serve as important organic carbon raw materials for the biosynthesis of important biomolecules like proteins, lipids and nucleic acids (Fathi et al., 2005; Afkar et al., 2010). In support of this, there was a significant positive correlation of chlorophyll *a* content with total proteins, total lipids and total carbohydrates in the present study. These changes in macromolecular allocation can reduce growth rates as a form of adaptation to stress conditions (Juneja et al., 2013). Contrary to our findings, some studies show that metal toxicity (10^{-6}–10^{-8} M) or N limitation results in reduced chlorophyll *a* and biomass production (Serra et al., 2010; Chia et al., 2014). This may be because in most studies, the microalgae are rarely acclimated to the nutrient conditions to be investigated. However, in agreement with our results, Soldo and Behra (2000), Omar (2002), La Rocca et al. (2009) and Okmen et al., 2011 showed that at sublethal metal (Ni, Zn, and Cd) concentrations, cell division inhibition occurs in microalgae and cyanobacteria, which results in cells having larger biomass and increased pigment content. The implication of this is that under chronic exposure conditions of combined nitrogen variation and Cd stress, acclimated microalgae will become adapted by increasing photosynthetic capabilities and selective biomolecule synthesis to help combat the toxic effects of metals.

4.2. Biochemical composition

As a physiological response to the effect of different environmental stresses, microalgae vary their biochemical composition. In this study, the increase in carbohydrate concentration showed a significant correlation with increasing Cd stress and N limitation. This is in agreement with the findings of Granum et al. (2002), Yang et al. (2012) and Bono et al. (2013) who showed that the exposure of microalgae to nitrogen limitation resulted in increased carbohydrate synthesis. According to Bono et al. (2013), during nitrogen limitation or starvation, microalgae undergo biochemical composition changes including an up-regulation of carbohydrate accumulation that accompanies an increase in cell biomass or size. The increase in carbohydrate production under combined nitrogen limitation and Cd stress is because the biomolecules serve as a sink for the excess fixed carbon generated from unbalanced carbon and N metabolism (Otero and Vincenzini, 2004; Shanab et al., 2012; Yang et al., 2012). However, our investigation showed that the combination of N limitation and Cd stress had an additive effect on carbon fixation because much higher carbohydrate concentrations were recorded in cultures maintained under combined stress than in either Cd stress or nitrogen limitation alone. This suggests that potentially complex interactions between environmental factors may determine carbohydrate production in *C. vulgaris* in aquatic ecosystems having variable and multiple stress factors interacting simultaneously.

Total lipids were accumulated under combined Cd stress and N limitation, which agrees with the results of Einicker-Lamas et al. (1996), Gushina and Harwood (2006), Widjaja et al. (2009) and Bhola et al. (2011). The accumulated lipids may be used as food reserves, thereby serving as alternative sources of energy needed to survive during environmental stress (Hu et al., 2008). The exposure to Cd stress and N limitation resulted in the inhibition of cell division and increase in cell biomass (dry weight), which caused physiological synchronization of the cells to produce higher lipid content (Yilancioglu et al., 2014) and other biomolecules as stated above. This increased lipid concentration per cell was previously attributed to increased neutral lipid production in response to combined Cd stress and N limitation (Chia et al., 2013a). Mock and Kroon (2002) and Hu et al. (2008) explain that the increase in microalgal cellular lipid content may enhance the maintenance of cellular redox homeostasis by acting as an electron sink. This helps the microalgae to combat possible oxidative damage that may be caused by reactive oxygen species produced under metal and related stress conditions in aquatic ecosystems (Mock and Kroon, 2002; Bonet et al., 2014).

Contrary to the usual observation that N limitation results in a decrease in microalgal protein content (Illman et al., 2000; Scragg et al., 2002; Chia et al., 2014), we observed higher total protein concentrations per cell under N limitation in combination with Cd stress. However, the increase in total protein obtained from our study is in agreement with that reported by Einicker-Lamas et al. (2002), Branco et al. (2010), Harish et al. (2011) and Shanab et al. (2012) after exposure of microalgae to sub-lethal Cd concentrations. This may imply that the accumulation of intracellular proteins in *C. vulgaris* following the decline in growth was also directed towards its survival and not cell division (Shanab et al.,

