UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Mixotrophy in *Chlorella sorokiniana* – Physiology, Biotechnological Potential and Ecotoxicology

ADRIANO EVANDIR MARCHELLO

São Carlos – 2017

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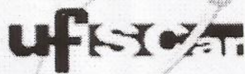
Orientadora: Profa Dra Ana Teresa Lombardi

Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais (PPGERN) como parte dos requisitos para obtenção do título de Doutor em Ecologia e Recursos Naturais.

São Carlos – 2017



UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ecologia e Recursos Naturais

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e meus sobrinhos Maressa e Lucca.

"Só Tu és o Senhor. Fizeste os céus, e os mais altos céus, e tudo o que neles há, a terra e tudo o que nela existe, os mares e tudo o que neles existe. Tu deste vida a todos os seres, e os exércitos dos céus te adoram."

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"O que sabemos é uma gota; o que ignoramos é um oceano."

Isaac Newton

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

α	Photosynthetic efficiency
e	Neperian logarithm
μ	Specific growth rate
μm^{-3}	Cubic micrometer
μmol	Micromol
τ	Parameters that determines the inflection point of the curve
%	Percentage
[Cd]	Cadmium concentration
~	Approximately
°C	Celsius degree
$^{1}O_2$	Singlet oxygen
AAP medium	Algal assay procedure medium
ACCase	Enzyme acetyl-CoA carboxylase
ADP	Adenosine diphosphate
Ag	Silver
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Enzyme that catalyzes the decomposition of ATP
A.U.	Arbitrary units
B	Maximum point of the curve with the same unit of y(t)
BG11 medium	Blue-green medium
C	Carbon
C16:0	Palmitic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
$C_6H_{12}O_6$	Glucose
Ca	Calcium
Cd	Cadmium
CFU	Colony forming units
Chl *a*	Chlorophyll *a*
cm	Centimeter
CNPq	National Council of Scientific and Technological Development
CO_2	Carbon dioxide
Cr	Chromium
Cu	Cooper
d	Diameter
d^{-1}	By day
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
e.g.	For example
et al	And collaborators
$FADH_2$	Flavin adenine dinucleotide
FAPESP	The São Paulo Research Foundaion

F*m*	Maximum fluorescence after dark adaptation
F_o	Basal fluorescence after dark adaptation
F*v*	Variable fluorescence after dark adaptation
F*v*/F*m*	Quantum maximum efficiency of Photosystem II
g	Grams
h	Hour
H^+	Hydrogen protons
H_2CO_3	Carbonic acid
H_2O	Water
H_2O_2	Hydrogen peroxide
Hg	Mercury
I_k	Irradiance saturation
K^+	Potassium cation
kJ	Kilojoule
L	Liters
LHC	Light harvesting complex
Ln	Natural logarithm
Log	Logarithm
m	Meters
mg	Milligram
min	Minutes
mL	Milliliter
mol	Measurement for amount of substance
mRNA	Messenger ribonucleic acid
mV	Millivolt
n	Number of replicas
N	Nitrogen
N:C	Nitrogen:carbon rate
$NADH_2$	Nicotinamide adenine dinucleotide
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate
Ni	Nickel
NPQ	Non-photochemical *quenching*
NPs	Nanoparticles
NP-TiO_2	Titanium dioxide nanoparticles
O_2	Oxygen gas
$O_2^{\bullet -}$	Superoxide radical anions
$OH^{\bullet}$	Hydroxyl
OM	Organic matter
p	p value
P:C ratio	Proteins:carbohydrates ratio
PAM	Pulse amplitude modulated fluorometry
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline solution
PCA	Plate count agar medium
pg	Picograms
pH	Potential of hydrogen
P_{max}	Maximum rate of photosynthesis

PSII	Photosystem II
*q*P	Photochemical *quenching*
Ref	Reference culture
rETR	Relative electron transport rate
RNA	Ribonucleic acid
$RO^{\bullet}$	Phenoxy
$ROO^{\bullet}$	Peroxy
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oxygenase
s	Second
s.d.	Standard deviation
SH	Sulfhydryl group
sp	Specie
SSU	Small-subunit
t	Time
TiO_2	Titanium dioxide
U.S.A.	United States of America
UV	Ultraviolet light
V	Biovolume
vs	Versus
y(t)	Chlorophyll *a* concentration ($mg.L^{-1}$) in the time
Zn	Zinc

ABSTRACT

In aquatic environments, phytoplankton consists mostly of photosynthetic microorganisms that serve as the basis of food chains. However, besides photoautotrophy, it is widely reported in the literature that many microalgae can take up dissolved organic matter present in the environment concomitantly with the photosynthesis, a metabolic pathway known as mixotrophy. Little is known about the ecophysiology of mixotrophy in microalgae, and almost all studies are focused on the use of this metabolic pathway to increase the production of algal biomass and stimulate the production of specific biomolecules. Another important issue, considering the current anthropic activity, is that most of the contaminants eliminated in aquatic environments, such as metals and nanoparticles, affect the phytoplankton. However, so far, no ecotoxicological study involving mixotrophic metabolism was found in the literature. To better understand mixotrophy in microalgae, this work chose the chlorophycean freshwater *Chlorella sorokiniana* as test organism. We divided the study into two parts: the first focused on the physiological/biotechnological interest through the study of growth, photosynthetic parameters, changes in cellular volume, and production of biomolecules (proteins, carbohydrates and lipids); the second part focused on the ecotoxicological effects of cadmium (Cd) and titanium dioxide nanoparticles (NPs-TiO_2). To stimulate mixotrophy, glucose (1.0 $g.L^{-1}$ or 5 x 10^{-3} $mol.L^{-1}$) was used as the organic carbon source. The results showed that during mixotrophy, the microalga *C. sorokiniana* presented higher population growth and production of biomolecules, such as chlorophyll *a* and lipids, when compared to photoautotrophic cultures. It was also observed that the photosynthetic parameters were affected by mixotrophy, although they did not interfere in the growth of the microalga, and that the presence of bacteria in the cultures acted as a stimulant factor in the production of algal biomass. Regarding the ecotoxicological effects of contaminants, microalgae in mixotrophy were more resistant to both Cd and NPs-TiO_2 than those in photoautotrophy, but with changes in the biochemical composition what can affected the energy transfer in the environment. In general, we can conclude that mixotrophy should be considered in studies with phytoplankton, since aquatic environments present a myriad of organic carbon that can be used by these microorganisms. As general conclusions, we can mention that organic carbon acted as an extra source of structural carbon and energy for microalgae, not

necessarily relying solely on photosynthesis to survive, so stimulating the growth and production of biomolecules of biotechnological interest, and increased cellular viability in environments contaminated with metals and nanoparticles. This study is a contribution to the understanding of mixotrophy and photoautotrophy metabolisms in a freshwater Chlorophyta with biotechnological potential.

Keywords: mixotrophy, biomass production, lipids, cadmium, nanoparticles.

RESUMO

Nos ambientes aquáticos, o fitoplâncton é formado basicamente de microrganismos fotossintetizantes que servem como base das cadeias alimentares. Entretanto, além da fotoautotrofia, é vastamente citado na literatura que muitas microalgas alimentam-se de matéria orgânica dissolvida presente no ambiente concomitantemente à realização da fotossíntese, uma via metabólica conhecida como mixotrofia. Sabe-se pouco sobre a ecofisiologia em metabolismo mixotrófico nas microalgas, sendo os estudos, em sua quase totalidade, voltados ao uso dessa via metabólica para aumentar a produção de biomassa algal e estimular a produção de biomoléculas específicas. Outra questão importante, considerando a atividade antrópica atual, é que a maioria dos contaminantes eliminados nos ambientes aquáticos, como metais e nanopartículas, são estudados em fitoplâncton sob metabolismo fotoautotrófico, não sendo encontrados trabalhos ecotoxicológicos envolvendo o metabolismo mixotrófico na literatura. Para entender melhor o metabolismo algal em mixotrofia, este trabalho escolheu a microalga Chlorophyta de água doce *Chlorella sorokiniana* como organismo-teste. Para melhor organizá-lo, foi dividido em duas partes: a primeira focou no interesse fisiológico/biotecnológico através do estudo do crescimento, parâmetros fotossintéticos, volume celular, e produção de biomoléculas (proteínas, carboidratos e lipídeos); a segunda parte focou nos efeitos ecotoxicológicos de cádmio (Cd) e de nanopartículas de dióxido de titânio (NPs-TiO_2). Para estimular a mixotrofia, glicose (1.0 $g.L^{-1}$ ou 5 x 10^{-3} $mol.L^{-1}$) foi utilizada como fonte de carbono orgânico. Os resultados mostraram que durante a mixotrofia, a microalga *C. sorokiniana* apresentou maiores crescimento populacional e produção de biomoléculas, como clorofila *a* e lipídeos, quando comparada com as culturas em fotoautotrofia. Também foi observado que os parâmetros fotossintéticos foram afetados em mixotrofia, porém não interferindo no crescimento da microalga, e que a presença de bactérias pode ter atuado como fator estimulante na produção de biomassa algal. Em relação aos efeitos ecotoxicológicos dos contaminantes, as microalgas em mixotrofia foram mais resistentes tanto ao Cd quanto às NPs-TiO_2 do que em fotoautotrofia, porém com mudanças na composição bioquímica, podendo afetar a transferência de energia nos ecossistemas aquáticos. De modo geral, podemos concluir que a mixotrofia deve ser considerada em estudos com fitoplâncton, visto que os ambientes aquáticos apresentam uma miríade de fontes de

carbono orgânico para esses microrganismos. Na mixotrofia, o carbono orgânico funciona como uma fonte extra de carbono estrutural e de energia para as microalgas, não dependendo obrigatoriamente somente da fotossíntese para sobreviver, estimulando o crescimento e produção de biomoléculas de interesse biotecnológico, além de aumentar a viabilidade celular em ambientes contaminados tanto com Cd quanto com NPs-TiO_2. Este estudo é uma contribuição ao entendimento dos metabolismos mixotróficos e fotoautotróficos em uma Chlorophyta de água doce com potencial biotecnológico.

Palavras-chave: mixotrofia, produção de biomassa, lipídeos, cádmio, nanopartículas.

INTRODUCTION

GENERAL INTRODUCTION

1. Mixotrophy

The diversity of organisms on Earth is a result of the evolution of adaptive strategies for survival of the species over millions of years. Based on rRNA analysis (Woese et al., 1990), nowadays the life is divided in three great domains: *Bacteria*, *Archaea* and *Eukarya* (Fig. 1). The diversity in each domain and environmental plasticity of the organisms led to a diversification of the metabolic process, many of which are not yet known (Pace, 1997; Raymann et al., 2015).

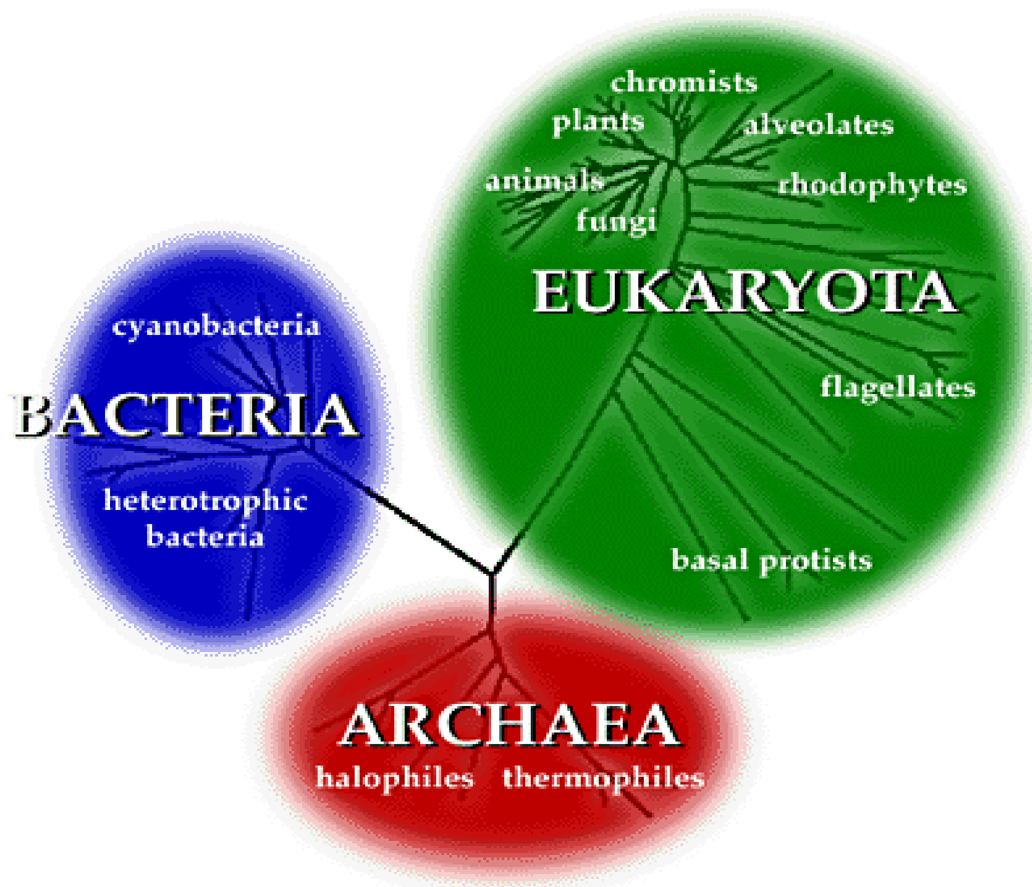

Figure 1 - Current phylogenetic tree of life based on small-subunit (SSU) rRNA sequences of the organisms (Woose et al., 1990).

Among the living creatures, the microorganisms and their metabolic reactions play key roles in sustaining life on Earth, and have a crucial importance for the support

of different aspects of human activities, such as the production of antibiotics, food, oxygen gas, nitrogen biofixation, poisons, etc (Madigan et al., 2016).

Classically, or at least didactically, the living beings are classified in two groups or categories: the *autotrophs* (produce their own organic matter using energy from the environment) and *heterotrophs* (use carbon and energy through the breakdown of organic matter from the biomass of other organisms; Dolan & Peréz, 2000; Tittel et al., 2003). These two categories define trophic levels and are the extremes of a continuum of survival strategies well illustrated among planktonic protists (Jones, 1994; Stoecker, 1998). Within these two extremes, many organisms have characteristics of both, *autotrophs* and *heterotrophs*, when they are categorized as *mixotrophs* (Tittel et al., 2003; Nelson & Cox, 2014; Madigan et al., 2016).

The mixotrophy is a special nutritional strategy and has been considered as a evolutionary successful to overcome stressing situations (Jones, 1994; Raven, 1997; Stoecker, 1998). Indeed, the mixotrophy is widely found in microorganisms, especially in protists (protozoans and algae), but also occurs in multicellular organisms, such as certain animals. For example, Valmalette et al. (2012) found an insect (*Acyrthosiphon pisum*) that electron transfer and ATP synthesis in carotenes is induced by light.

Mixotrophic protists differ both qualitatively and quantitatively in their dependence on feeding, light and uptake of dissolved nutrients (Stoecker, 1998). The mixotrophy in protists can be facultative or obligate (McManus et al., 2012). The costs and benefits of mixotrophy in different taxa of protists and environments are still largely a subject of speculation (Boëchat et al., 2007).

Many protozoans, for example, either sequester plastids derived from their prey (*kleptoplastids* or *chloroplast retention*), such as the *Perispira ovum* when it eats *Euglena proxima* (Johnson et al., 1995; McManus et al., 2012), or keep algal endosymbionts, such as the *Paramecium bursaria* that phagocytes the microalga *Chlorella* sp (Dolan, 1992). Some microalgae species have the ability of switching between phototrophic and heterotrophic metabolism depending on environmental conditions or perform both, mixotrophy (Kaplan et al., 1986; Lee, 2004).

Microalgae are among the most diverse organisms on Earth, considering both the evolutionary and ecological viewpoints (Shama & Rai, 2011; Hildebrand et al., 2013; Gimpel et al., 2015). There are at least 40,000 – 70,000 species, but some estimates propose that there could be up to eight times that amount if undiscovered or

unclassified species are accounted for (Norton et al., 1996; Bhattacharya et al., 2004; Guiry, 2012).

The plasticity of microalgae, of interest in Biotechnology and Ecology (Fig. 2), is a result of environmental selection during the course of evolution (Keeling, 2010) and to possess a rich source of genetic (nuclear, chloroplast and mitochondrial genomes) and chemical diversity (Specht et al., 2010; Gimple et al., 2012).

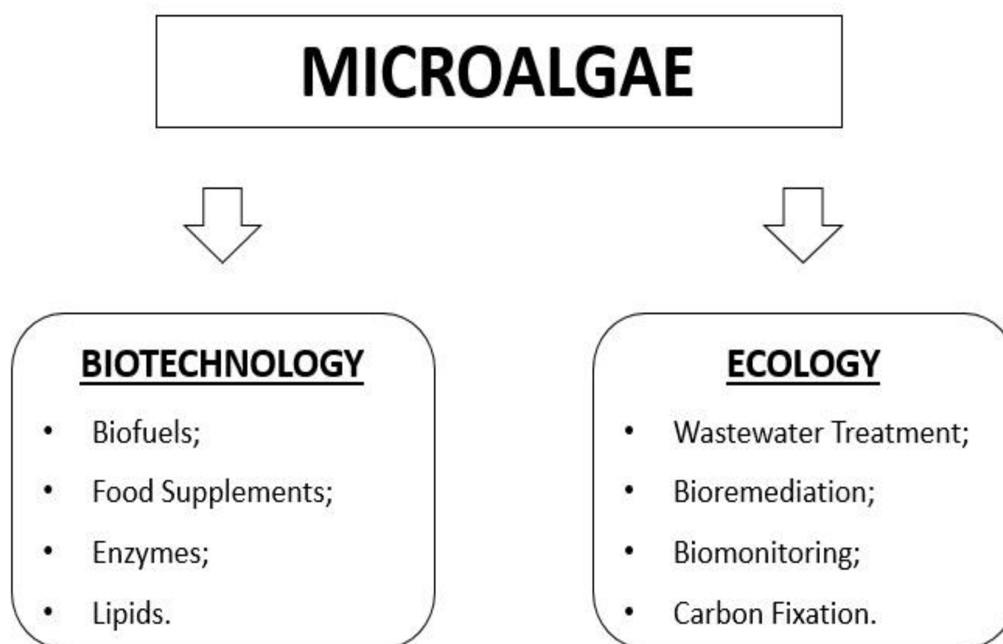

Figure 2 – Some potential applications of microalgae both in Biotechnology and in Ecology segments of interest. Souce: author.

2. Biotechnological aspects of mixotrophy in microalgae

Microalgae have the capacity to produce several chemical compounds of biotechnological interest, as food supplement, lipids, enzymes, biomass, polymers, toxins, pigments, hydrogen gas, etc (Perez-Garcia et al., 2011; Arbib et al, 2014). When a microalga culture is kept under controlled conditions, optimization of the production of chemical compounds of interest can be obtained (Davis et al., 2011; Gimple et al., 2015). Currently, there is great interest in the production of biofuels from microalgal biomass because it has some advantages in comparison with biofuel produced from

terrestrial plants biomass. The microalgae related advantages are higher photosynthetic efficiency, fast growth, direct carbon dioxide mitigation, and growth in non-arable lands (Juntila et al., 2005; Marchello et al., 2015). In mixotrophic growth conditions, Li et al. (2014) found the production of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) by the microalga *Chlorella sorokiniana*, similar to the fatty acids composition in soybean oil.

From this point of view, the culture of microalgae in mixotrophic conditions (i.e., supplemented with organic carbon) has been greatly used in production of biomass and secondary metabolites (Li et al., 2015). The mixotrophic culture of microalgae has some advantages in relation to autotrophic and heterotrophic ones (Juntila et al., 2005; Li et al., 2014).

Throughout the autotrophic cultures (energy and carbon sources are light and carbon dioxide, respectively), both the increase in number of cells and the formation of biofilm at flask become so dense that create self-shading, reducing drastically the light penetration for photosynthetic activity (Li et al., 2013). Instead, in heterotrophic cultures, the only source of energy and carbon for cells is the organic carbon supplemented in the medium, increasing the production costs (Juntila et al, 2005; Perez-Garcia et al., 2011). Another problem is the growth of other microorganisms, as bacteria, that may consume the organic carbon so fast that limits the microalgal growth, and produce organic acids, such as the lactic and malic, compromising (and inhibiting in some cases) the microalgae growth (Giovanardi et al., 2013).

In mixotrophic cultures of microalgae, the cells can use light and carbon dioxide during photosynthesis and organic carbon during respiration, thus increasing the energy and biomass produced in a short period of time when compared with autotrophic and heterotrophic cultures; an increase the relation cost-benefits is observed in the mixotrophic algae growth (Wan et al., 2011).

The uptake of glucose by microalgae, for example, occurs due to a co-transport system with protons. As can be seen in Figure 3, the hexose/H^+ symport system involves the consumption of one ATP molecule per hexose/H^+ transported via membrane (Komor, 1973; Komor & Tanner, 1974). During mixotrophy in glucose, for example, the microalgae can utilize energy (ATP) from the cyclic photophosphorylation in chloroplasts and/or oxidative chain reaction in the mitochondria to take up the carbohydrate from the environment.

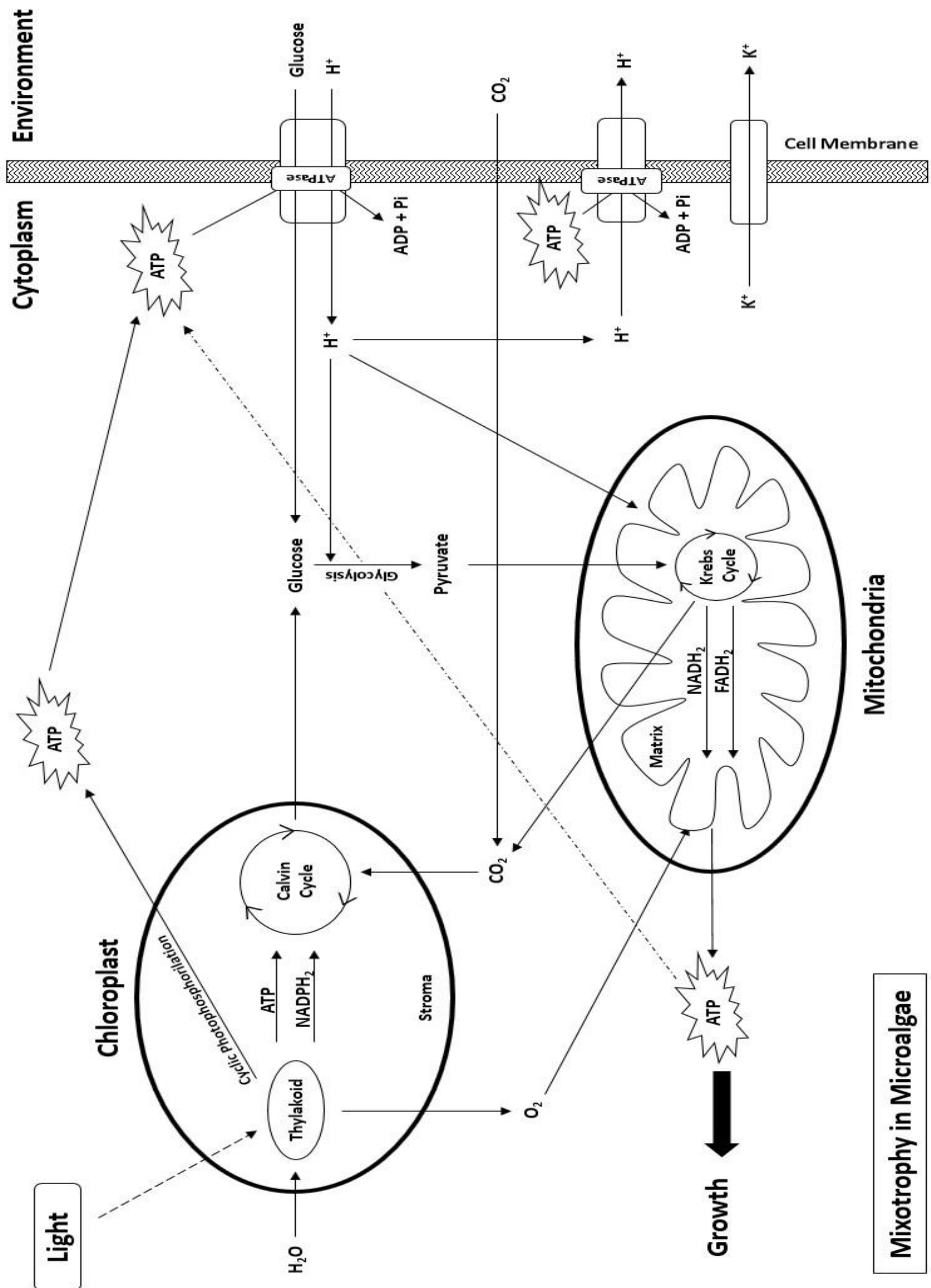

Figure 3 - Mixotrophy scheme inside the algal cell showing the biochemical and biophysical processes of this nutritional strategy. Source: author.

Literature concerning mixotrophic cultures of microalgae has shown that different species behave differently, what is positive, because it shows the genetic diversity of this group of microorganisms in relation to the environmental conditions (Rodrígues-Lopoz, 1966; Giovanardi et al., 2014; Juntila et al., 2015).

3. Ecological aspects of mixotrophy in microalgae

Environmentally, the microalgae have a crucial role in aquatic ecosystems, because they are the main components of the phytoplankton, i.e., the basis of aquatic food webs (Reynolds, 2006). During photosynthesis, microalgae produce oxygen gas and organic matter that are used by aerobic heterotrophic organisms for their maintenance and growth (Reynolds, 2006).

The classical theories of food web in ecology do not consider the role of mixotrophy in the relations among the organisms (Elton, 1927; Odum, 1957). However, mixotrophy can influence the food web structure and function (Boëchat et al., 2007; Wilken et al., 2014), taking to competition for dissolved organic matter between bacteria and microalgae in microbial loops (Ptacnik et al., 2004; Bell, 2012). It also allow certain species of phytoplankton to occasionally dominate, and perhaps disrupt microbial food web structure (Stoecker, 1998), particularly in the dark or at low irradiance (Kamjunke et al., 2008).

For example of the mixotrophic role in the structure of the aquatic food web, Boëchat et al. (2007) showed that the microalga *Ochromonas* sp. during mixotrophy, can change their fatty acids composition, limiting grazing by zooplankton, changing the biochemical matter composition and energy through food webs (Müller-Navarra et al. 2004).

Based in some studies, as Ptanick et al. (2004), Boëchat et al. (2007), Hartmann et al. (2013), Wilken et al. (2014), and Cropp & Norbury (2015), a possible scheme of an aquatic microbial food web considering the mixotrophy nutrition in phytoplankton (purely speculative scheme) has been generated and is shown in Figure 4. Phytoplankton, in this case, can phagocytes bacteria, protozoans and other algae (Hartmann et al., 2013; Wilken et al., 2014), or take up, by osmotrophy, dissolved organic carbon (DOC) derived from cell lysis (from autochthonous or allochthonous sources) or feces of protozoan, for example. Many groups of photosynthetic

microorganisms potentially utilize organic carbon, such as Cyanobacteria, Chrysophyte, Chlorophytes, Dinophytes, Bacillariophytes, and Xanthophytes (Droop, 1974).

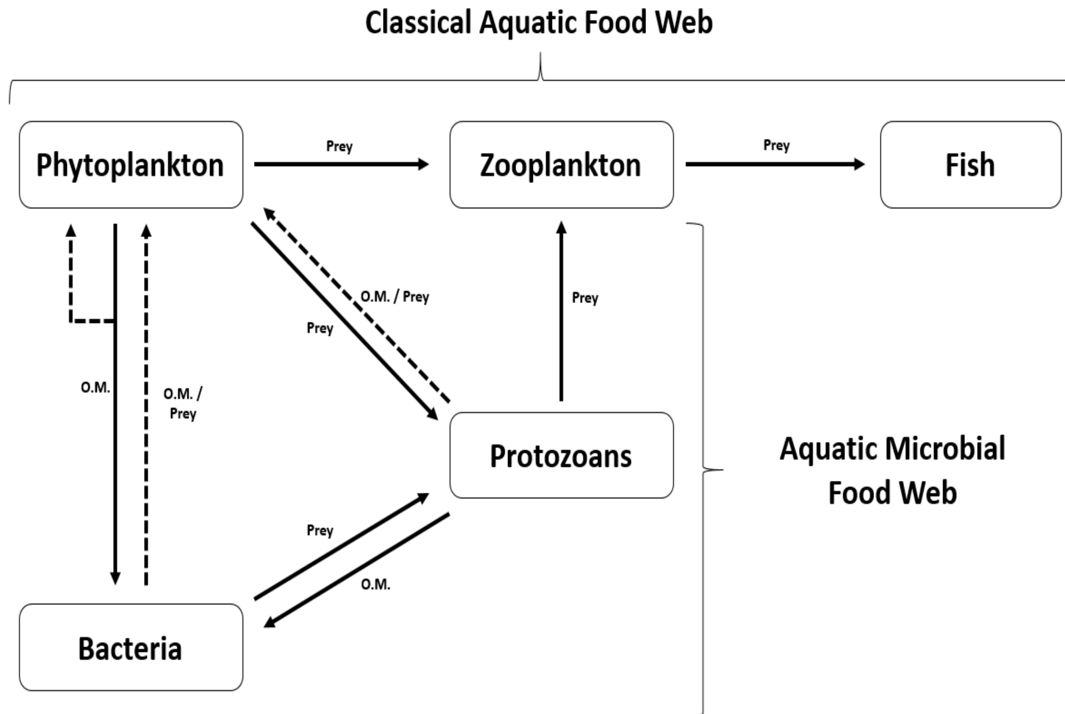

Figure 4 - Scheme of aquatic microbial food web with phytoplankton performing mixotrophy. Mixotrophy (dashed lines) by microalgae may occur via phagotrophy or, most commonly, osmotrophy of organic matter excreted by other organisms, such as bacteria, protozoans, and phytoplankton itself. At the top of the figure, a classical aquatic food web is represented. O.M. means organic matter (lysis of cytoplasm and/or feces). Source: author.

In ecological studies, microalgae have been used in wastewater treatments, bioremediation of metal contaminated environments, atmospheric carbon dioxide fixation, assessment water quality, and others (Fischer et al., 1984; Mehta & Gaur, 2005; Arbib et al., 2014; Marchello et al., 2015).

Our knowledge about the interactions of microalgae with metal ions or other toxic agent is largely based on laboratory studies using microalgae cultures exposed to autotrophic conditions. However, this does not necessarily represent the reality in natural environments (Monteiro et al., 2011), since a myriad of organic compounds are normally present. These compounds can both affect the toxicant speciation and the algal metabolism. A search of literature revealed few studies that investigated the responses of microalgae to toxic agents in the presence of organic substrate for algae nutrition, so

stimulating the mixotrophic growth. So far, one investigation (Subashchandrabose et al., 2013) looked at cyanobacteria and microalgae under mixotrophic growth (glucose, acetate, ethanol, and glycerol as organic carbon source) as distinctive biological agents for the degradation of xenobiotic organic pollutants.

4. *Chlorella sorokiniana*

The freshwater green microalga *Chlorella sorokiniana* is non-motile and unicellular (Shihira & Kraus, 1965; Huss et al., 1999). Figure 5 shows a photomicrograph of the species, its tallus is formed by spherical or ellipsoidal cells of approximately 3 – 5 µm diameter, but grown on glucose it can became larger (Sorokin, 1959), and glucosamine as a dominant cell wall component (Huss et al., 1999).

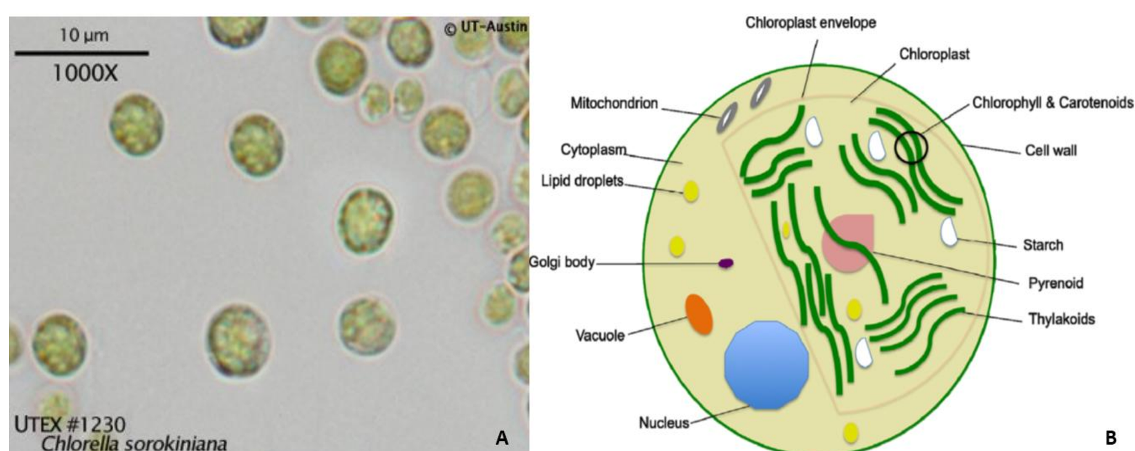

Figure 5 – The freshwater microalga *Chlorella sorokiniana* observed in: A. microscopy with resolution of 1000x (Photo from Culture Collection of Algae at The University of Texas at Austin, https://utex.org/products/utex-1230. Accessed in May 2017), and B. schematic ultrastructure of genus *Chlorella* representing different organelles (Safi et al., 2014).

In relation to biotechnological potentials of this microalgae, Sorokin (1959) showed that *C. sorokiniana* does not produce norspermidine, a natural chemical that is produced by some algae, bacteria, and terrestrial plants, with antitimoral properties. However, *C. sorokiniana* can produce lutein. Also, secondary carotenoids are never produced, but this algae shows hydrogenase activity (Huss et al., 1999). It turns white in old inorganic cultures, and even more quickly on glucose media (Sorokin, 1959).

Reproduction (Fig. 6) occurs exclusively by asexual cycle by means of autospores production (Kessler & Huss, 1992; Safi et al., 2014).

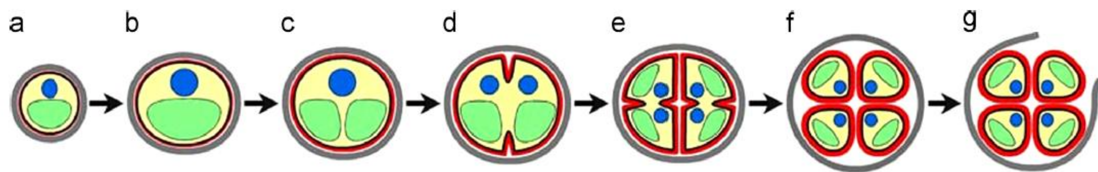

Figure 6 – Reproduction phases of genus *Chlorella* sp: a. early cell-growth phase; b. late cell-growth phase; c. chloroplast dividing phase; d. early protoplast dividing phase; e. late protoplast dividing phase; f. daughter cells maturation phase; g. hatching phase (Safi et al., 2014).

In accordance with international algal taxonomy (http://www.algaebase.org, accessed in May 2017), the specie *C. sorokiniana* is classified as:

Empire Eukaryota
Kingdom Plantae
Subkingdom Viridiplantae
Infrakingdom Chlorophyta
Phylum Chlorophyta
Subphylum Chlorophytina
Class Trebouxiophyceae
Order Chlorellales
Family Chlorellaceae
Genus *Chlorella*

This microalga has an amazing environmental plasticity, can grow over a wide range of temperature, tolerates high light intensities (Juntila et al., 2015), and has been widely used in mixotrophic studies, especially with glucose as organic carbon source, as reported in Table 1 together with other microalgae that also grow in mixotrophic conditions.

Table 1 - Studies of mixotrophy in some microalgae species and the organic carbon used in the cultures.

Microalgae Species	Organic Carbon Source	Reference
Chlorella sorokiniana	Glucose	Wan et al. (2011) Li et al. (2014; 2015); Juntila et al. (2015)
Chlorella vulgaris	Glucose Glucose and glycerol	Mayo & Noike (1994); Liang et al. (2009)
Chlorella pyrenoidosa	Glucose	Yang et al (2000); Kamjunke et al (2008)
Chlorella regularis	Glucose, galactose, acetic acid, etanol, acetaldehyde and pyruvic acid	Endo et al. (1973)
Chlorella minutissima	Glucose, glycerol and acetate	Higgins & VanderGheynst (2014)
***Chlamydomonas* sp**	Glucose	Kamjunke et al (2008)
Neochloris oleoabundans	Glucose	Giovanardi et al. (2013); Baldisserotto et al. (2014); Giovanardi et al. (2014)
Pavlova lutheri	Acetate and bicarbonate	Guihéneuf et al. (2009)
Tisochrysis lutea	Glycerol	Alkhamis & Qin (2016)
Isochysis galbana	Glycerol	Alkhamis & Qin (2015)
Botryococcus braunii	Glucose	Wan et al. (2011)
***Scenedesmus* sp**	Glucose, glycerol and acetate	Dittamark et al, (2014)
Phaeodactylum tricornutum	Glucose, glycerol and acetate	Liu et al. (2009)
***Nannochloropsis* sp**	Glucose	Cheirsilp & Torpee (2012)

It is in reason to its robustness and plasticity, and ability to grow mixotrophically, that we have chosen *C. sorokiniana* for the present research. In the pages that follow, we explore the physiology and relationship of the freshwater microalgae *C. sorokiniana* grown under mixotrophy (glucose as organic nutrition) with toxicants. Doing this, we expected to approach more closely what happens in natural environments and contribute to fulfill a gap in the scientific literature.

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OBJECTIVES

GENERAL OBJECTIVES

Considering the importance of mixotrophy in the ecosystems and in biotechnology of microalgae, and the lack of information about this metabolic pathway, the present thesis aimed at studying the influence of mixotrophic metabolism in the freshwater microalga *Chlorella sorokiniana* (Chlorophyta) in relation to its physiology, biomass production and toxicity responses in medium with organic carbon addition. Thus, our results fill an important gap in the area of phytoplankton's ecophysiology.

Specific Objectives

To accomplish our goal, we divided this thesis into 4 chapters, each one as being one specific objective and are in article format. These specific objectives are presented as follow:

- *Chapter 1* – Comparative study of growth and photosynthetic responses of *Chlorella sorokiniana* (Chlorophyta) under photoautotrophic and mixotrophic conditions;
- *Chapter 2* – An investigation onto the effects of glucose on the biochemical composition of the freshwater microalga *Chlorella sorokiniana*;
- *Chapter 3* – An investigation onto Cd toxicity to chlorella sorokiniana in mixotrophy and photoautotrophy: a Bayesian approach;
- *Chapter 4* – Effects of TiO_2 nanoparticles in different metabolic pathways in the freshwater microalga *Chlorella sorokiniana*;

Hypothesis

Based on the review on microalgae mixotrophy, we hypothesize that the physiological responses of *Chlorella sorokiniana* differ if it is either in mixotrophic or photoautotrophic growth. For instance, we recall that in natural environments,

microalgae are exposed to all sorts of dissolved organic materials, so in reality it may exchange from photoautotrophic to mixotrophic metabolism in different moments, resulting in different physiological responses. This ca be an effect of the extra source of energy and structural carbon of the dissolved organic materials present in the environment.

CHAPTER 1

Comparative Study of Growth and Photosynthetic Responses of *Chlorella sorokiniana* (Chlorophyta) Under Photoautotrophic and Mixotrophic Conditions

ABSTRACT

The variety of products derived from microalgae has stimulated studies in the optimization of their growth and cultivation, especially, in mixotrophic conditions. This study aimed at investigating the biomass production and photosynthetic activity of freshwater microalga *Chlorella sorokiniana* under mixotrophic (1.0 $g.L^{-1}$ glucose), the photo-mixotrophic (glucose added in stationary phase) and photoautotrophic conditions (no glucose). The results showed pH changes after glucose addition, reaching pH 11.62 in mixotrophic and 10.47 in photo-mixotrophic cultures, which limited the microalgal growth. Highest biomass was obtained in the mixotrophic culture in comparison with the photo-mixotrophic one. Rapid light saturation curves showed that α (photosynthetic efficiency, 1.69) and rETR (relative electron transport rate, 565.61) were higher in the mixotrophic cultures, whereas the highest I_k (irradiance saturation, 386.68) was obtained in the photoautotrophic ones. In the photo-mixotrophic cultures, photosynthetic activity varied during glucose consumption, decreasing the maximum quantum yield F_v/F_m after glucose addition, indicating change in metabolism, from photoautotrophy to mixotrophy by the microalga.

Key word: physiology, mixotrophy, photosynthetic efficiency, microalgae cultivation.

INTRODUCTION

In the last five decades, the interest in microalgae biomass has grown due to their biotechnological potential (Rodríguez-López, 1966; Yang et al., 2000; Perez-Garcia et al., 2006; Giovanardi et al., 2014) and products they synthesize. Microalgae are microorganisms that can convert light energy into sugar and further in different molecules, such as proteins, lipids, vitamins, polysaccharides, pigments, polymers and

others (Perez-Garcia et al., 2011). They can be grown in cultures that do not require large areas of arable lands, being possible to grow them in non-arable land, and depending on species robustness, it can be cultivated in wastewaters and other residual effluents. This can help reduce the costs of their production and decrease the eutrophication potential of effluents (Wan et al., 2011; Li et al., 2014; Marchello et al., 2015).

During photoautotrophic growth, the microalgae perform photosynthesis and take up inorganic carbon (CO_2) from the environment as carbon source while using light (natural or artificial) as energy source (Perez-Garcia et al., 2011). However, as photoautotrophic cultures get denser, their growth become light limited due to self-shading caused by the neighbor microalgae cells (Yang et al., 2000; Perez-Garcia et al., 2011). Considering that some species have the capacity to grow in the absence of light using organic carbon, such as glucose or acetate as carbon and energy sources, these species can eventually reach higher biomass in cultures than strict photoautotrophic ones (Juntila et al., 2015). However, heterotrophic cultures can have increased costs due to the addition of an organic carbon source at the same time that it can stimulate the growth of undesirable microorganisms (Ip et al., 1982; Giovanardi et al., 2014; Li et al., 2014) that can impair the algal growth.

Due to the capacity of microalgae to adjust their metabolism, most studies have considered mixotrophic cultivation as a method for achieving high biomass densities in a short period of time (Giovanardi et al., 2014; Li et al., 2014). Mixotrophy is a process whereby CO_2 and organic carbon are simultaneously used by the microalgae as carbon source. In mixotrophy, oxidative processes, like photosynthesis and respiration, can occur concurrently, generating energy for the microalgae (Heredia-Arroyo et al., 2010; Gionavardi et al., 2014). In several studies, microalgae cultures in mixotrophic growth outperformed the sum of photoautotrophic and heterotrophic conditions (Li et al., 2014).

Mixotrophy is a complex mechanism through which carbon and energy are obtained. Microalgae maintain its photosynthetic apparatus and the enzymes of the Calvin cycle, and at the same time that they require membrane transporters for the

uptake of organic carbon for the production of ATP (adenosine triphosphate) by oxidative phosphorylation (Heredia-Arroyo et al., 2010).