2012). Protein accumulation in combined stressed *C. vulgaris* provides a means of abolishing the toxic effects of Cd, by increasing carbohydrate utilization for energy production from enhanced respiration (Osman et al., 2004; Afkar et al., 2010). In addition, an increase in total protein production increases the concentration and subsequently the activity of important antioxidant enzymes for combating reactive oxygen species, and phytochelatins for chelating metals (Suárez et al., 2010; Bonet et al., 2014). Therefore, based on the synergistic effect recorded on total protein production by *C. vulgaris* in the present study, the use of only metal toxicity or nutrient limitation alone is not sufficient to predict the stress conditions that are prevalent in aquatic ecosystems. In support of this assumption, a study by Spijkerman et al. (2007) showed that significantly higher total protein production composed primarily of heat shock proteins (Hsp) was obtained in *Chlamydomonas acidophila* incubated in metal-enriched lake water than in metal-enriched artificial medium. This variation was related to the multiple stress factors associated with the lake water, a condition that was not observed with the artificial medium.

Amino acids are essential biomolecules representing a pool for both proteosynthesis and synthesis of other vital cellular metabolites (Moe, 2013). The increase in isoleucine, phenylalanine and tyrosine under combined Cd and N stress may be because they are both glucogenic and ketogenic amino acids, while proline, histidine, glutamine, valine and asparagine on the other hand are glucogenic amino acids (Moe, 2013; Johnson and Alric, 2013). The nature of the different amino acids and their possible roles in lipid and carbohydrate biosynthesis through the tricarboxylic acid cycle (TCA cycle) (Johnson and Alric, 2013) may explain the positive relationship they had with total lipid and carbohydrates production in *C. vulgaris.* Thus, the increase in total carbohydrates and lipids per cell may have been related to the up-regulation of the biosynthesis of these amino acids under conditions of combined Cd stress and N limitation. The variations in the composition and proportions of these amino acids can be used as important biological markers for multiple stress factors in aquatic ecosystems, since they had significant correlations with Cd stress and N variation.

The increase in the synthesis of some amino acids under Cd stress and N limitation may also be related to their possible integration into phytochelatins to serve as metal chelators (Jetley et al., 2004; Kumar et al., 2010; Kovacik et al., 2010). Although the role of some of the amino acids accumulated during exposure to combined N limitation and Cd stress are presently not known for any microalgae, sarcosine, glycine, valine, thioproline, methionine, phenylalanine, glutamine and ornithine may contribute to complexation of Cd in *C. vulgaris.* This is because in agreement with our study, Omar (2002) reported an accumulation of methionine, phenylalanine, valine and glycine after exposure of *Scenedesmus quadricauda* and *S. obliquus* to toxic Zinc concentration. Another accumulated amino acid especially at the highest Cd concentration in combination with N limitation was glutamine, which serves as a metabolic precursor for glutathione (GSH) (Piotrowska-Niczyporuk et al., 2012; Chia et al., 2014). This may be related to the efficiency of GSH in reactive oxygen species (ROS) reduction, since it contains a sulfhydryl residue which is easily oxidizable (Johnson and Alric, 2013). Furthermore, the increase in the concentration of another amino acid proline was not surprising because it is widely regarded as an osmoregulatory solute (Kovacik et al., 2010; Çelekli et al., 2013). This amino acid acts as a stabilizer for cellular structures, a free radical scavenger and electron sink, thereby alleviating the damaging effects associated with metal pollution (Mehta and Gaur, 1999; Tripathi et al., 2006; Sharma and Dietz, 2006; Choudhary et al., 2007; Kovacik et al., 2010; Çelekli et al., 2013). On the other hand, even though the detoxification function of histidine and asparagine in microalgal response to metal or nutrient associated stress conditions is not known, their accumulation in higher plant cells have been linked to metal tolerance because they show equimolar concentrations with metal exposure levels (Sharma and Dietz, 2006). Therefore, they can be said to have played a minimal role in combating Cd stress in *C. vulgaris* because their levels were lower as Cd concentration increased.

5. Conclusion

Significant variations of *C. vulgaris* growth, biomass production and biochemical composition were observed with varying N and Cd concentrations in culture medium. A decrease in growth rate and cell density was the general trend with increasing Cd stress and N limitation. Dry weight, chlorophyll *a,* total lipids, carbohydrates and proteins per cell were accumulated as both Cd stress and N limitation increased. Although, the concentration of some amino acids decreased under N limitation, there were noticeable increases in the accumulation of ketogenic and glucogenic amino acids when N limitation was combined with Cd stress. The increase in proline and glutamine may be related to the role they play in protecting the cell from the damaging effects of metal and associated environmental stresses. The changes in amino acid composition recorded in the present study can be used as sensitive biomarkers for Cd stress and N limitation in *C. vulgaris* and possibly other microalgae.

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