Mixotrophic growth is less susceptible to photoinhibition and photooxidative damages, especially in closed bioreactors that accumulate oxygen (Li et al., 2014), and light determines the consumption of organic carbon during mixotrophy (Rodríguez López, 1966). In some species, as *Chlorella sorokiniana*, cells grew faster under light intensities between 100 and 500 µmol photons m^{-2} s^{-1} (Li et al., 2014), however, in *C. vulgaris*, the blue light can inhibits the expression of the hexose/H^+ symport protein genes (Perez-Garcia et al., 2011). One of the advantages of mixotrophy in microalgae cultures is that the excess of oxygen gas released through photosynthesis in closed bioreactors can be used by the cells during the aerobic respiration of the organic carbon added to the medium, reducing the photooxidative damages (Chojnacka & Noworyta 2004).

In spite of the potential of the mixotrophy approach for microalgae production, studies focusing in the physiology mechanisms during mixotrophy in these organisms are scarce (Wijffels & Barbosa, 2010; Giovanardi et al., 2014). Among the variety of organic carbon sources used for both mixotrophy and heterotrophy in microalgae that can be found in literature, glucose ($C_6H_{12}O_6$) has been the most used one (Mayo et al., 1994; Giovanardi et al., 2014). Glucose molecule carries more energy than other sources of organic carbon, for example, 1 mol of glucose generates approximately 2.8 $kJ.mol^{-1}$ of energy for the cell compared to ~ 0.8 $kJ.mol^{-1}$ from acetate (Perez-Garcia et al., 2011). In *Chlorella* sp, glucose changes the metabolic pathways of carbon assimilation, the size of the cells and the volume of storage material (starch, lipids, protein, chlorophyll, RNA and vitamins; Martinez et al., 1991). *Chlorella* sp possess a hexose/H^+ symport system in its membrane responsible for the uptake of glucose from the medium, creating an eletrogenic potential (Tanner, 1969; Komor, 1973; Komor & Tanner, 1974, 1976).

Another aspect that influences the health of microalgae and their production is the photosynthetic efficiency. This can be determined by the use of variable chlorophyll *a* fluorescence (Schreiber et al., 2002). It is known that light energy hitting chlorophyll *a* molecule can either be absorbed to develop photochemical reactions or re-emitted as

heat, or fluorescence. According to Schreiber et al. (2002), the proportion of energy used for photochemical reactions and the amount of fluorescence emission from chlorophyll *a* is inversely related. To determine the photosynthetic efficiency in microalgae, a rapid and non-invasive technique known as pulse amplitude modulated (PAM) fluorometry (Schreiber et al., 2002; White et al., 2011) has been widely used. This technique allows the determination of the maximum quantum efficiency of photosystem II, PSII (F_v/F_m, the maximum total energy absorbed by chloroplasts when exposed to photosynthetically supersaturating light), the photochemical energy (*q*P, energy that is directed to photochemical reactions), and non-photochemical energy (NPQ, energy that is dissipated mostly as heat, not used in photosynthesis) by microalgae cells (Müller et al., 2001; Hendrickson et al., 2004).

Among the microalgae commonly investigated in mixotrophy, the genus *Chlorella* dominates; because it is robust, shows fast growth and has great importance in the food industry, as production of lipids (Rodríguez-López, 1966; Rosemberg et al., 2014; Li et al., 2015). Among the species of this genus, it has been shown that *Chlorella sorokiniana* is a good candidate for mixotrophic growth because of its ability to grow in organic carbon sources (Wan et al., 2011; Rosenberg et al., 2014). However, details of the physiology of this microalga under mixotrophic conditions are important to optimize its potential for biotechnological applications (Wijffel & Barbosa, 2010).

In the present study, the green microalgae *Chlorella sorokiniana* (Chlorophyta) was cultivated under controlled laboratory conditions in three different growth situations (photoautotrophic, mixotrophic and photo-mixotrophic). Our objective was to understand the photosynthetic activity of this microalga during mixotrophic growth. The present results are a contribution to the improvement of the production of *Chlorella sorokiniana* biomass for biotechnological interests.

MATERIAL AND METHODS

Algal cultures and experimental design

The microalga *Chlorella sorokiniana* (Chlorophyta); it was obtained from the freshwater microalgae culture collection at Federal University of São Carlos, Brazil (WDCM 835). Unialgal cultures were performed in 1000 mL Erlenmeyer flasks containing 500 mL of modified rich nutrient BG11 medium (Rippka et al., 1979). Controlled conditions of temperature (24 ± 1 °C), light intensity (190 µmol photons m^{-2} s^{-1}) and photoperiod (12 h light/12 h dark) were used throughout. Illumination was applied at the bottom of the flasks, with an optical path inside the flasks of ~ 7 cm, and the intensity used is among the range proposed by Li et al. (2014) for growth of *C. sorokiniana*, e.g., 100 – 500. Shaking was performed four times daily by hand. The initial inoculum (10^5 cells.mL^{-1}) was obtained from exponentially growing cultures acclimated in the same conditions as they would be submitted to. The inoculum, which was cultivated in photoautotrophic conditions, showed F_v/F_m (maximum quantum efficiency) of 0.70. This value suggesting healthy cells and agrees with previous literature (Kumar et al., 2014).

Three culture conditions (photoautotrophic, mixotrophic and photo-mixotrophic) with three experimental replicates were performed. The photoautotrophic cultures contained just the modified BG11 (inorganic) medium according to that described in Rippka et al. (1979). In the mixotrophic culture conditions, glucose was added in the concentration of 5 x 10^{-3} mol.L^{-1} (1 g.L^{-1}) as organic carbon source in the beginning of experiment. This glucose concentration is reported in the literature as one that results in high growth and optimum algae performance (Liang et al., 2009; Kong et al., 2011), and previous studies were also carried out with glucose, sucrose and fructose, with the media enriched with glucose presenting better results than the other organic sources used. The third condition, photo-mixotrophic (two stage batch culture), was tested to stimulate the biomass increase in stationary phase in photoautotrophic conditions, e.g., when light limiting conditions would prevent further cell growth in the BG11 medium used. So, for the photo-mixotrophic condition (a culture with two-stages, photo and

mixotrophic), the culture was grown first under the photoautotrophy up to stationary phase (4th culture day), after which, 5×10^{-3} $mol.L^{-1}$ glucose was added.

pH and Chlorophyll *a*

Samples were taken daily and at the same time in the morning. pH was determined with a pH-meter (HANNA® Instruments, USA), while chlorophyll *a* concentration was determined by *in vivo* fluorescence using a fluorimeter (Turner Designs, Model Trilogy – U.S.A.). The concentration of chlorophyll *a* was obtained from a calibration curve performed by plotting fluorescence intensity *vs* concentration of chlorophyll *a* from exponentially growing cultures of *Chlorella sorokiniana*. This resulted in a linear curve that was adjusted through linear regression and the equation used for calculating the concentration of chlorophyll *a* in the samples.

Cell Density and Specific Growth Rate

The number of cells in the culture was quantified daily in a cytometer Muse® Cell Analyzer (Merck Millipore) and the results expressed in $cell.mL^{-1}$. The specific growth rates (μ) were calculated through graphic representation of the natural logarithm of the number of cells per mL as function of time. The linear regression from the straight line so obtained was calculated for the exponential growth phase. In this case, the angular coefficient represents the specific growth rate. For its calculation, we used the first 4 points of figure 1.1B, e.g., data points at days 0, 1, 2 and 3.

To determine if the growth curves showed sigmoidal behavior, the Boltzman adjustment (1872) was used, using chlorophyll a ($mg.L^{-1}$) data measured daily, in accordance with the equation I:

$$y(t) = [B/1 + e^{-(t-\tau).\mu}] + A \qquad (I)$$

where y(t) is the chlorophyll *a* concentration ($mg.L^{-1}$), B is the maximum point of the curve with the same unit of y(t), A is the initial value with the same unit of y(t), t is time, τ is the parameters that determines the inflection point of the curve, and μ is the growth rate ($time^{-1}$).

Photosynthesis Parameters

Chlorophyll fluorescence was obtained daily with a pulse amplitude modulated fluorimeter, Phyto-PAM (Heinz-Walz Effeltrich, Germany). Variable fluorescence (F_v) was determined by the difference between F_o and F_m, and the maximum quantum yield (F_v/F_m) was measured after 15 min of dark adaptation of the samples (Schreiber et al., 1986; Lombardi & Maldonado, 2011) in accordance with the equation II:

$$F_v/F_m = (F_m - F_o)/F_m \qquad \text{(II)}$$

where F_v/F_m is the maximum quantum yield, F_m is the maximum effective fluorescence, and F_o is the initial effective fluorescence.

F_o represents chlorophyll *a* fluorescence emission produced by the excitation of the light harvesting complex (LHC) before energy transference to PSII reaction center (Krause & Weis, 1991), while F_m represents the maximum fluorescence when primary electron acceptor of PSII is reduced and the reaction center remains unable for charge separation (Nedbal et al., 2000).

For the light-adapted state, cells were exposed to actinic light at 128 μmol photons m^{-2} s^{-1} for 10 minutes. Variable fluorescence ($F_v' = F_m' - F_o'$), photochemical *quenching* (qP), non-photochemical *quenching* (NPQ) (Roháček, 2002) were then obtained during exponential phase of growth. The fluorescence parameters were normalized in relation to F_o according to Roháček & Barták (1999).

The relative electron transport rate (rETR) was obtained using the model of Eilers & Peeters (1988) with the following the equation II.

$$\text{rETR} = \text{PAR} / (a\,\text{PAR}^2 + b\,\text{PAR} + c) \qquad \text{(II)}$$

where PAR is the irradiance (μmol photons m^{-2} s^{-1}) and a, b and c are the adjustment parameters. With these parameters the initial slope ($\alpha = c^{-1}$), where only light limits photosynthesis, the maximum photosynthesis rate ($P_{max} = [b + 2\,(ac)^{1/2}]^{-1}$) and the optimal irradiance saturation (I_k) were calculated in the exponential phase of growth.

Statistical Analysis

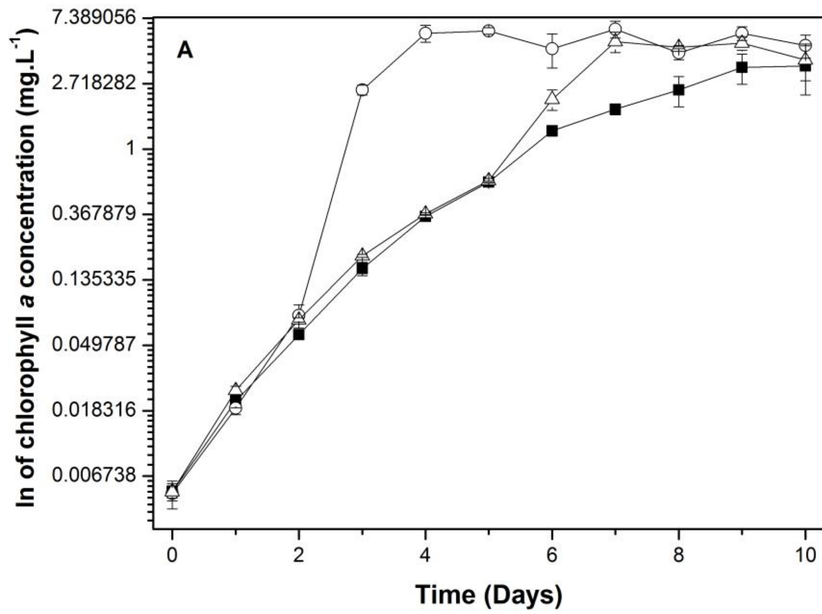
The results were tested for normality and homogeneity, and significant differences between means of each variable ($p < 0.005$) were tested by one-way ANOVA and Tukey's post-hoc analysis. Data were analyzed using Origin Pro (version 8.5.0) and Assistat (7.7 beta) software.

RESULTS

Growth Parameters

Figure 1.1A shows the concentration of chlorophyll *a* in the cells throughout the experimental time. It increased for all treatments, but a sharper increase was observed for the mixotrophic condition up to the 4th day, after which no extra increase in chlorophyll *a* was observed for this culture. The values in the photo-mixotrophic cultures followed the photoautotrophic one until the 5th, but increased after the addition of glucose.

As observed in Figure 1.1B, the population density (cell.mL^{-1}) increased exponentially in all cultures, especially in the mixotrophic one whose exponential phase ended in the 3rd day. After glucose addition in the photo-mixotrophic culture a new exponential growth phase was detected. After the 8th culture day, no differences in the number of cells among the three cultures were observed.



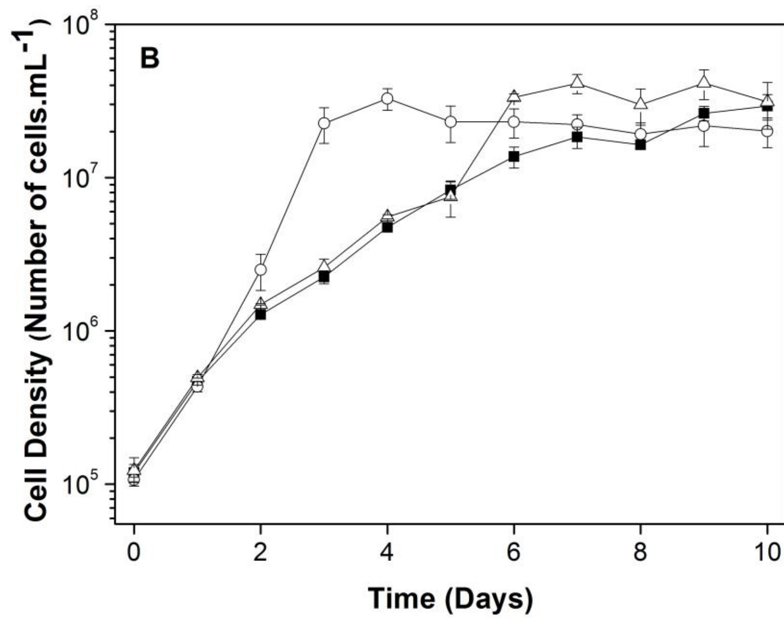


Figure 1.1 – Ln values of chlorophyll *a* concentration in mg.L^{-1} (A) and density in cells.mL^{-1} (B) in *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circle) and photo-mixotrophic (empty triangle) growth conditions. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Growth curves (Fig. 1.2) based on the concentration of chlorophyll *a* ($mg.mL^{-1}$), show that the three cultures were similar until the second day, from which the mixotrophic culture (open circles) presented exponential growth with a rate of 1.56 d^{-1}. The photoautotrophic (full squares) and photo-mixotrophic (open triangles) growth was similar until day 4, when glucose was added in the latter, which presented an exponential phase with a rate of 1.26 day^{-1} between days 5 and 7, remaining until the end of the experiment. There were no statistical differences ($p = 0.0181$) between the cultures with and without glucose between the 7th and 10th days. However, the photoautotrophic culture did not present a characteristic exponential phase, with a slow growth (0.07 d^{-1}) but without statistical differences ($p = 0.3763$) with the other treatments on the last day of the experiment.

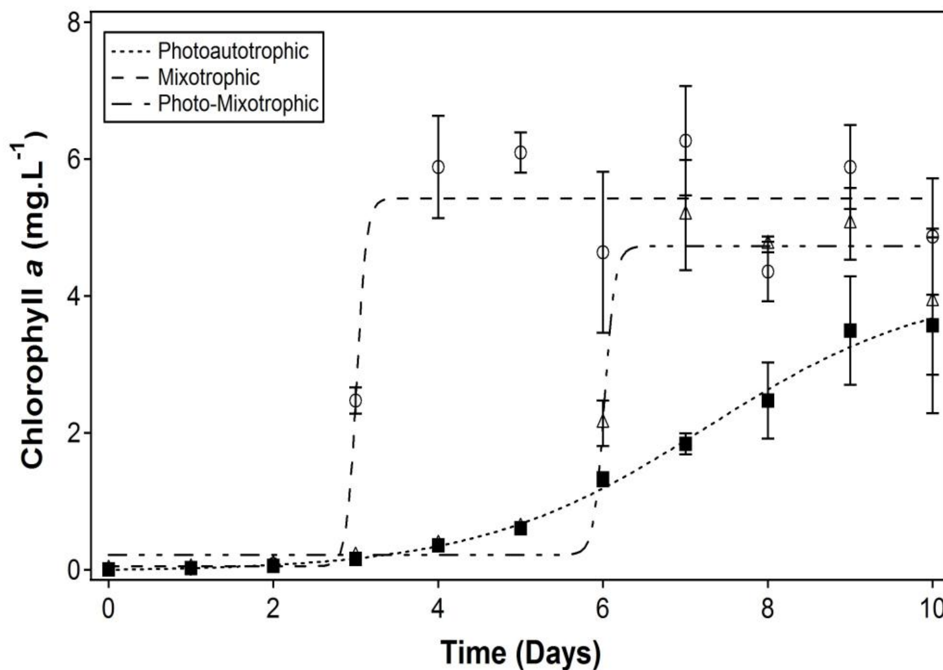


Figure 1.2 – Sigmoid growth curves based on chlorophyll *a* concentration ($mg.L^{-1}$) of *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circle) and photo-mixotrophic (empty triangle) growth conditions. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

When the same data are plotted in the form of sigmoid curves (Fig. 1.2), we can observe that both mixotrophic and photo-mixotrophic cultures followed the sigmoid model, with growth rates of 16.81 d^{-1} and 15.52 d^{-1}, respectively. However, when the data of the photoautotrophic culture were plotted following the sigmoid model, the curve formed followed a linear growth, confirming that there was no exponential growth in this culture during the experimental time.

pH

The values of pH (Figure 1.3) were similar for all treatments up to the third experimental day in the cultures, but differed significantly ($p = 0.0001$) after glucose addition, with decrease in pH in mixotrophic and photo-mixotrophic cultures. It steadily increased for the photoautotrophic culture up to pH 9.6 in the last day of experiment. Photo-mixotrophic culture pH was similar to photoautotrophic until glucose addition, and the mixotrophic culture showed the highest pH (~ 11.4).

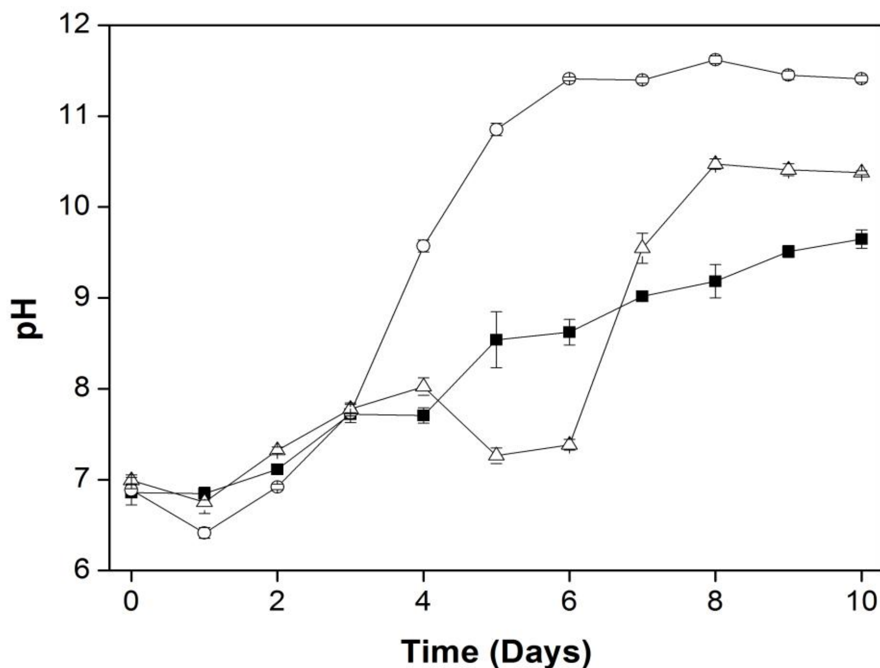


Figure 1.3 - Time-course pH values in *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circle) and photo-mixotrophic (empty triangle) growth conditions. Error bars

mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Photosynthetic Parameters

Immediately after the inoculation (day 0), there was a decrease in F_v/F_m values in all cultures (Figure 1.4). In the photoautotrophic and photo-mixotrophic cultures, it increased during 24 hours. Again, we observed differences between the photoautotrophic and photo-mixotrophic cultures after glucose addition. In the later, F_v/F_m decreased to 0.56 and increased after the 6th day (with maximum value of 0.74), decreasing thereafter. Meanwhile, the values of F_v/F_m in the photoautotrophic condition was maintained around 0.70. A different behavior was observed for the mixotrophic culture, where F_v/F_m decreased after inoculation (0.57), but reached the maximum value of 0.74 in the 3rd day, after which it decreased (0.60).

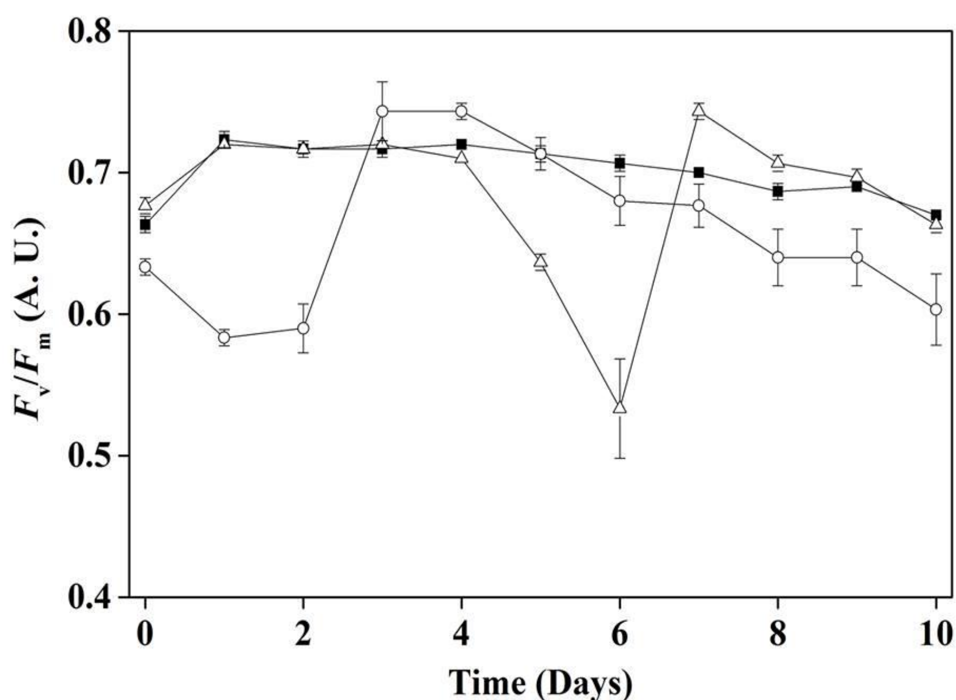


Figure 1.4 – Time-course of maximum quantum efficiency (F_v/F_m ratio) in *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circles) and photo-mixotrophic (empty triangle) grown conditions. A.U. means Arbitrary Unit. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

The parameters of photosynthesis-irradiance curve and *quenchings* (Table 1.1) showed that there were differences among the three cultures. The mixotrophic culture showed the highest values for rETR and α, while the photoautotrophic culture showed the highest value for I_k. The photo-mixotrophic cultures showed the lowest values for rETR and I_k, and an intermediary value for α.

Table 1.1 – Parameters of photosynthesis-irradiance curve ($rETR_{max}$, I_k and α) and *quenchings* (*q*P and NPQ) of *Chlorella sorokiniana* under photoautotrophic, mixotrophic and photo-mixotrophic growth conditions. For each sample, values are means ± s.d. (n = 3). Different letters means statistical difference by Tukey test, with 5% of significance.

Cultures	**$rETR_{max}$ (P_{max})** (µmol electrons m^{-2} s^{-2})	**I_k** (µmol photons m^{-2} s^{-2})	**α** (mg C. mg Chl a^{-1} [µmol photons m^{-2} s^{-1}])	***q*P**	**NPQ**
Photoautotrophic	542.77 ± 2.20 ^{b}	386.68 ± 5.31 ^{a}	1.41 ± 0.02 ^{c}	0.88 ± 0.05 ^{a}	0.04 ± 0.01 ^{c}
Mixotrophic	565.61 ± 4.21 ^{a}	339.85 ± 3.05 ^{b}	1.69 ± 0.05 ^{a}	0.44 ± 0.03 ^{b}	0.40 ± 0.04 ^{a}
Photo-Mixotrophic	476.60 ± 9.65 ^{c}	316.53 ± 14.25 ^{c}	1.51 ± 0.04 ^{b}	0.83 ± 0.06 ^{c}	0.36 ± 0.03 ^{b}

In the present research, the photochemical energy (*q*P) used by the microalgae for photosynthetic activity during exponential growth phase reached 0.88 and 0.83 in photoautotrophic and photo-mixotrophic cultures, respectively. The energy dissipation as heat (NPQ) was higher in both mixotrophic (0.40) and photo-mixotrophic (0.36) cultures after glucose supplementation, but lower (0.04) in the photoautotrophic condition.

DISCUSSION

The addition of glucose stimulated the growth of *C. sorokiniana*. This is in accordance with the results in White et al. (2011) and Giovanardi et al. (2014), where the authors suggest that microalgae use mixotrophic metabolism to grow in nutrient rich media. The organic carbon source supplies the cells with extra energy and carbon for the construction of new cellular structures and reproduction (Rodrígues-López, 1966; Martínez et al., 1997). Cheirsilp & Torpee (2012) reported that *Chlorella* growing in mixotrophy with glucose as a carbon source was better (final biomass ~ 1.3 $g.L^{-1}$) than in photoautotrophic conditions (final biomass ~ 0.6 $g.L^{-1}$). The difference between cultures with glucose addition can be due to the amount of organic carbon available per cell unit (10^{-8} g $glucose.cell^{-1}$ in mixotrophic growth) compared to photo-mixotrophic, which, because of its higher density, the amount of glucose per cell was about 100 times lower (1.8 x 10^{-10} g $glucose.cell^{-1}$).xvie

Yang et al. (2000) found increased chlorophyll *a* concentration and cell density during mixotrophic cultivation of *Chlorella pyrenoidosa* – original name of *Chlorella sorokiniana* (Rosenberg et al., 2014) – with glucose as organic carbon source. However, it should be noted that other factors may contribute to the biomass and chlorophyll *a* increase, such as low pH and light intensities between 100 and 200 µmol photons m^{-2} s^{-1} (Ip et al., 1982; Mayo et al., 1994; Li et al., 2014). Light intensity in our experiment (190 µmol photons m^{-2} s^{-1}), may have contributed to the increase in growth parameters (chlorophyll *a* concentration and cell density). Under our experimental conditions, mixotrophic cultures showed the best growth in shorter time in comparison with the photoautotrophic and photo-mixotrophic cultures. Probably, the refixation of CO_2 released by the aerobic respiration in the presence of glucose by microalgae and bacteria (Li et al., 2014), through photosynthesis has also been proved to be critical for biomass synthesis in mixotrophic conditions (Martínez et al., 1997), which provides higher carbon availability to produce energy storage products.

The increase in chlorophyll *a* concentration in the mixotrophic culture may be due to the need of the cells to maximize light energy capture, since the cultures were at high cellular densities, limiting the available light per cell unit (auto-shading) to photosynthetic activity (Schenk et al., 2008; Giovanardi et al., 2014). In cultures

without glucose addition, chlorophyll *a* production was lower, which was also observed by Rosenberg et al. (2014) in the cultivation of different species of *Chlorella*.

According to the microalgae species and the organic carbon source, the concentration of chlorophyll *a* may vary (Ip et al., 1982; Liu et al., 2009; Alkhamis & Qin, 2016). For example, Ip e al. (1982) and Wan et al. (2011) reported a reduction in chlorophyll *a* concentration in mixotrophic cultures with glucose in *Chlorella zoofingiensis* and *Botryococcus braunii*, respectively. However, Alkhamis & Qin (2016) verified an increase in chlorophyll *a* concentration in mixotrophic cultures of *Tisochrysis lutea* supplemented with glycerol as an organic carbon source.

The high values of growth rates in the mixotrophic and photo-mixotrophic cultures in relation to the photoautotrophic ones indicate that the addition of glucose stimulated the growth of microalgae, as also observed by Rodríguez-López (1966), Martínez et al. (1997) and White et al. (2011). Similar to that found in the present study, Rosenberg et al. (2014) observed that glucose increased the growth rate of *C. sorokiniana*, and Baldisserotto et al. (2014) reported a seven-fold increase in the growth rate in mixotrophic cultures of *Neochloris oleoabundans*. In the present study, the growth rates considering the sigmoid model for mixotrophic and photo-mixotrophic cultures were about 23 and 17 times higher, respectively, than photoautotrophic one, which confirms the action of glucose on population growth.

The pH decrease observed after the addition of glucose in both mixotrophic and photo-mixotrophic cultures is in accordance with the results of Kong et al. (2011), in which mixotrophic cultures of *Chlorella vulgaris* with glucose as organic carbon source also presented pH decrease. According to Komor et al. (1989) and Giovanardi et al. (2014), such pH decrease after glucose addition is due to the consumption of carbohydrates and more respiratory than photosynthetic activity by the microalgae, increasing CO_2 in the medium rather than its uptake.

Komor & Tanner (1974; 1976) studied the eletrogenic mechanisms of hexose transport by membranes in microalgae of the genus *Chlorella* and showed that there is a co-transport system involving glucose and H^+. On contact with glucose there is a stimulation of gens in microalgae through which synthesis of hexose/H^+ symport system proteins occurs (Komor & Tanner, 1971; Perez-Garcia et al. 2011). Doing this, the

microalgae can take up sugar and H^+, always following the stoichiometry of one H^+ per sugar molecule taken up (Komor & Tanner, 1974).

The hexose/H^+ symport system involves the consumption of one ATP molecule per hexose/H^+ transported via membrane. The energy sources for this can be the cyclic photophosphorylation in the thylakoids and the aerobic respiration in mitochondria (Komor & Tanner, 1974). Other carbohydrates, as sucrose and starch, increase the O_2 consumption per carbohydrate molecule and the ATP used to, approximately 1.5 ATP per molecule of carbohydrate (Decker & Tanner, 1972; Komor & Tanner, 1974). Inside the mixotrophic cell, the glucose can follow different pathways: approximately 85% is converted to oligosaccharides (~ 50% of sucrose) and polysaccharides (~ 30% of starch) and the rest remains as free glucose, which is used for the production of ATP through Krebs cycle or glyoxalate cycle (Yang et al., 2000; Perez-Garcia et al., 2011).

The entry of protons along with glucose tends to reduce intracellular (cytoplasmic) pH, which affects physiological processes. In order to avoid this, the cell has homeostasis maintenance mechanisms (Jones et al., 1975; Ullrich-Eberius et al., 1978), in which part of the protons that enter the cells are eliminated via the proton pump through the action of the enzyme H^+-ATPase and, the other part, about 10%, is used in both glycolysis and cellular respiration. To keep the membrane polarized and compensate for these 10% of protons sequestered in metabolic processes, the cell sends to the external medium K^+ with ATP consumption (Trombala, 1981; Komor et al., 1989; Taylor et al., 2012). This proton-buffering intracellular system increases respiration and elimination of CO_2 into the medium during glucose uptake, which probably explains the reduction in pH (Fig. 1.3) in the first 24 hours after addition of glucose in both the mixotrophic (empty circle) and in the photo-mixotrophic (empty triangle).

The expected PSII maximum quantum yield (F_v/F_m) around 0.70 for the photoautotrophic and photo-mixotrophic cultures, is an indication that microalgae are healthy and not nutrient or light-limited (Kromkamp & Peene 1999). The decreased F_v/F_m in the mixotrophic cultures that occurred after glucose addition can be probably due to the reduction of photosynthesis under mixotrophy. In mixotrophic cultures, cells can assimilate organic carbon to produce energy leading to a lesser dependence on light,

increasing their respiratory rate (Decker & Tanner, 1972; Yang et al., 2000; Heredia-Arroyo et al., 2010; White et al. 2011) and decreasing the F_v/F_m without losses in cell yield (Baldisserotto et al., 2012).

The decrease in photosynthetic activity in mixotrophic conditions observed in our results is widely described in literature (Oesterhelt et al., 2007; Liu et al., 2009). According to Komor et al. (1989) glucose is rapidly consumed by the algae, being completely assimilated in the first 24 hours, which complements the pH data and justifies the reduction of Fv/Fm. However, after that period, the increase in Fv/Fm indicates photosynthetic activity and, consequently, alkalinization of the culture medium. This confirms that photosynthesis returned to be the main growth and energy gain process for the alga, an effect also observed by Baldisserotto et al. (2014). After the third and seventh days for mixotrophic and photo-mixotrophic cultures, respectively, the Fv/Fm values exceeded 0.7 and were slowly reduced, but the chlorophyll *a* and cell density remained stable (without statistical differences, $p = 0.0181$; Fig. 1.1). The Fv/Fm 0.7 for the photoautotrophic cultures indicate that they were healthy and without nutritional limitation, which may be due to the culture medium used, modified BG11, which is rich in inorganic nutrients (Kromkamp & Peene, 1999).

The high rETR in mixotrophic cultures indicates an effect in the electron transport chain (White et al., 2011) in comparison with the photoautotrophic culture (controls). In this case, irradiance saturation (I_k) was obtained at ~ 387 µmol photons m^{-2} s^{-1}, but in mixotrophic cultures only at ~ 340 µmol photons m^{-2} s^{-1}. The cells in photoautotrophic cultures have the unique option to invest in photosynthetic activity (Falkowski & Raven, 2013), while in mixotrophic cultures, glucose is another source of energy (Komor & Tanner, 1971; Decker & Tanner, 1972). Mixotrophic cultures showed the best photosynthetic efficiency ($\alpha = 1.69$), what is in accordance with biomass production (chlorophyll *a* concentration and cell density). This can be related with the production of ATP via cyclic photophosphorylation by the microalgae cell, energy that is used for the uptake of glucose from the medium (Komor & Tanner, 1974). The present results showed that during mixotrophic growth, the *C. sorokiniana* presented the highest photosynthetic efficiency in comparison with photoautotrophic and photo-mixotrophic growth. According to Gacia et al. (1996) who studied the effects of light in

Caulerpa sp, such behavior can be due to less need for light and are expected in benthonic algae and shadow adapted plants.

The increase in *q*P in these cultures can indicate that more light energy was directed to photosynthesis during cell growth. This is expected in healthy microalgae cells, with no light and/or nutrients limitations (Müller et al., 2001). The low *q*P (0.44) in mixotrophic cultures can be due to the mixotrophic metabolism *per se*, in which the microalgae cells used energy from oxidative process in the mitochondria to produce ATP, depending less on the light energy for growth and reproduction (Baldisserotto et al., 2014). Overall, it indicates that energy generated by phosphorylation of glucose is more economic to microalgae than that produced through photosynthetic activities, what is supported by literature results (Komor & Tanner, 1971; Decker & Tanner, 1972).

The high value of NPQ (0.40), in mixotrophic cultures, with excess light intensity, can be due to a regulation mechanism that balances the absorption and utilization of light energy (NPQ), thereby minimizing the potential for photooxidative damage (Müller et al., 2001; Hendrickson et al., 2004; Vredenberg et al., 2009). According to Müller et al. (2001), this can occur through the regulation of the size of light-harvesting pigment antennae as a consequence of changes in gene expression and/or proteolysis. Furthermore, in cultures with glucose, the less need of photosynthetic activity can explain the higher NPQ by microalgae cells (Komor & Tanner, 1971; Decker & Tanner, 1972).

CONCLUSIONS

The present results showed that mixotrophic culture of *Chlorella sorokiniana* presented the highest growth and photosynthetic parameters values (even with irradiance saturation at ~ 340 µmol photons m^{-2} s^{-1}), resulting in higher biomass production in four days in comparison with photoautotrophic and photo-mixotrophic conditions. During mixotrophy, microalgae rapidly produced biomass using as energy sources both the organic carbon from glucose and light *via* photosynthesis. The addition of glucose when the culture enters in stationary phase was not as effective in time as glucose addition since the initial growth, in relation to time.

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CHAPTER 2

An Investigation onto the Effects of Glucose on the Biochemical Composition of the Freshwater Microalga *Chlorella sorokiniana*

ABSTRACT

The manipulation of the culture medium and environmental conditions may stimulate microalgae to synthesize biomolecules in different proportions. The present work aimed at investigating the growth and production of biomolecules by the microalga *Chlorella sorokiniana* in three different culture conditions: photoautotrophic (synthetic medium and light), mixotrophic (light, synthetic medium and 1 g.L^{-1} glucose) and photo-mixotrophic (1 g.L^{-1} of glucose when entering the stationary growth phase in synthetic medium). Because the cultures were not axenic, bacterial density was monitored daily. Variables such as chlorophyll *a*, cell density, biovolume and biochemical composition (proteins, carbohydrates and lipids) were determined, and proteins:carbohydrates (P:C) ratios were calculated. The results showed that the mixotrophic cultures had higher production of chlorophyll *a* (6.26 mg.L^{-1}), cell density (6.62 x 10^{7} cell.mL^{-1}), and lipids (0.06 pg.µm^{-3}). Photo-mixotrophic cultures showed the highest biovolume (360.5 µm^{-3}) and total carbohydrates (0.026 pg.µm^{-3}). The protein concentration decreased in the photoautotrophic and photo-mixotrophic cultures, but remained without significant alterations in mixotrophic ones. The reduction in P:C ratio in mixotrophic cultures can be due to a lack of equilibrium between of nitrogen in the medium and the added carbon. The bacterial density increased along with the microalgae in the cultures with addition of glucose, but with the stabilization of the growth of the microalgae, the bacterial density reduced.

Key-words: mixotrophy, lipid, protein, carbohydrates, colony forming unit.

INTRODUCTION

Microalgae are photoautotrophic microorganisms able to convert solar energy into chemical energy through photosynthesis (Li et al., 2014; Juntila et al., 2015). Besides the carbohydrates production during photosynthesis, microalgae can also

produce other energy-rich molecules (lipids, proteins, pigments, vitamins), which have biotechnological applications, such as in bioenergy, nutraceuticals, food, pharmaceutical and cosmetic industries (Yang et al., 2000; Baldisserotto et al., 2014; Giovanardi et al., 2014; Li et al., 2015).

The production of microalgal biomass aiming at the extraction of molecules of biotechnological interest (Baldisserotto et al., 2014), liquid cultures are employed with inorganic nutrients and light as energy source, while carbon sources can depend on the diffusion of atmospheric carbon dioxide (CO_2) into the medium (Perez-Garcia et al., 2011; Li et al., 2014). However, this photoautotrophic cultivation can end up being limited by light, because the higher biomass content causes self-shading that reduces the light availability to microalgal growth (Cheirsilp & Torpee, 2012). In these cultures, the excess of oxygen gas released by the microalgae can cause oxidative damages to the cells if kept in closed photobioreactors (Li et al., 2014).

Another mode of biomass production is the use of heterotrophy by microalgae for growth, replacing the fixation of atmospheric CO_2 with organic carbon sources dissolved in the culture media (Perez-Garcia et al., 2011; Cheirsil & Torpee, 2012). This mode of cultivation eliminates the use of light and shows fast growth and biomass production (Rosemberg et al., 2014). However, the heterotrophic cultivation has some disadvantages: not all microalgal species can rely on heterotrophy, the high costs of the organic carbon, inhibition caused by excess of organic substrate, and competition of substrate with other microorganisms, such as bacteria and yeasts (Cheirsil & Torpee, 2012; Li et al., 2014; Rosemberg et al., 2014).

Mixotrophy comes as an alternative to microalgae production reducing the disadvantages of photoautotrophy and heterotrophy (Sanders, 1991; Böechat et al., 2007). Mixotrophy is a metabolic process common among planktonic algae and protozoa (Stoecker, 1998). During mixotrophic growth, microalgal cells can utilize both inorganic and organic carbon as source of carbon for the production of biomolecules and ultra-cellular structures, and light and chemical energy as source of energy to metabolic activities (Yang et al., 2000; Giovanardi et al., 2014; Li et al., 2015). Some advantages of mixotrophy are high densities of microalgae cultures, high growth rate,

high dry biomass, high DNA generated, high lipid and protein content (Perez-Garcia et al., 2011; Cheirsil & Torpee, 2012).

As organic carbon source, the mixotrophic cultures can utilize glucose, fructose, sucrose, acetate and organic wastes from wastewater treatment plants and effluents of industrial food production (Rodrígues-López, 1966; Kaplan et al., 1986; Lee, 2004; Perez-Garcia et al., 2011; Giovanardi et al., 2013). Among them, glucose is the most common carbon source used, because it have more energy per mol than other organic sources (Kamjunke & Tittel, 2009; Perez-Garcia et al., 2011). The transport of glucose through the plasmatic membrane is well known, being the result of a glucose/H^+ symport system (Komor et al., 1989).

Some works have demonstrated that microalgae cells show changes in their morphology during mixotrophic growth conditions (Rodríguez-López, 1966; Endo et al., 1974; Baldisserotto et al., 2014; Giovanardi et al., 2014). These changes depend on the microalga specie and organic carbon source (Baldisserotto et al., 2012; Giovanardi et al., 2013). During cultivation of *Chlorella* sp. with different source of organic carbon, Rodríguez-López (1966) described giant and discolored forms due to the excess of starch in their cytoplasm, and Endo et al. (1974) reported that glucose cause morphological changes in this microalga, as cell size and volume densities of storage material (starch, lipids, protein, chlorophyll, RNA and vitamins). In mixotrophic cultures of *Scenedesmus* sp, Dittamart et al. (2014) observed that cells became swollen and some coenobic colonies separated into single cells.

Microalgae can accumulate proteins, carbohydrates and lipids, which vary among microalgal species and cultivation conditions (Xu et al., 2004; Garcia et al., 2005; Alkhamis & Qin, 2016). Usually, microalgae cells contains 15 – 52% proteins, 5 – 12% carbohydrates, and 4 – 70% lipids (Muller-Feuga et al., 2003). Depending of microalgae species, type and concentration of organic carbon, light intensity and nutrients, changes in the biochemical composition of the cells can occur (Wan et al., 2011; Cheirsilp & Torpee, 2012; Giovanardi et al, 2013; Alkhamis & Qin, 2016). Algae can be induced to synthesize more proteins in the optimal growth condition while the accumulation of lipids and carbohydrates are enhanced under unfavorable conditions

(Das et al., 2011), making them an ideal organism with vast biotechnological applications.

Among the biomolecules produced by microalgae, the interest in lipid production has increase in the last decades to find renewable fuels in substitution of petroleum, as ethanol, methane, hydrogen and biodiesel (Giovanardi et al., 2013). However photoautotrophic cultures have lower lipid content (< 20% of dry weight), whilst during stress condition, as nutritional deficiency or decrease in light intensity, photoautotrophic microalgae may increase the lipid content, reaching ~ 70% of dry weight (Das et al., 2011; Juntila et al., 2015; Alkhamis & Qin, 2016). During mixotrophic conditions, the addition of organic carbon, generally glucose, shift the nitrogen:carbon ratio towards the carbon, causing a similar effect caused by nitrogen starvation, reducing cell division rate and increasing the accumulation of lipids (Baldisserotto et al., 2014; Giovanardi et al., 2014; Li et al., 2014; Li et al., 2015).

A problem resulting from the addition of organic carbon in mixotrophic cultures is a possible bacteria contamination (Perez-Garcia et al., 2011; Higgins & VanderGheynst, 2014). These microorganisms can compete with microalgae for organic carbon, and, sometimes, limiting the growth of the microalgae, reducing the substrate or producing organic acids (Kamjunke & Tittel, 2009; Giovanardi et al., 2014). However, some environmental factors, such as alkalinity, photosynthetic activity, light intensity and oxygen concentration may limit the bacterial growth (Cordero et al., 2010; Amengual-Morro et al., 2012; Marchello et al., 2015) whether or not containing organic carbon in the medium. Some microalgae, as the genus *Chlorella* sp, limit the bacterial growth and others organisms by the production of substances that has bactericidal properties, as chlorelin (Pratt, 1944; Ryther, 1954). However, to use the potentiality of mixotrophic condition for biotechnological applications of microalgae, much has still to be known and understood about this metabolism under culture conditions.

The aim of this work was to understand the changes in biochemical composition (proteins, carbohydrates and lipids) and morphology of the cells of the freshwater microalga *Chlorella sorokiniana* during mixotrophy. The bacterial density in the culture medium also was analyzed to identify if bacteria influence the microalgal growth in mixotrophy.

MATERIAL AND METHODS

Algal cultures and experimental design

Non-axenic cultures of freshwater microalgae *Chlorella sorokiniana* were kept in 1000 mL Erlenmeyer flasks containing 500 mL of modified BG11 medium (Rippka et al., 1979). The cultures were carried out in controlled conditions of temperature (24 ± 1 °C), light intensity (190 µmol photons m^{-2} s^{-1}) and photoperiod (12 h light/12 h dark). Illumination was applied at the bottom of the flasks, with an optical path inside the flasks of ~ 7 cm. The initial inoculum (10^5 cells.mL^{-1}) was obtained from exponentially growing cultures acclimated in the same conditions of the experimental cultures.

Three culture conditions were performed: photoautotrophic (just inorganic medium), mixotrophic (inorganic medium and glucose as organic carbon) and photo-mixotrophic (the culture first grown under photoautotrophic conditions up to stationary phase, after which, glucose was added). For the photo-mixotrophic culture, glucose addition was performed in the 4th day. Both in mixotrophic and photo-mixotrophic culture conditions, glucose was added in the concentration of 5 x 10^{-3} mol.L^{-1} (1 g.L^{-1}) as organic carbon source.

Chlorophyll *a* and cell density

The chlorophyll *a* concentration was obtained from a calibration curve performed using a fluorimeter (Turner Designs, Model Trilogy – U.S.A.) by plotting fluorescence intensity *vs* concentration of chlorophyll *a* extracted from exponentially growing cultures of *Chlorella sorokiniana*. This resulted in a linear curve that was adjusted through linear regression and the equation used for calculating the concentration of chlorophyll *a* in the samples. The number of cells in the cultures, expressed as cell.mL^{-1}, was quantified daily in a cytometer Muse® Cell Analyzer (Merck Millipore).

Cytomorphology

For the analysis of the cytomorphology, samples were taken daily, fixed with formaldehyde (4% final concentration) until the moment of analysis. The cells (mean of 25 chosen randomly) were visualized under optic microscopy (Nikon Eclipse Phase Contrast 0.90 Dry) with a camera attached and the diameter measured using the software NIS – Elements F 4.00.00. The biovolume was calculated using equation I.

$$V = \pi/6 \times d^3 \qquad \text{(I)}$$

where, V is the biovolume and d is the diameter of the cell (Hillebrand et al., 1999).

Biochemical composition

Biochemical composition was determined during the exponential (3rd day) and stationary (5th day) phases to understand the differences in biomolecules production in two growth phases in batch growth (exponential and stationary). Samples for total proteins (50 mL) and carbohydrates (20 mL) analysis were taken, centrifuged and frozen until analysis. Proteins were determined according to the method of Bradford (1976) with extraction based in Rausch (1981), and carbohydrates followed the methodology described in Albalasmeh et al. (2013). Lipid concentration was determined using the methodology described in Lombardi (1990) and Parrish (1999). Due to the changes in cell biovolume, the biochemical composition was expressed in picograms per μm^3 for best comparisons, as proposed in Kilham et al. (1997).

Bacterial presence

Bacterial colonies in the cultures were monitored daily. Culture samples (1 mL) were taken from each culture and diluted in an isosmotic phosphate buffered saline solution (PBS) to the decimal scale 10^4. Each dilution was inoculated in duplicates into sterile and disposable Petri dishes containing PCA medium (Merck KGaA, Germany). The Petri dishes were then incubated under controlled conditions at 30 °C for 48 h in the dark. The colonies were counted and the results expressed as colony forming units per volume ($CFU.mL^{-1}$).

Statistical Analysis

The results were tested for normality and homogeneity, and significant differences ($p < 0.05$) between means of each variable were tested by one-way ANOVA and Tukey's post-hoc analysis. Data were analyzed using Origin Pro (version 8.5.0) and Assistat (7.7 beta) software.

RESULTS

Chlorophyll *a*

Figure 2.1 shows the concentration of chlorophyll *a* in the photoautotrophic, mixotrophic and photo-mixotrophic treatments during the experimental time. The concentration of chlorophyll *a* increased in all treatments, but the highest increase was observed in the mixotrophic culture that passed from 0.07 (2nd day) to 2.47 $mg.L^{-1}$ (3rd day), reaching 5.88 $mg.L^{-1}$ on the 4th day, a thousandfold increase in relation to the beginning of the experiment. After day 4, the concentration of chlorophyll *a* in the mixotrophic culture did not show statistically significant differences. The concentration of chlorophyll *a* in the photoautotrophic and photo-mixotrophic cultures increased without differences ($p > 0.05$) up to the 5th day. After this, the photo-mixotrophic cultures increased dramatically, from 0.61 to 5.18 $mg.L^{-1}$ on the 7th day, remaining without differences ($p > 0.05$) until the end of the experiment. In the meantime, the photoautotrophic cultures increased slowly, reaching a maximum of 3.57 $mg.L^{-1}$ on the 10th day.

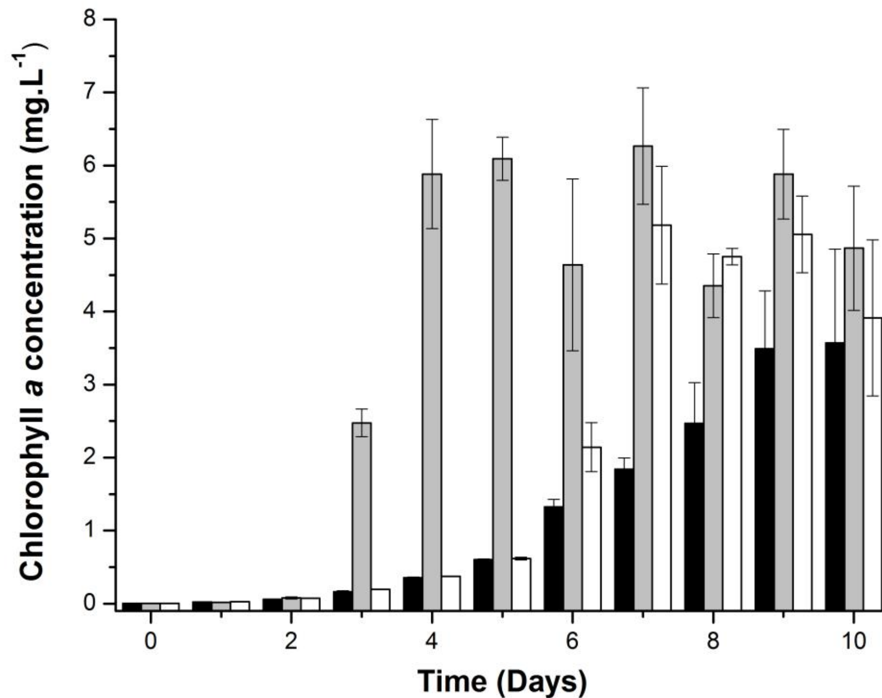

Figure 2.1 - Values of chlorophyll *a* concentration in mg.L^{-1} in *C. sorokiniana* under photoautotrophic (black column), mixotrophic (grey column) and photo-mixotrophic (white column) growth conditions during experimental time. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Cell Density

As observed in Figure 2.2, the cell density followed the same pattern observed for chlorophyll *a* concentration in all treatments. It can be observed that by the end of the experiment, cell density was similar for all treatments (~ 6.20 x 10^7 cell.mL^{-1}; ANOVA $p > 0.05$), however, this value was achieved earlier for the mixotrophic condition (3rd culture day), than for the others (6th culture day for the photo-mixotrophic and 10th day for the photoautotrophic).

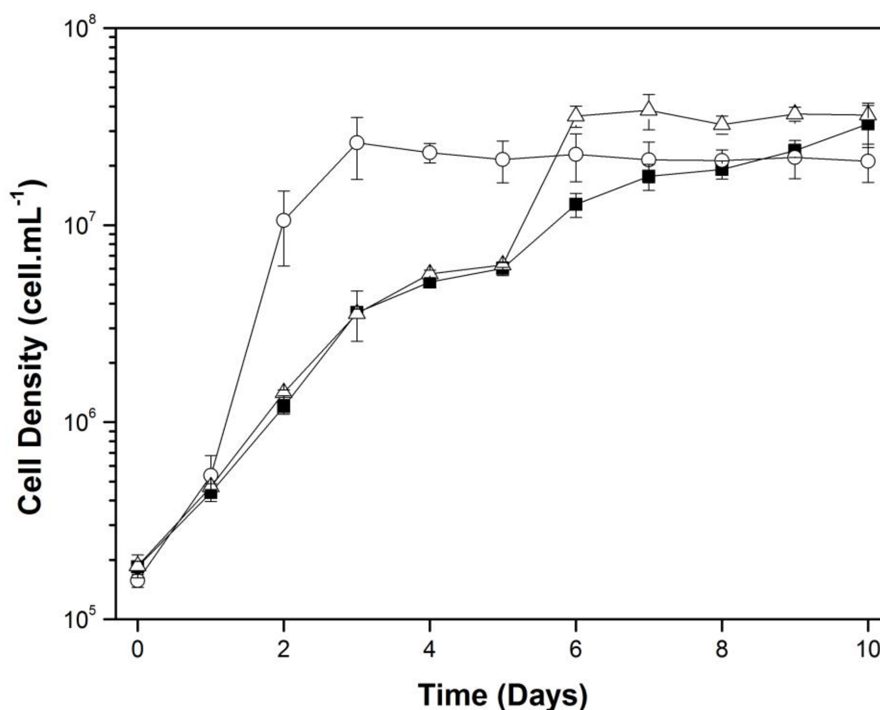

Figure 2.2 – Values of cell density (cells.mL^{-1}) in *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circle) and photo-mixotrophic (empty triangle) growth conditions during experimental time. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Cytomorphology

There were great differences among the cultures in relation to biovolume (Fig. 2.3), which increased in all cultures in different intensities. In the photoautotrophic cultures, the initial biovolume was of 33.9 µm³ and increased slowly until the end of experiment, reaching a maximum value of 214 µm³, an increase of ~ 6.4 times. The biovolume of the photo-mixotrophic cultures also increased slowly until the 4th day (99.8 µm³). From this day, when glucose was added, the biovolume increased exponentially (~ 9.4 times), reaching the maximum value of 360.5 µm³ in the 7th day. In the mixotrophic cultures, the biovolume increased ~ 8.4 times, reaching a maximum value of 334.2 µm³ at 6th day, decreasing thereafter.

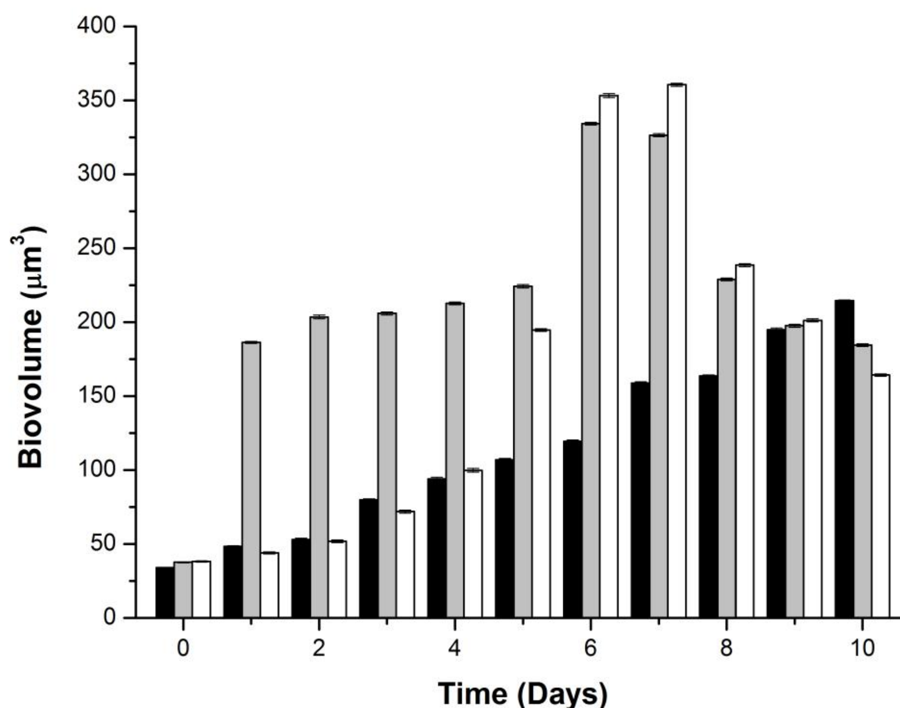

Figure 2.3 - Values of cell biovolume (µm^3) in *C. sorokiniana* under photoautotrophic (black bars), mixotrophic (grey bars) and photo-mixotrophic (white bars) growth conditions during experimental time. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Biochemical Composition

Figure 2.4A shows that photoautotrophic and photo-mixotrophic cultures showed the highest protein content per cell volume on the 3rd day, but a decrease of approximately 3.2- and 2.4-fold on 5th day, respectively. However, no differences in the protein content between days 3 and 5 during mixotrophic growth conditions ($p > 0.05$) were detected; there was also no significant difference ($p > 0.05$) in biovolume values in these two days. In relation to carbohydrates content per cell volume (Fig. 2.4B), there was no difference in the 3rd culture day among treatments ($p > 0.05$), but a large increase in the photo-mixotrophic was observed.

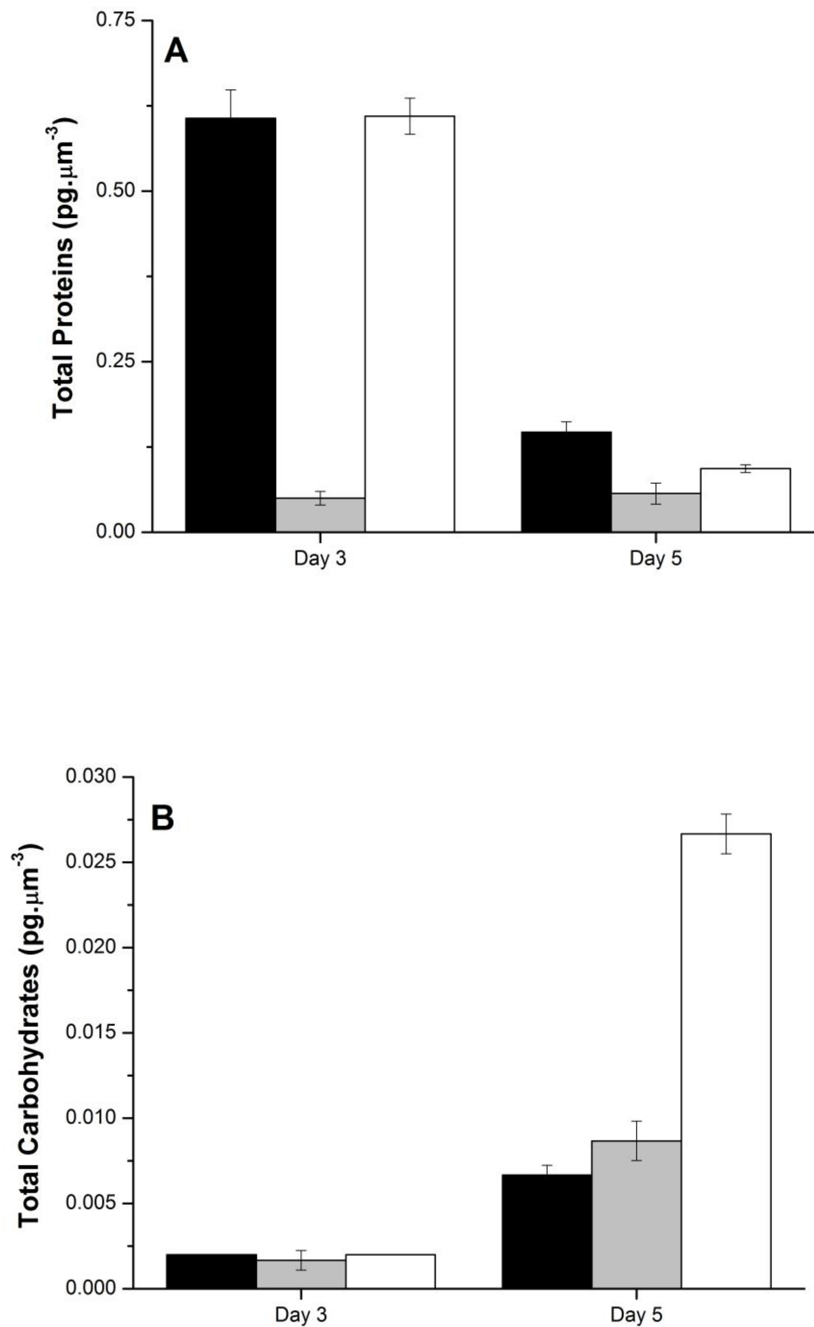

Figure 2.4 – Total proteins (A) and carbohydrates (B) in pg.µm^{-3} of *C. sorokiniana* under photoautotrophic (black column), mixotrophic (grey column) and photo-mixotrophic (white column) growth conditions at 3rd and 5th days. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

In Figure 2.5, the differences in proteins:carbohydrates ratio (P:C ratio) between exponential (3rd day) and stationary (5th day) growth phases can be visualized. The P:C ratio decreased in all three treatments during growth, with a reduction of, approximately, 3.0-, 4.0-, and 10-fold, in photoautotrophic, mixotrophic and photo-mixotrophic cultures, respectively. The lowest P:C ratio was obtained, during exponential growth, in the mixotrophic condition.

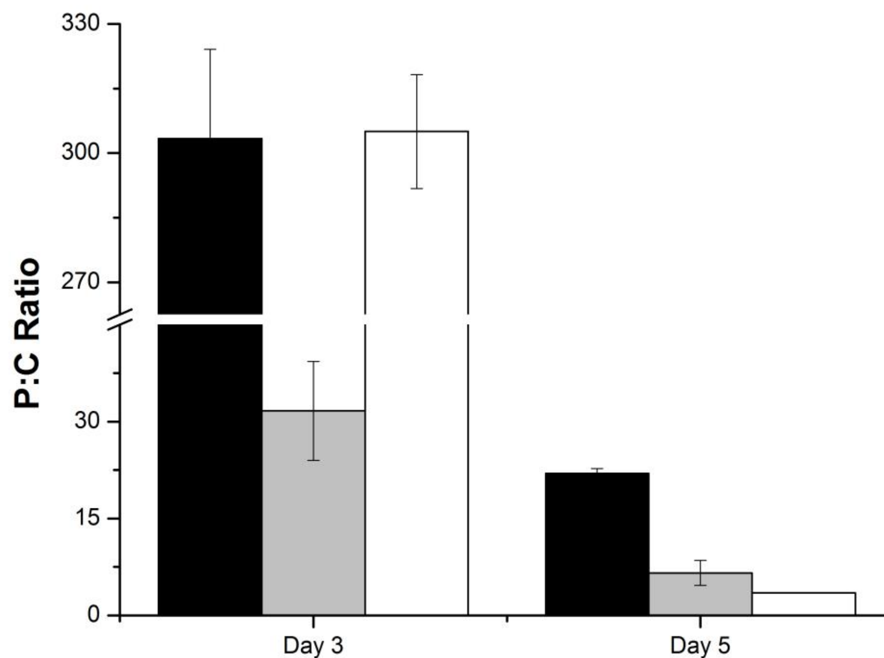

Figure 2.5 – Proteins:carbohydrates ratio (P:C ratio) of *C. sorokiniana* under photoautotrophic (black column), mixotrophic (grey column) and photo-mixotrophic (white column) growth conditions at 3rd and 5th days. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Figure 2.6 shows the total lipids concentration per cell volume in the photoautotrophic, mixotrophic and photo-mixotrophic cultures. The mixotrophic cultures presented the highest lipids content per cell volume in relation to the other cultures during exponential growth. However, on the 5th day, the concentration of total lipids per cell volume decreased for all cultures, at about 10-, 30-, and 10-fold, in the

photoautotrophic, mixotrophic and photo-mixotrophic cultures, respectively. Even though glucose was furnished on the 4th day, photo-mixotrophic cultures did not present statistical differences ($p > 0.05$) in relation to the others on the 5th day.

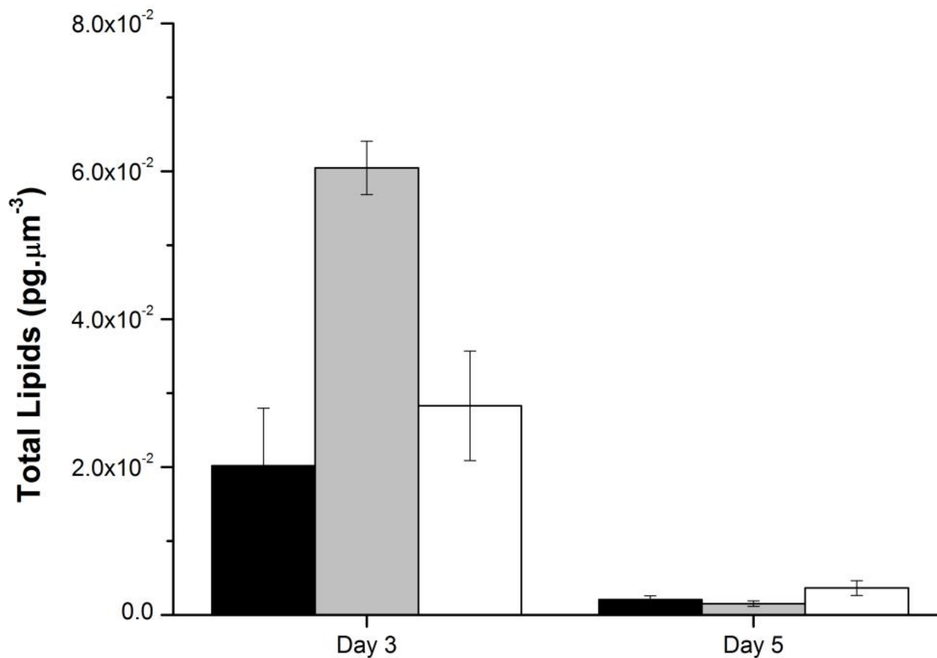

Figure 2.6 - Total lipids per cell volume (pg.μm^{-3}) of *C. sorokiniana* under photoautotrophic (black column), mixotrophic (grey column) and photo-mixotrophic (white column) growth conditions at 3rd and 5th days. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

Bacterial Density

Figure 2.7 shows the bacterial density in the culture medium during the growth of microalga *C. sorokiniana*. As expected, after glucose addition in the mixotrophic (day 0) and photo-mixotrophic (day 4) cultures, the bacterial density increased exponentially until day 5 and 7, respectively. After the 5th day, there was an abrupt decrease in bacterial density in mixotrophic cultures until the 7th day, remaining constant until the last day of experiment (day 10). Before glucose addition, photo-mixotrophic culture showed the same growth of photoautotrophic one, with increased bacterial density until the 6th day, decreasing thereafter.

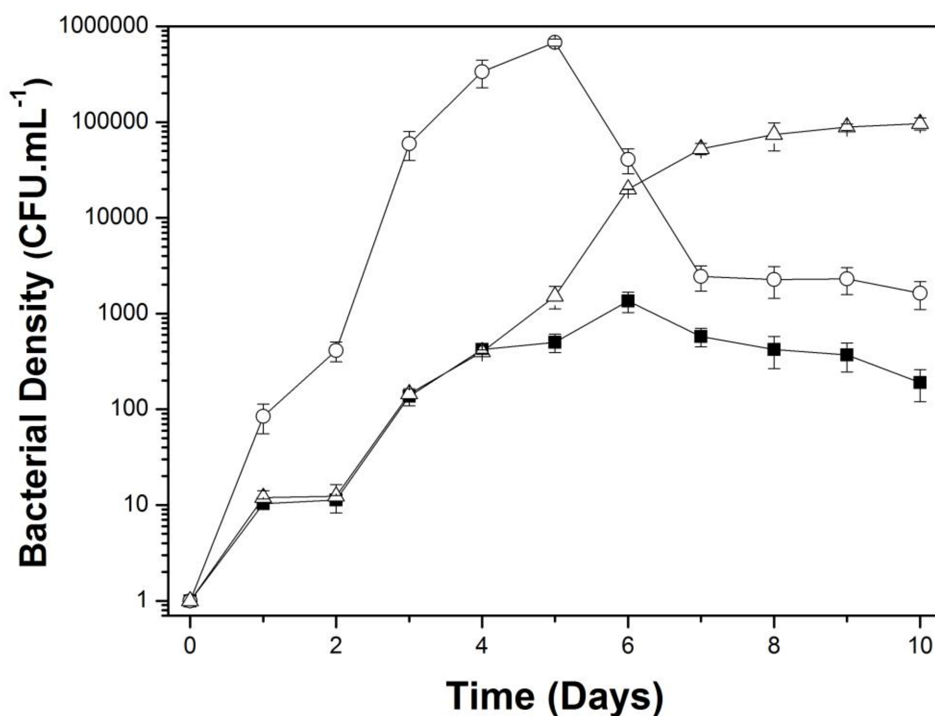

Figure 2.7 - Bacterial density, expressed as colony forming units per mL, in photobioreactors of microalga *C. sorokiniana* under photoautotrophic (full squares), mixotrophic (empty circle) and photo-mixotrophic (empty triangle) growth conditions during experimental time. Error bars mean standard deviation from the mean (n = 3). Glucose was added at the beginning (mixotrophic cultures) and at 4th day (photo-mixotrophic cultures) of experiment, respectively.

DISCUSSION

The rapid increase in chlorophyll *a* concentration and number of cells after the addition of glucose in mixotrophic and photo-mixotrophic cultures are in accordance with literature results. White et al. (2011) and Giovanardi et al. (2014) showed that glucose supplies the cells with extra energy and organic carbon to build new cellular structures (Rodrígues-López, 1966), thus rapid increase in some physiological parameters are observed. Mixotrophic cultures of the genus *Chlorella* sp. having glucose as organic carbon source has been widely investigated and has best performance in growth parameters than photoautotrophic ones (Yang et al., 2000; Kong et al., 2011; Cheirsilp & Torpee, 2012). Besides the glucose addition, light intensity may contribute to the biomass increase, stimulating the light harvesting complex (LHC) antenna

production (Li et al., 2014), which is important in high light intensities, in order to maximize the light capture efficiency (Schenk et al., 2008; Giovanardi et al., 2014). Another factor that has been shown to also contribute to increase in biomass is the refixation of CO_2 released by the aerobic respiration in the presence of glucose during photosynthesis. This can offer carbon source to the biochemical synthesis and stimulates the cellular division (Wan et al., 2011; Li et al., 2014).

It has been observed in literature that the chlorophyll *a* concentration in mixotrophic cultures vary depending on the microalga species and organic carbon source (Cheirsilp & Torpee, 2012; Alkhamis & Qin, 2016). Fang et al. (2004), for example, showed a decrease in the chlorophyll *a* content in mixotrophic cultures of *Nannochloropsis* sp., while Alkhamis & Qin (2016) reported an increase in mixotrophic cultures of *Tisochrysis lutea*.

Over time, the biovolume of cells increased, especially in the cultures with glucose. The increase of biovolume in photoautotrophic cultures may be due to the production and storage of starch and/or increase in vacuolization, linked to cell age (Baldisserotto et al., 2012; Giovanardi et al., 2014). However, in mixotrophic and photo-mixotrophic cultures, the increase in biovolume of cells is due to the excess of starch and lipids storage inside cells because of the excess of glucose in the medium (Scott et al., 2010; Wan et al., 2011; Baldisserotto et al., 2012). The excess of glucose causes a disequilibrium in N:C ratio, decreasing the synthesis of nucleic acids and proteins, inhibiting cell division (so cells increase in size) and stimulating the storage of biomolecules, as carbohydrates and lipids (Rodríguez-López, 1966; Giovanardi et al., 2014). Rodríguez-López (1966) found giant and discolored forms of *C. sorokiniana* in mixotrophic cultures with 11 different organic carbon sources, and attributed the results to the high concentration of starch and lipids inside the cell, which completely disorganizes the chloroplasts and distributes the pigment in a greater cellular volume.

The reduction of biovolume over time suggest that microalgae consumed starch and lipids in order to support their growth, after the glucose in the medium had been consumed. Giovanardi et al. (2013; 2014) found similar results in mixotrophic cultures of *Neochloris oleoabundans* with carbon-rich waste source from apple vinegar production.

Protein is part of the structural and genetic material in cells and the highest concentration, especially during exponential growth we observed, indicates that cells were healthy (Kilham et al., 1997; Li et al., 2014). The reduction in protein content in photoautotrophic cultures can indicate a nutrient limitation, as nitrogen sources (Kilham et al., 1997; Rocha et al., 2014; Alkhamis & Qin, 2016), but in this study, it can also be related to the increase in cell biovolume. Alkhamis & Qin (2016) reported a 2.5-fold increase in protein content in mixotrophic cultures of *T. lutea* in relation to photoautotrophic ones. The great increase in carbohydrate concentration in mixotrophic and photo-mixotrophic cultures can be due to the addition of glucose in the medium, which stimulates the storage of this sugar as starch inside the cells (Rodrígues-López, 1966; Giovanardi et al., 2013; Baldisserotto et al., 2014), probably a mode of luxury consumption as occurs in bacteria (Skoog et al., 2002). Kong et al. (2013) reported the increase in carbohydrate production in *Chlorella vulgaris* under mixotrophic growth conditions, both glucose and glycerol.

The P:C ratio is considered a physiological indicator in microalgae (Kilham et al., 1997). An increase in this ratio, and consequently, of proteins content, indicates that microalgae are healthy, while the reduction in this ratio (increase in carbohydrates content) indicates stressing conditions, mostly related with nutrients (Lai et al., 2011; Li et al., 2015). In this work, a decrease in P:C ratio in all treatments at the 5th day was observed. In the cultures with glucose addition, the decrease in P:C ratio can be due the excess of carbohydrates stored as starch in the microalgal cytoplasm, as also observed by Giovanardi et al. (2013) and Baldisserotto et al. (2014). Alkhamis & Qin (2016) reported increase in P:C ratio during both photoautotrophic and mixotrophic growth, but the authors added urea as nitrogen source, which did not limit the synthesis of proteins by the microalga *T. lutea*.

In the present results, higher lipid content was observed in mixotrophy than in photoautotrophic and photo-mixotrophic conditions. During mixotrophy, the microalgae can obtain energy from both photosynthesis and oxidation of organic carbon compounds (Dittamart et al., 2014). Some of this energy is directed towards growth, while the rest is stored as carbohydrates and, especially, lipids, which makes the cell swollen, increasing its volume (Dittamart et al., 2014; Giovanardi et al., 2014; Juntila et al., 2015) and lipid content. Increase in lipids concentration during mixotrophic cultures is widely reported

in literature: Rodríguez-López (1966), Liang et al. (2009), Giovanardi et al. (2014), Li et al. (2014), Juntila et al. (2015). Giovanardi et al. (2014) observed differences in lipids storage since their 3rd day between cultures photoautotrophic and mixotrophic with 2.5 $g.L^{-1}$ of glucose.

Wan et al. (2011) reported that during mixotrophy, cytosolic enzyme acetyl-CoA carboxylase (ACCase) is overexpressed, suggesting that fatty acids precursors of lipids synthesis are derived from glycolysis of exogenous sugars rather than the carbon fixed through photosynthesis.

According to Li et al. (2015), the production and storage of starch and lipids occurs under conditions of stress or nutrient starvation. In the present study, the lack of equilibrium on N:C ratio (excess of carbon due the glucose addition) can cause a stress in microalgae, increasing the storage of starch and lipids. In addition, Li et al. (2015) found that the accumulation of starch and lipids is strongly dependent of the linear electron flow of photosynthesis. In our case, after the exhaustion of the organic carbon source, the microalga returned photosynthetic activity only, consuming its reserves (carbohydrates and lipids) for growth so reducing its volume (Giovanardi et al., 2013), as confirmed by the results of Figure 2.3.

Comparing the concentration of carbohydrates (Fig. 4B) and lipids (Fig. 6), we can observed that the values were inversely proportional. This is in accordance with Goodson et al. (2011) and Li et al. (2015), where the authors studied *C. sorokiniana* and *Chlamydomonas reinhardtii*, respectively, and reported that lipid synthesis was largely dependent of starch degradation, because less starch in chloroplasts creates more physical space for storage lipids bodies.

As expected, bacterial density was higher in the cultures with glucose addition (mixotrophic and photo-mixotrophic) than in the inorganic medium (photoautotrophic). This is due to necessity of the organic carbon for the bacterial metabolism and cell division (Tittel & Kamjunke, 2004; Kamjunke & Tittel, 2009). However, the increase in bacterial density followed similar pattern as that of the microalgal cell density, both in inorganic and in organic medium, which can suggest a beneficial relationship between *C. sorokiniana* and bacteria (Higgins & VanderGheynst, 2014).

The present results are in agreement with literature and suggest that co-culture of microalgae and bacteria (non-axenic cultures) can be used to stimulate microalgae biomass production (de-Bashan et al., 2002; Croft et al., 2005; de-Bashan, 2008). It is known that bacteria can secrete phytohormones (indole-3 acetic, indole-3 propionic and indole-3 butyric acids) and co-factors (vitamins B_{12}) that enhance microalgal growth (Croft et al., 2005; de-Bashan, 2008). However, the pH variation during the day/night cycle (photosynthesis x respiration), light intensity, dissolved oxygen, nutrients and other factors can limit the bacterial growth (Cordero et al., 2010; Amengual-Morro et al., 2012; Marchello et al., 2015), in addition to the production of a bactericide chloreline by *Chlorella* (Pratt, 1944; Ryther, 1954).

CONCLUSION

According to the results, we can concluded that the mixotrophic cultures of the microalga *Chlorella sorokiniana* presented the highest growth and production of lipids in comparison to the photoautotrophic and photo-mixotrophic cultures. In the photoautotrophic cultures, the microalgae grew slowly, taking 10 days to produce the same biomass as in the cultures with glucose. In mixotrophy, the cells increased their volume due to the storage of biomolecules, especially lipids. Therefore, mixotrophic cultures are efficient in the production of biomass and biomolecules in a short time, which is interesting for the biotechnological industries. Photo-mixotrophic cultures were not as efficient as mixotrophic ones, considering that it had taken twice the time for the same biomass. The bacteria present in the culture media did not appear to have negatively affected the growth of the microalgae, instead it could have contributed to the growth of *C. sorokiniana* with the production of stimulant substances.

ACKNOWLEDGEMENTS

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CHAPTER 3

An Investigation onto Cd Toxicity to *Chlorella sorokiniana* in Mixotrophy and Photoautotrophy: a Bayesian approach

ABSTRACT

Aquatic ecosystems are composed by a myriad of substances, including natural (and artificial) dissolved organic materials that can be assimilated by microalgae. If at the same time that microalgae assimilate organic compounds, they can perform photosynthesis, the metabolism is refereed as mixotrophy. However, ecotoxicological tests with metals usually consider only the photoautotrophic metabolism. This can lead to misinformation about the action of these contaminants on phytoplankton cells and, consequently, the whole ecosystem. This research investigated if there are toxicological differences between the photoautotrophy and mixotrophy in *Chlorella sorokiniana* exposed to cadmium (Cd). The parameters chlorophyll *a*, photosynthetic efficiency (F*v*/F*m*), cell viability, biochemical composition and pH were used to monitor possible toxic effects at 72 hours cultures. As a source of organic carbon, glucose (1 $g.L^{-1}$) was added so stimulating the mixotrophic metabolism. To evaluate the probability of the photoautotrophic culture being more affected by Cd than the mixotrophic one (or the inverse), Bayesian analysis was performed for all variables. The results showed that photoautotrophic cultures were more affected by the Cd than the mixotrophic ones, with reduction of all evaluated parameters, except for protein concentration, which increased. However, in mixotrophic cultures, no changes in protein concentration and proteins:carbohydrates ratio were observed, and chlorophyll *a*, F*v*/F*m* and cell viability were only affected at higher concentrations of Cd (range ln -11.5 to -9.4). However, both mixotrophy and photoautotrophy had the same probability of having the carbohydrates concentration affected by Cd. From the data obtained, we concluded that the microalgae in mixotrophy were more resistant to the action of Cd. In addition, we showed that F*v*/F*m* was affected by both the metal in photoautotrophy and by glucose in mixotrophy. The reduced photosynthetic capacity under mixotrophy can end up reducing the release of oxygen gas in the environment, which can compromise the entire aquatic ecosystem.

Key word: mixotrophic growth, cadmium, glucose, biochemical composition, *Chlorella sorokiniana*.

INTRODUCTION

Continental aquatic environments represent important sources of water to the humanity (Wetzel, 2001). These environments are the final destination of rainwater that falls on the earth's surface and which carries terrestrial material to rivers and lakes (Guenet et al., 2014). In addition to the natural materials carried by the rains, anthropic activities also release their wastes into the aquatic environments (Pempkowiak et al.; 1998; Qian et al., 2009; Sisman-Aydin et al., 2013), causing pollution, reducing the biodiversity and the water quality (Liu et al., 2011; Rodgher et al., 2012).

Usually, the pollution of aquatic environments has various sources, such as domestic and industrial effluents (Zhou et al., 2006; Rodgher et al., 2012; Marchello et al., 2015). The industrial effluents can increase the amount of metals in the water column and in the sediments (da Costa & França, 2003; Bajguz, 2011; Liu et al., 2011). Unlike organic compounds that can be degraded by the action of microbial enzymes, metals cannot, being accumulated in the environment (Pempkowiak et al.; 1998), or incorporated by the organisms, and transferred through the food chain. This can cause biochemical damage in organisms, reducing their populations and, altogether affecting all the ecosystem equilibrium (Qian et al., 2009; Rodgher et al., 2012; Chia et al., 2015).

Among the metals discharge in aquatic environments, cadmium (Cd) has high toxicity, persistence, and a greater solubility in water compared to other metals, which determines its wide distribution and the bioaccumulation potential (Lockwood, 1976; Taylor, 1983; Zhou et al., 2006). In addition, it has low affinity to dissolved organic matter, which increases its availability as free Cd in aquatic ecosystems (Tonietto et al., 2016).

Metals, as Cd, can enter in the food chain through phytoplankton, the primary producers and base of the food chains in aquatic ecosystems (Ting et al., 1991; Wetzel, 2001; Sisman-Aydin et al., 2013). These microorganisms synthesize organic materials

by the assimilation of carbon dioxide and water during photosynthesis (Rodgher et al., 2012; Chia et al., 2015), using sunlight as energy source (photoautotrophy metabolism).

Another form that phytoplankton can improve its growth is through mixotrophy (Stoecker, 1998; Juntila et al., 2015). Mixotrophy is a metabolic pathway whereby carbon dioxide (CO_2) and organic carbon (e.g., glucose, sucrose, acetate and others) are simultaneously assimilated and both mitochondrial respiration and photosynthesis operates concurrently as energy source for cell growth (Boëchat et al., 2007; Juntila et al., 2015). Mixotrophic metabolism in phytoplankton organisms can occurs when the environment contains organic matter and/or few inorganic nutrients (Stoecker, 1998; Boëchat et al., 2007), and in benthic algae, when the light scarce (Gacia et al., 1996).

In contact with the microorganism, metals adsorb onto cell wall due to electrostatic interactions, a physicochemical phenomenon where functional groups in the cell wall, such as amino, carboxylic, sulfhydryl, phosphate and thiols, bind with metals ions (Kaplan et al., 1995; Tonietto et al., 2016). An initial fast uptake occurs due to the surface adsorption on the cell wall with a subsequent slow uptake due to the membrane transport of the metal ions into the cytoplasm of the microalgal cell (Ting et al., 1991; Pokora et al., 2014).

During evolution, microorganisms have developed a variety of strategies to avoid metals toxicity (Perales-Velas et al., 2006; Bajguz, 2011; Pokora et al., 2014). Microalgae can produce and release metal chelating agents such as peptides, known as phytochelatins (with low molecular weight and negatively charged that form organometallic complexes), extracellular polysaccharides, or accumulating ions inside intracellular vacuoles (Kaplan et al., 1987; Ahner & Morel, 1995; Kaplan et al., 1995; Neis, 1999; Perales-Velas et al., 2006; Fauziah, 2011). However, in high metals concentrations, the detoxifying mechanisms of microalgae may not be enough and the organism be injuried (Fauziah, 2011). An exception may observed in the marine diatom *Thalassiosira weissflogii* that can use Cd as an enzymatic cofactor replacing Zn, for example (Prince & Morel, 1990; Lane et al., 2005; Sisman-Aydin et al., 2013).

It is known that Cd toxicity for phytoplankton results mainly from blocking functional sulfhydryl groups (-SH), but also from the displacement of essential metal ions in structural proteins and enzymes, affecting photosynthesis, respiration, cell

division, ATPase activity, proteins, chlorophylls and carotenoids synthesis, nitrogen and carbohydrates metabolisms (Sanitá di Toppi & Gabrielli, 1999; Zhou et al., 2006; Bajguz, 2011; Monteiro et al., 2011; Chia et al., 2015). However, all toxicity tests reported so far were performed in photoautotrophy, there are no data in the literature about the toxicity effects of Cd during mixotrophic growth, what is likely to be present in the environment in reason to the myriad of organic materials there present, and may present different responses to presence of Cd in relation to photoautotrophy.

In order to get new insight on the toxicity of Cd in freshwater microalgae, we studied the effects of Cd contamination on the growth, photosynthesis activity and biochemical composition in the microalga *Chlorella sorokiniana* under photoautotrophy and mixotrophy, with the aim to observe the toxic effects during two different metabolic strategies.

MATERIAL AND METHODS

Algal Culture and Experimental Design

Cultures of microalga *Chlorella sorokiniana* were performed in 1000 mL polycarbonate flasks, containing 250 mL of AAP Medium (EPA, 2012) with no EDTA. The cultures was maintained in controlled conditions of temperature (24 ± 1 °C), light intensity (130 µmol photons m^{-2} s^{-1}) and photoperiod (12 h light/12 h dark). The initial inoculum (10^5 cells.mL^{-1}) was obtained from exponentially growing cells in the same culture conditions.

Two different growth metabolisms were studied, photoautotrophic (just inorganic medium) and mixotrophic (inorganic medium and organic carbon source). Glucose was the organic carbon source for mixotrophic cultures and was added in the concentration of 5 x 10^{-3} mol.L^{-1} (1 g.L^{-1}). Cadmium (standard solution of $CdCl_2$ Titrisol Merck) was added in twenty different nominal concentrations ranging from 10^{-8} to 10^{-5} (Table 3.1), no replicates, during 72 hours.

Table 3.1 – Cadmium concentrations ($mol.L^{-1}$) used in the culture medium of *Chlorella sorokiniana* grown either under photoautotrophy or mixotrophy. Cadmium concentrations (nominal values), represented by [Cd], are reported in $mol.L^{-1}$.

[Cd]	ln [Cd]	[Cd]	ln [Cd]	[Cd]	ln [Cd]	[Cd]	ln [Cd]
1×10^{-8}	-18.4	1×10^{-7}	-16.1	1×10^{-6}	-13.8	1×10^{-5}	-11.5
2×10^{-8}	-17.7	2×10^{-7}	-15.4	2×10^{-6}	-13.1	2×10^{-5}	-10.8
4×10^{-8}	-17.0	4×10^{-7}	-14.7	4×10^{-6}	-12.4	4×10^{-5}	-10.1
6×10^{-8}	-16.6	6×10^{-7}	-14.3	6×10^{-6}	-12.0	6×10^{-5}	-9.7
8×10^{-8}	-16.3	8×10^{-7}	-14.0	8×10^{-6}	-11.7	8×10^{-5}	-9.4

Growth Parameters

The pH was determined with a pH-meter, chlorophyll *a* concentration by *in vivo* fluorescence using a fluorimeter (Turner Designs, Model Trilogy – U.S.A.); its concentrations were obtained from a calibration curve performed through fluorescence intensity *vs* concentration of chlorophyll *a* extracted from exponentially growing cultures of *Chlorella sorokiniana*. The cell viability (determined by chlorophyll fluorescence) was measured by a cytometer Muse® Cell Analyzer and the results are expressed in percentage (%).

The maximum fluorescence of photosystem II (F*v*/F*m* of PSII) was obtained in 20-min dark-adapted cells using a pulse amplitude modulated fluorimeter, Phyto-PAM (Heinz Walz Effeltrich, Germany). This parameter can be used to infer about the physiological status of photosynthetic microalgae (Lombardi & Maldonado, 2011).

Proteins were determined according to the methodology of Bradford (1976) with extraction based in Raush (1981), while carbohydrates followed the method of Albalasmeh et al. (2013). All biochemical composition data are reported in picograms per cubic micrometer ($pg.\mu m^{-3}$) and not per cell because mixotrophic cells are larger than the photoautotrophic ones.

Statistical Analysis

The statistical analysis was performed under the Bayesian paradigm using software R. In this case, the parameters are considered random variables, which allow us to compare them via probabilities. We used appropriate non-linear regression models for each variable to explain the relation of the interested variable and the natural logarithm of Cd concentration during the two metabolism scenarios, photoautotrophy or mixotrophy. Considering these two scenarios, we calculated the probability of the cadmium's effect being more intense during photoautotrophy than mixotrophy at each cadmium concentration.

RESULTS

Chlorophyll *a*

For low concentration of Cd, the photoautotrophic cultures showed 100% probability (Fig. 3.1C) of being more affected by Cd than the mixotrophic cultures, however, from Cd concentration of 4×10^{-7} mol.L^{-1} (ln -14.7) and above, this behavior inverts: mixotrophy was more affected, probably due to the absence of chlorophyll *a* in photoautotrophic conditions to perform the comparisons. These results can be well visualized by the shape of the curves in Figure 3.1C, indicating that the photoautotrophic (Fig. 3.1A) microalgae are more sensitive in the lower concentrations of Cd than in mixotrophy (Fig. 3.1B). Regardless of the concentration of the metal, mixotrophic cultures always presented higher chlorophyll *a* concentrations in relation to the photoautotrophic ones.

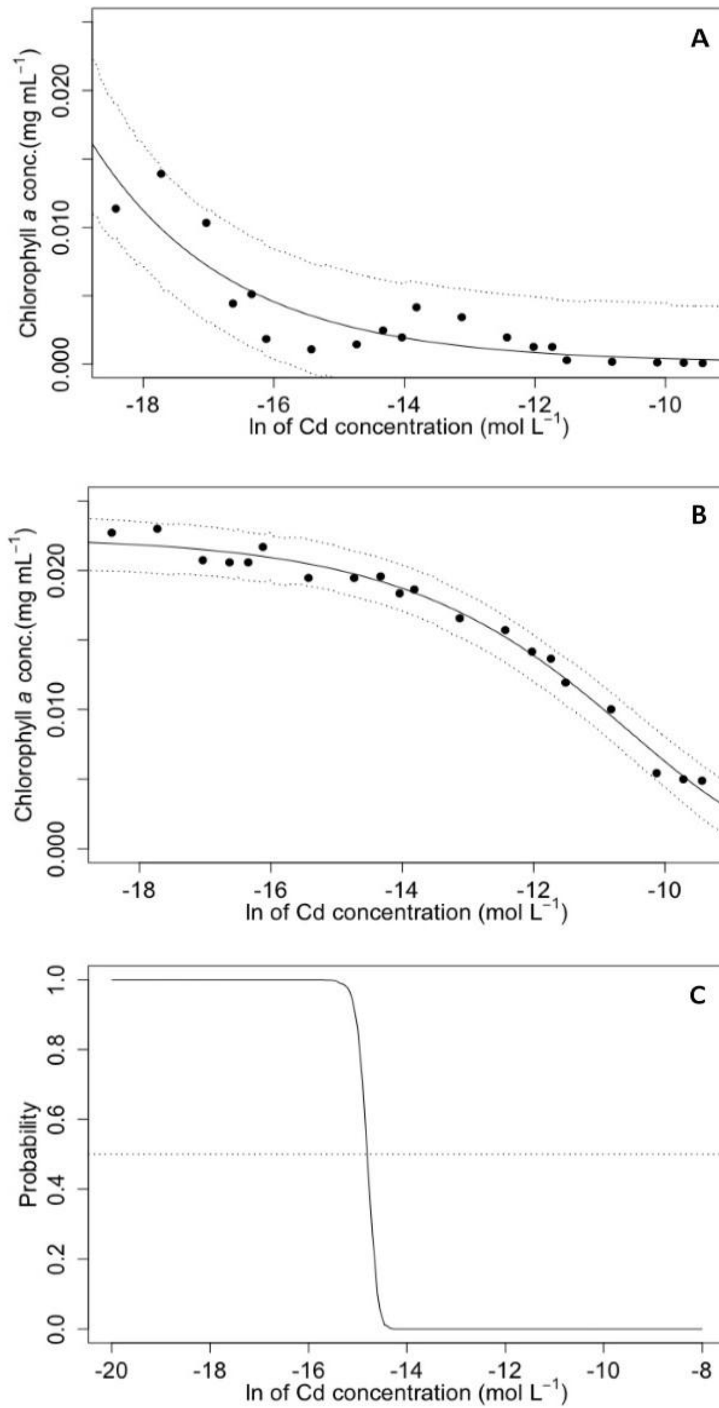

Figure 3.1 – Chlorophyll *a* concentration (mg.mL^{-1}) in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (C).

Photosynthetic Activity

The photosynthetic activity (F*v*/F*m*) in the mixotrophic condition (Fig. 3.2B) was maintained around 0.4 up to 10^{-5} mol.L^{-1} (ln -11.5) Cd concentration, whereas in the photoautotrophic condition, it decreased. From this concentration on, an opposite behavior was observed, while in mixotrophy there was a decrease, in photoautotrophy it maintained equal to zero. This behavior is translated through the probabilities in Figure 2C. The probability of the photosynthetic activity being more affected by Cd than mixotrophic activity is 100% up to 10^{-5} mol.L^{-1} (ln -11.5) Cd concentration and 0% from than on because the lack of photosynthesis.

Cell Viability

Figure 3.3 shows that in photoautotrophic cultures, cell viability decreased abruptly at 6 x 10^{-6} mol.L^{-1} (ln -12.0) Cd concentration and above, while in mixotrophic cultures, the reduction was subtle and occurred only at 4 x 10^{-5} mol.L^{-1} (ln -10.1). Until the concentration 6 x 10^{-6} mol.L^{-1} (ln -12.0), the effect of Cd on cell viability was more intense on mixotrophy (Fig. 3.3B) than on photoautotrophy (Fig. 3.3A), although in both conditions, the effect was almost null. When the intense decrease starts for photoautotrophic conditions, the probability of it being more accentuated than for mixotrophic condition is 100%.

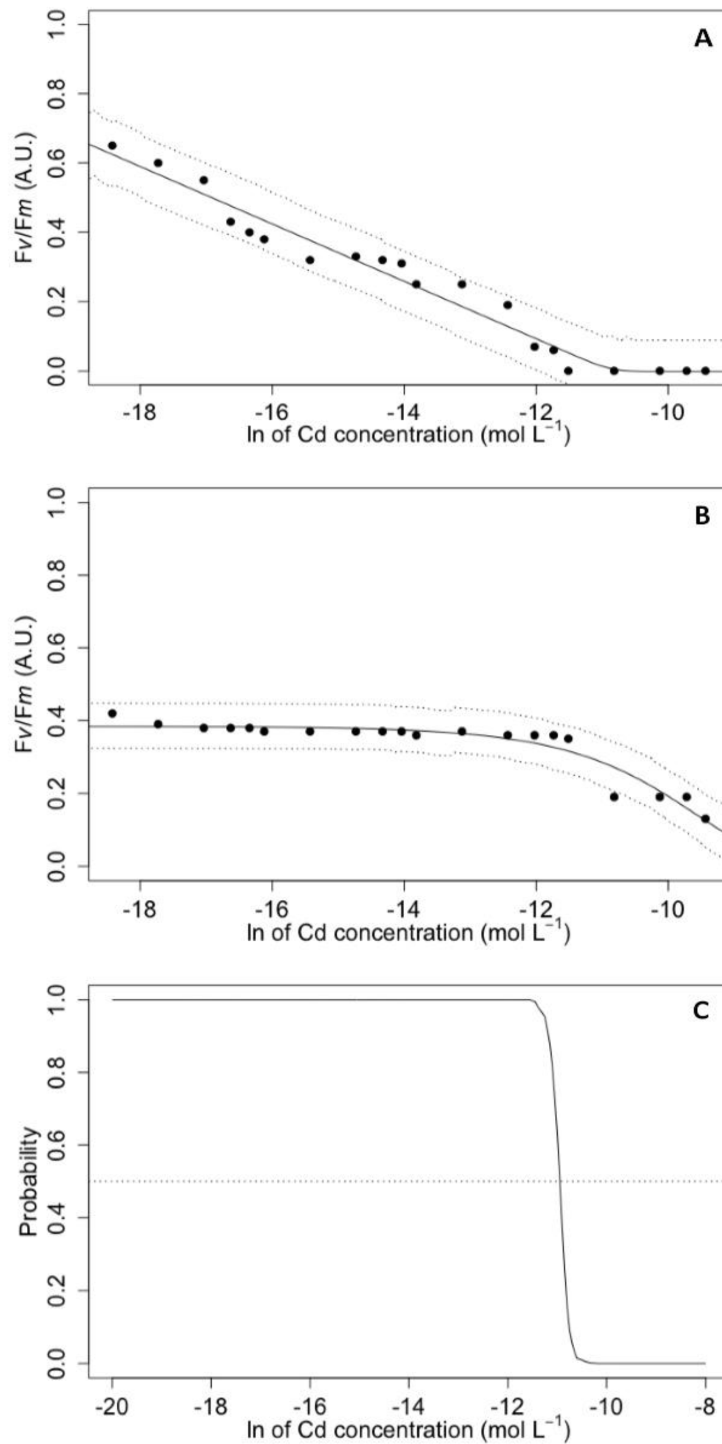

Figure 3.2 – Photosynthetic activity (F*v*/F*m*) in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. PAM measurement is reported as arbitrary units (A.U.). The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (C).

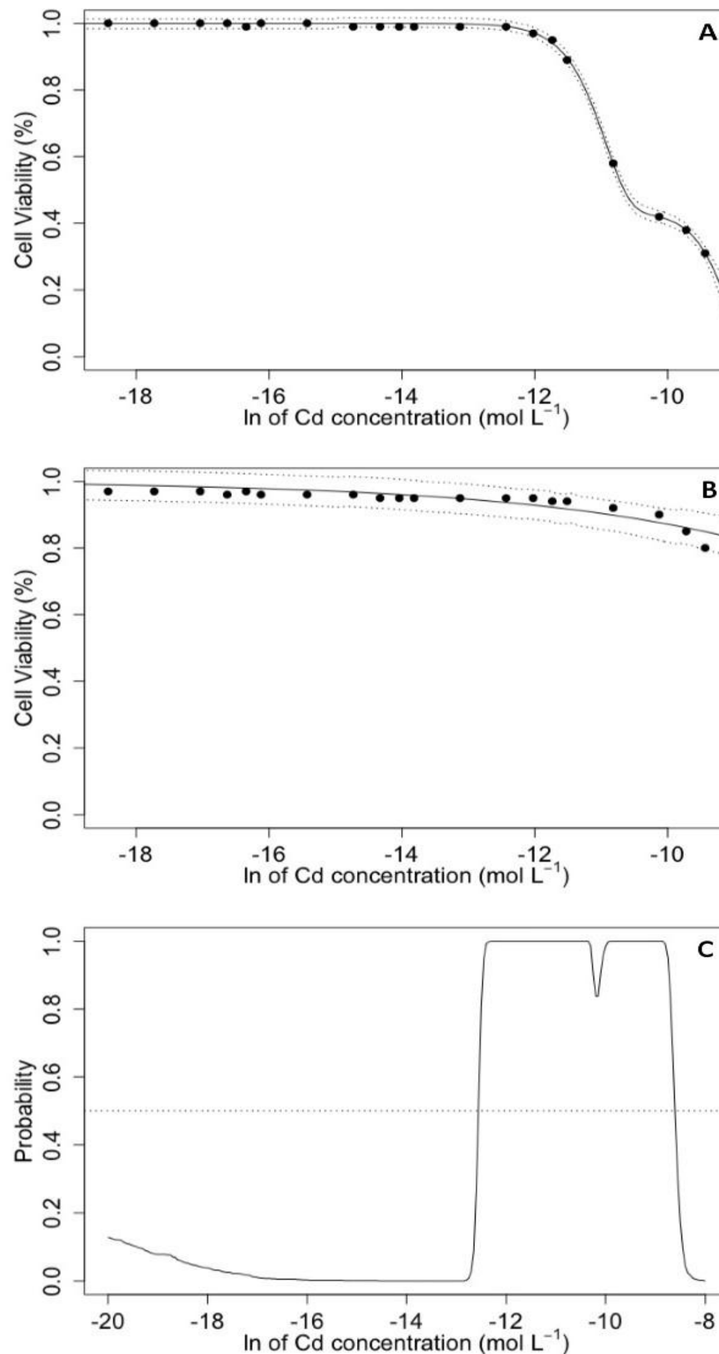

Figure 3.3 – Cell viability, expressed in percentage (%), in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (C).

Proteins

As observed in Figure 3.4, the concentration of proteins increased in the photoautotrophic cultures at Cd concentrations of 6 x 10^{-6} mol.L^{-1} (ln -12.0) and above,

with 100% probability of proteins production being more affected by the metal in photoautotrophy than in mixotrophy. For lower Cd concentration, mixotrophy presented a steeper increase with 100% probability since photoautotrophy remained constant. The photoautotrophic condition had total proteins concentration above 8 pg.µm^{-3} in the highest Cd concentration (8 x 10^{-5} mol.L^{-1}; ln -9.4), while in the mixotrophic metabolism, proteins remained low without significant increase as function of Cd.

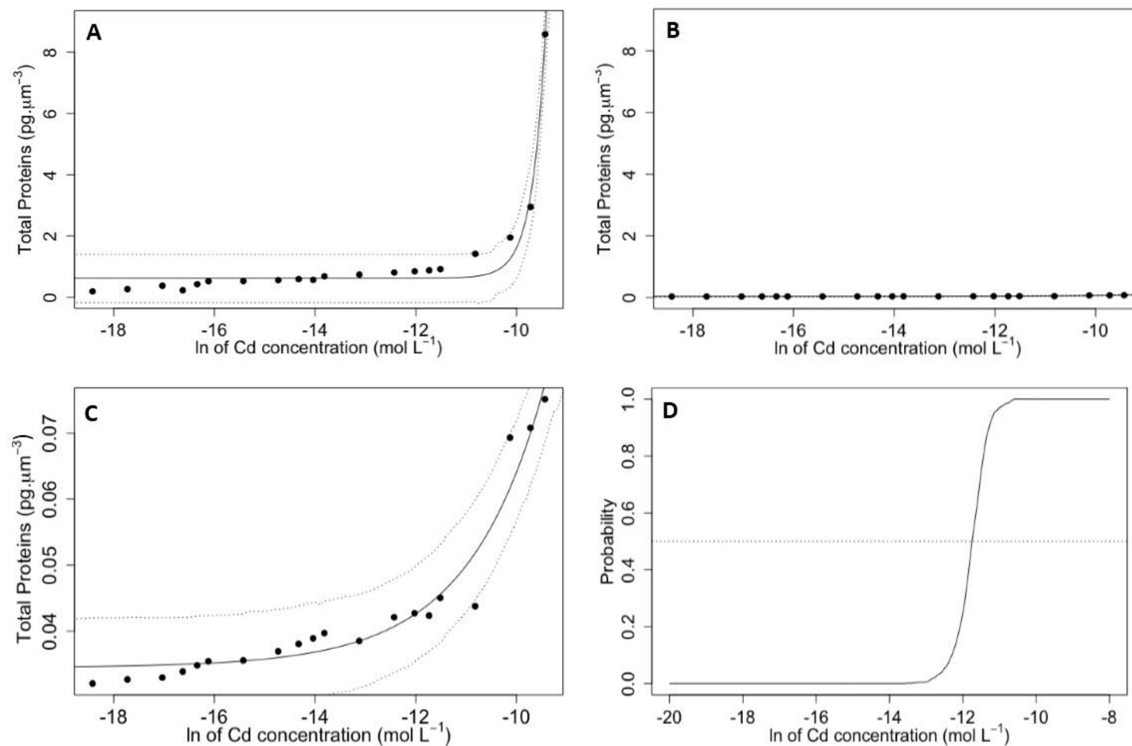

Figure 3.4 – Total proteins content, expressed in pg.µm^{-3}, in photoautotrophic (A) and mixotrophic (B and C) growth conditions at 72 hours as function of the natural log of Cd concentrations. Graph B represents mixotrophic condition on a comparable scale to photoautotrophy (A). The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (D).

Carbohydrates

Figure 3.5 shows that the mixotrophic cultures presented higher carbohydrates concentrations than the photoautotrophic ones, but in both a decrease in relation to the initial value was observed with the increase of the concentration of Cd in the medium. For all Cd concentrations, the rate of decrease was higher in the photoautotrophy than in

the mixotrophy with 0% probability, that is, Cd's effect is more intense for mixotrophy than for photoautotrophy with 100% probability.

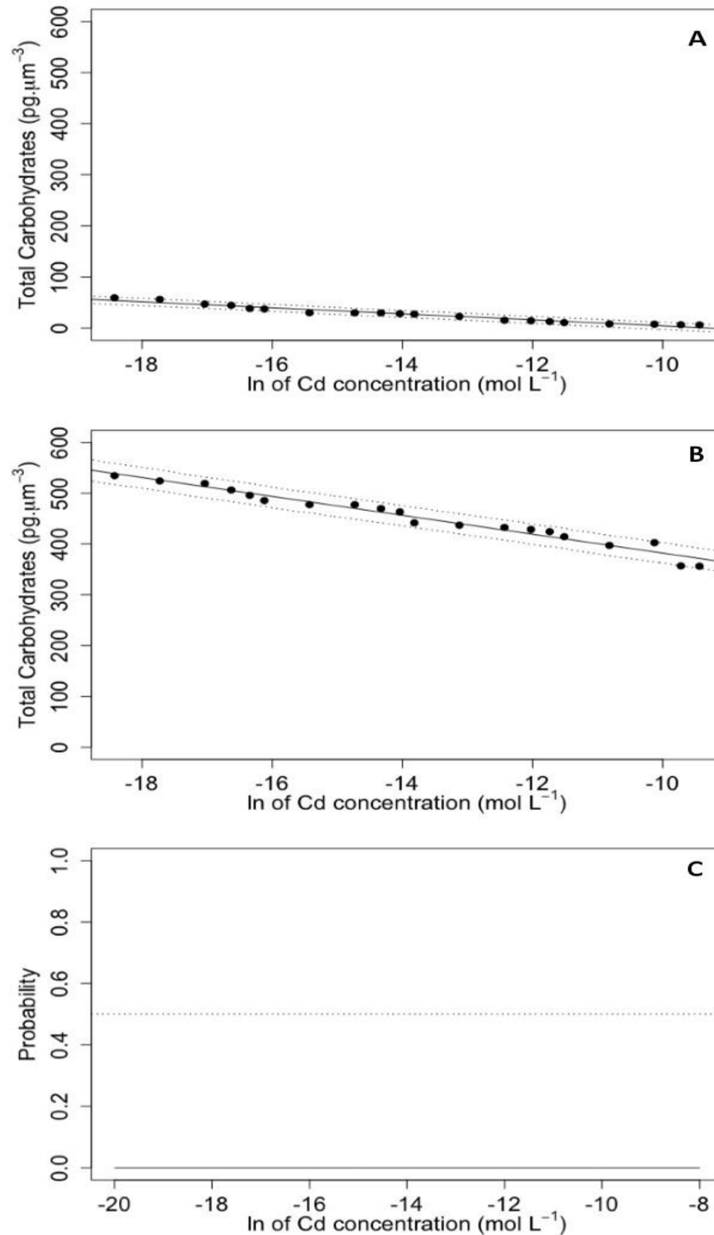

Figure 3.5 - Total carbohydrates content, expressed in pg.µm^{-3}, in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (C).

P:C Ratio

Figure 3.6 shows the ratio between proteins and carbohydrates (P:C ratio) in the photoautotrophic and mixotrophic cultures. It is observed that there was an increase in the P:C ratio for both the photoautotrophic and mixotrophic cultures with the increase in Cd concentration, but the increase on P:C ratio for mixotrophy was barely perceptible when compared to the increase in photoautotrophy. Under these conditions, the photoautotrophic cultures had 100% probability of being more affected by cadmium than the mixotrophic ones.

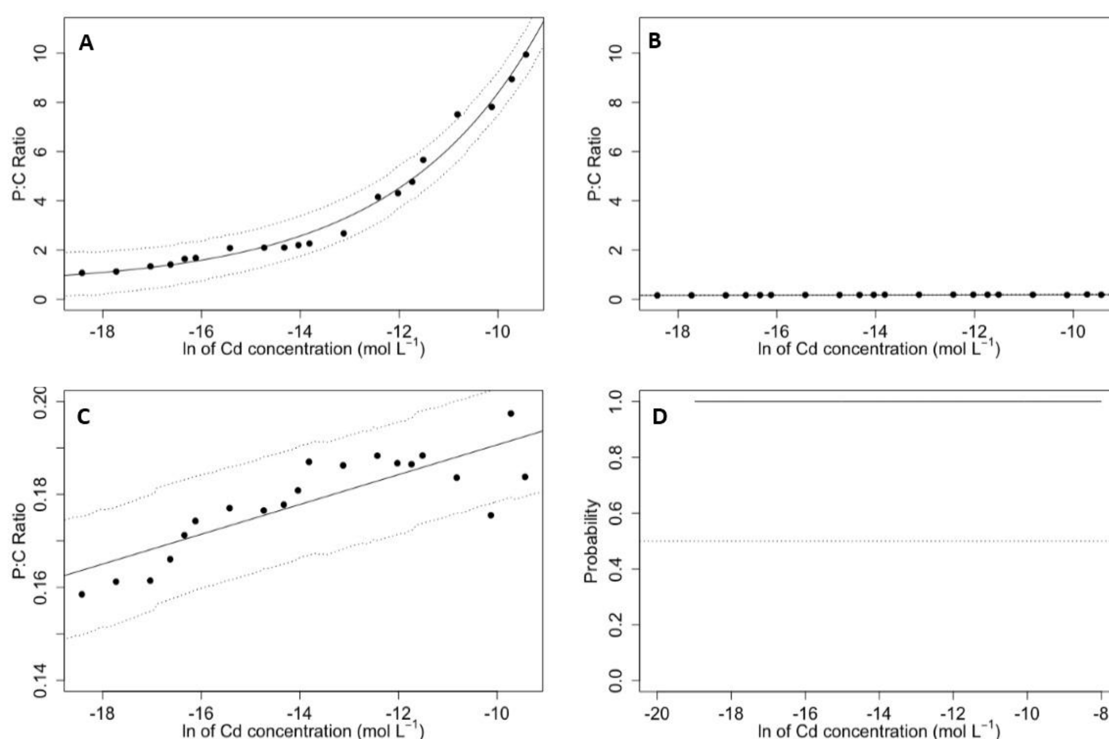

Figure 3.6 – P:C ratio in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. Graph B represents mixotrophic condition on a comparable scale to photoautotrophy (A). The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (D).

pH

The pH values (Fig. 3.7) decreased as function of the concentration of Cd in both photoautotrophic and mixotrophic cultures. However, photoautotrophic cultures showed

70% probability of being more affected by Cd at any concentration than the mixotrophic cultures.

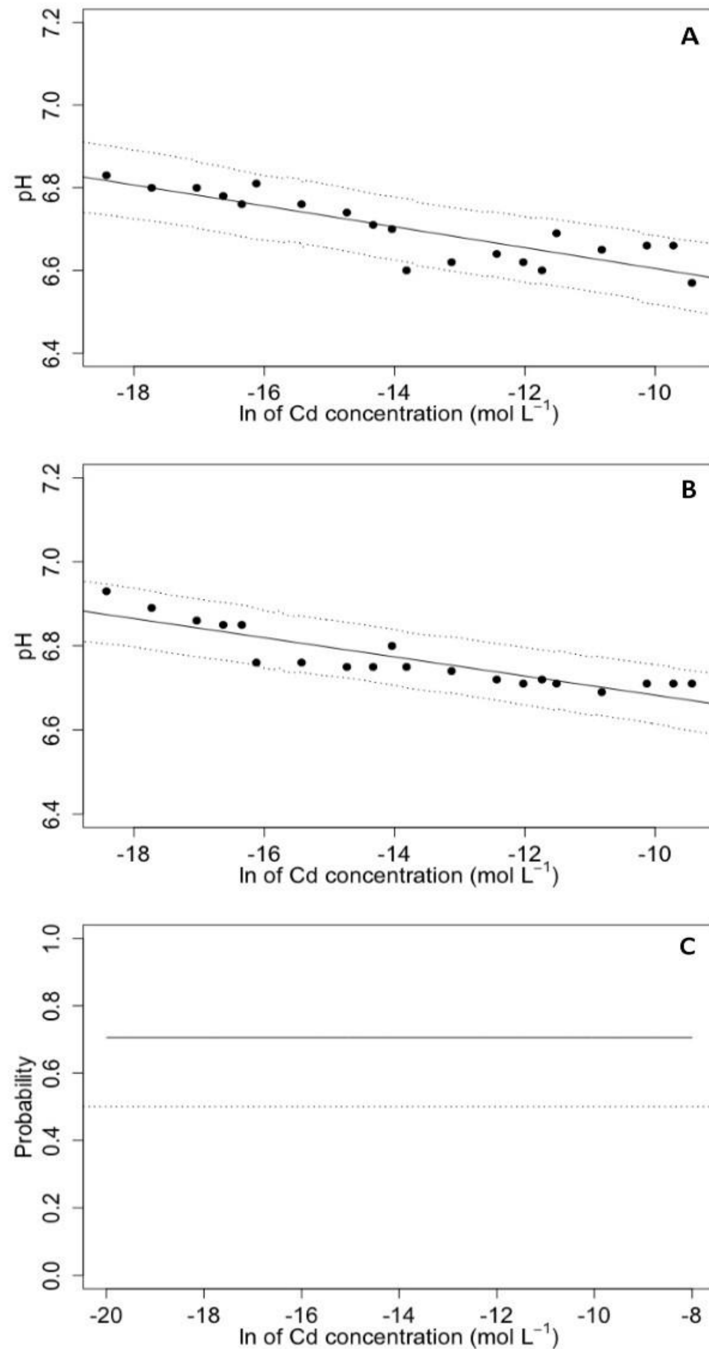

Figure 3.7 - Values of pH in photoautotrophic (A) and mixotrophic (B) growth conditions at 72 hours as function of the natural log of Cd concentrations. The probability of photoautotrophy being more affected than mixotrophy for each Cd concentration is shown in (C).

DISCUSSION

The reduction of chlorophyll *a* in both growth conditions (photoautotrophy and mixotrophy) can, according to the results of Qian et al. (2009), be related to a decline in the antenna size of the photosynthetic reaction center complexes caused by Cd, which can substitute other metal ions (mainly Zn, Cu and Ca) in metalloenzymes affecting the cell metabolic activities, especially in photoautotrophic cultures. Similar to our results, Qiu et al. (2006) showed a decrease in chlorophyll *a* in *Chlorococcum* sp. and correlated it to the increasing Cu and Cd concentrations. Wong & Chang (1991) reported structural alterations in thylakoid membranes of *Chlorella* sp. in the presence of the metals Cu, Cr and Ni.

The higher chlorophyll *a* content in mixotrophic cultures in comparison with the photoautotrophic ones can be due to the added organic carbon, which stimulate the production of chlorophyll *a* in some species of microalgae, as reported by Alkhamis & Qin (2016) in mixotrophic cultures of *Tisochrysis lutea* cultivated with glycerol. Glucose consumption probably increased the resistance of mixotrophic cells in lower Cd concentrations; however, we cannot affirm that there were effects in the higher Cd concentrations because there was no chlorophyll in photoautotrophy at the same concentrations for an adequate Bayesian comparison.

The decrease in F*v*/F*m* we obtained in the photoautotrophic cultures has also been showed in other studies (Neelam & Rai, 2003; Qian et al., 2009). Such behavior has been attributed to an inhibition of the PSII by metal ions. The reduction of F*v*/F*m* observed in the last four Cd concentrations may not result from the metal per se, but from the lack of photosynthesis in photoautotrophy, which prevents comparisons between the two treatments. The reduction of F*v*/F*m* in mixotrophy (~ 0.4) was a result of the oxidation of organic carbon for the production of energy, thus reducing the photosynthetic activity (Giovanardi et al., 2013; Baldisserotto et al., 2014). Giovanardi et al. (2014) pointed out that glucose plays an inhibitory effect in photosynthesis, reducing the apparent affinity for CO_2 during CO_2 fixation or limiting the synthesis of the enzyme RuBisCO (ribulose-1,5-biphosphate carboxylase/oxygenase).

Cadmium can affect the activity of the oxygen-evolving complex, causing the disassembly of PSII, down-regulating PSII proteins (Qian et al., 2009), and arrest the photosynthetic electron flow (Voigt & Nagel, 2002). It can also inhibit the water-splitting complex of the oxidizing site of PSII (Mallick & Mohn, 2003). Qian et al. (2009) reported that the inhibition of the gene psbA mRNA transcript may contributes to the decrease in the activity of PSII and electron transfer rates in *Chlorella vulgaris*. High concentrations of Cd affected chlorophyll *a* and photosynthesis in *C. sorokiniana* possibly due to the production of reactive oxygen species (ROS) inside the chloroplasts, such as hydroxyl $OH^{\bullet}$, phenoxy $RO^{\bullet}$, peroxy $ROO^{\bullet}$, superoxide radical anions $O_2^{\bullet-}$, singlet oxygen $^1O_2$, and hydrogen peroxide H_2O_2 (Kumar et al., 2008). These radicals can produce gradual peroxidation of lipid structures, oxidative DNA damage, and photosynthetic apparatus damages (Baryla et al., 2000; Kasprzak, 2002; Dewez et al., 2005).

The higher sensitivity of the photoautotrophic cells to higher Cd concentrations in comparison with the mixotrophic ones can be possibly be due to the toxicity of Cd to the photosynthetic apparatus (Kumar et al., 2008; Qian et al., 2009). As pointed out by Kulacki et al. (2002), Tang & Dobbs (2007), and Barreto & Lombardi (2016), specific growth rates and total cell densities were not as sensitive as cell viability in ecotoxicological tests. The cell viability can be considered as a measurement of the physiological state of the culture (Pouneva, 1997). Barreto & Lombardi (2016) reported a decrease in the viability of cells of the microalga *Scenedesmus bijugus* exposed to TiO_2 nanoparticles.

Metals, such as cadmium, bind to the hydroxyl groups of microalgae cell walls, pass through the cell membrane and accumulated inside the cell (Fauziah, 2011). Once inside the cells, Cd may bind to physiologically inert sites (no effect), physiologically active sites (possible metabolic effects), or be transported by facilitated diffusion or active uptake to organelles, such as chloroplasts (Fisher et al., 1984; Campbell et al. 2002). In addition, as a result of its high affinity to sulfhydryl groups (-SH), Cd can block essential enzymes, resulting in the inhibition of various metabolic processes in the cells (Sanitá Di Toppi & Gabrielli, 1999), for example, cell division (Monteiro et al., 2011; Chia et al., 2015). According to Franklin et al. (2002), reduction in population

parameters, as cell viability, can cause changes in population dynamics, species succession, and community structure and function in aquatic environments.

The increase in proteins concentration in the photoautotrophic cultures at the highest Cd concentrations can be related to a detoxification mechanism (Olafson et al., 1979; Li et al., 1980; Gaur & Rai, 2001). Carfagna et al. (2013) explain that proteins in microalgae cells are negatively charged, while Cd is positively charged; so, if they bind to each other, the inactivation of the metabolic proteins, as enzymes, can occur. To avoid problems in their metabolic activity, microalgae synthesize specific proteins to chelate the Cd (Kaplan et al., 1995; Perales-Velas et al., 2006; Chia et al., 2015) so increasing their protein content.

Kaplan et al. (1995) showed that microalgae, as the genus *Chlorella*, synthesize phytochelatins in response to Cd. In addition, Ahner et al. (1994) and Perales-Velas et al. (2006) pointed out that the production of metallothioneins with sulfhydryl groups can be a detoxification mechanisms in microalgae. Howe & Merchant (1992) reported that metallothioneins sequestered approximately 70% of cytosolic Cd in *Chlamydomonas reinhardtii*. Chia et al. (2015) reported the increase of proteins in *Chlorella vulgaris* cultivated in different Cd concentrations after growth decline, suggesting that the proteins were used for the microalgae survival (production of antioxidant enzymes to combat ROS) and not for cell division. Carfagna et al. (2013) suggested that the microalga uses proteins of degraded chlorophylls and proteins to synthesize chelating proteins to keep the cell alive. Both heavy metal-binding proteins, phytochelatins and metallothioneins, will be accumulated in vacuoles (Nies, 1999).

During mixotrophy, the lack of protein concentration (when compared in concentration) increase is probably a result of Cd being more toxic to photosynthesis (Zhou et al., 2006; Qian et al., 2009) than to the assimilation and oxidation of organic carbon, whereas in photoautotrophy, cells needed to produce protein chelators to try to maintain their homeostasis and preserve the photosynthetic activity (Kaplan et al., 1995).

The decrease in carbohydrates content along with the increase in Cd concentration can be explained by the toxic effects of Cd in the photosynthetic activity (Qian et al., 2009). Consequently, without production by photosynthesis, the cells used

the stored carbon to survive (Juntila et al., 2015). In mixotrophic cultures, the cells used the extra carbon added (increasing respiration) in the culture as storage starch (Giovanardi et al., 2013; Baldisserotto et al., 2014), explaining the higher carbohydrates content in mixotrophic cells. Contrary results were found by Chia et al (2015), who reported an increase in carbohydrates concentration in cultures with Cd, but these cultures were under nitrogen limitation, which may explain the differences between the two studies.

Generally, the P:C ratio is used as a parameter that tells about the physiological status of microalgae, in which ratios smaller than 1.0 indicate nutrient-limited cells (Ganf et al., 1986; Kilham et al., 1997). In the present study, higher values were found in photoautotrophic cultures (Fig. 6A), than in mixotrophic ones. Considering that the photoautotrophic cultures could have produced chelating proteins, these may be responsible for the high P:C ratios. Therefore, the P:C ratio does not demonstrate nutritional limits in ecotoxicological experiments, and its use is recommended under great caution to avoid false interpretations.

The decrease in pH values with the increase in Cd concentration in both metabolic conditions, being 70% higher in photoautotrophy than in mixotrophy, can be a result of the reduction in photosynthesis (Fig. 3.2) and increase in respiration of the intracellular stored carbon (Fig. 3.5). The increase in the organic carbon oxidation increases the release of CO_2 (Juntila et al., 2015) and the reduction of photosynthesis decreases the fixation of CO_2 (Giovanardi et al., 2014) from the environment, consequently reducing the pH by the dissociation of the carbonic acid (H_2CO_3) produced by the reaction of the water with CO_2 (Larsdotter, 2006).

CONCLUSION

The results obtained in this work show that the metal Cd, known to present high toxicity to phytoplankton, caused more harm to *Chlorella sorokiniana* in photoautotrophy than in mixotrophy. The statistical analyzes showed that the cells in photoautotrophy were more likely to be affected by Cd than those in mixotrophic growth, except for the highest concentrations tested. This higher sensitivity of the photoautotrophic microalgae may be due to the greater effect of Cd on photosynthesis

than on respiration, consequently, the microalgae with glucose were more tolerant because they obtained energy to survive from a source other than light. Therefore, we can conclude that in aquatic environments the microalgae are less affected in mixotrophy than in photoautotrophy. In the environment, a reduction in photosynthesis can reduce the release of oxygen gas, increasing the acidity and reducing the respiratory activity of others aquatic organisms. This can affect the stability of aquatic ecosystems.

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CHAPTER 4

Effects of TiO_2 Nanoparticles in Different Metabolic Pathways in the Freshwater Microalga *Chlorella sorokiniana*

ABSTRACT

The products that employ nanoparticles (NPs) in their composition has increased since the beginning of NPs production, hence their availability in the environment, especially in aquatic ecosystems, tends to increase. In these ecosystems, the phytoplankton is immersed in a complex matrix of nutrients, excreted materials and other chemical compounds, which can influence the metabolic strategy of microalgae. One of the metabolic via is mixotrophy, situation whereby microalgae perform photosynthesis and use dissolved organic carbon at the same time. Most toxicity evaluations do not consider such a metabolic route, but this can represent a preferential metabolism in natural environments. The present study aimed at evaluating the effects of titanium dioxide nanoparticles (NP-TiO_2) at a concentration range of 7.9 x 10^{-4} (log -3.10) to 7.9 mg.L^{-1} (log 0.89), on photosynthesis, growth, viability and biochemical composition of the green microalga *Chlorella sorokiniana* during photoautotrophic and mixotrophic growth (glucose, 1 g.L^{-1}, as the organic carbon source). The results showed lower chlorophyll *a* and photosynthetic activity (F*v*/F*m*) in mixotrophy in comparison with photoautotrophy, which can be due to a decreased need for photosynthesis in this metabolic pathway; however, there were no changes in the values with the increase in NPs concentration in the medium. Photoautotrophy cultures were sensitive to NPs, reaching 39% of viability at 7.9 mg.L^{-1} (log 089), while in mixotrophy, cell viability was not affected by NPs. The biochemical composition and cell density changed as function of NPs concentrations, with increase in proteins:carbohydrates ratio in both treatments. The results showed that the microalga *C. sorokiniana* is more resistant to NPs during mixotrophic growth, but with changes in biochemical composition, whereas the photoautotrophic cultures were more sensitive to the increase of NP concentrations in the media.

Key word: titanium dioxide nanoparticles, biochemical composition, phytoplankton, mixotrophy, cell viability.

INTRODUCTION

Nanoparticles, defined as particles less than 100 nm in size in more than one dimension (Nowack & Bucheli, 2007; Navarro et al., 2008; Yang et al., 2012), are widely recognized as having versatile applications in a variety of areas as textiles, electronics, pharmaceutics, cosmetics, anti-fouling paints, food products, environmental remediation (Navarro et al., 2008; Cardinale et al., 2012; Melegari et al., 2013). The global investments in NPs increased from US$ 10 billion in 2005 to US$ 1 trillion since 2011, and the production increased from 10,000 in 2004 to 88,000 tons per year after 2010 (Navarro et al., 2008; Sharma, 2009; Barreto & Lombardi, 2016).

Among the most abundant NPs manufactured, titanium dioxide (TiO_2) nanoparticles are the second highest globally produced, approximately 3,000 tons annually (Gottschalk et al., 2009; Zhu et al., 2011; Piccinno et al., 2012; Yang et al., 2012), and with an estimate production of 2.5 x 10^6 ton per year until 2025 in the United States (Robichaud et al., 2009). This high production is associated with the photocatalytic activity, induced by UV-light, of this NP-TiO_2, which has been used in paints, solar technologies, pharmaceuticals, cosmetics and sunscreens (Hund-Rinke et al., 2006; Hartmann et al., 2010; Cardinale et al., 2012; Kulacki & Cardinale, 2012).

The use of NP-TiO_2 as personal care products (e.g. sunscreens), coating and paints due to their UV-light absorption efficiency, transparency to visible light that increases with decreasing particle size (Franklin et al., 2007), environmental contamination seem to be inevitable (Zhu et al., 2011).

Kaegi et al. (2008) reported that runoff under heavy rainfall can contain concentration as high as 3.5 x 10^8 nanoparticles.L^{-1}, which are discharged into aquatic ecosystems, and concentration of less than 1 $\mu g.L^{-1}$ has been found in river surface waters (Sharma, 2009; Dalai et al., 2013). However, once NP-TiO_2 enters aquatic habitats, these tend to interact with ions, organic matter and organisms, as phytoplankton, but literature is contradictory regarding the toxicity of NPs-TiO_2 (Cardinale et al., 2012). Until now, it is known that the toxicity of NPs is related to their physical and chemical properties, such as particle size, shape, aggregation status, surface coating, ionization (Nel et al., 2006; Beer et al., 2012; Yang et al., 2012) and the generation of reactive oxygen species (ROS) inside the cells (Kadar et al., 2012).

In aquatic ecosystems, the phytoplankton is responsible for the primary production, using sunlight and inorganic carbon to synthesize energy-rich organic matter, a process called photosynthesis, which sustains the aquatic food web (Reynolds, 2006). Due its role as primary producers in these environments, many researches have been carried out with the objective of evaluating the entry of toxic compounds in the food chain via phytoplankton (Araujo & Souza-Santos, 2013; Dalai et al., 2013).

Any change in its metabolic activity caused by toxic compounds can affect the organisms in higher trophic levels as well as the ecosystem as a whole (Kahru & Dubourguier, 2010). For example, Cardinale et al. (2012) studying the effects of NP-TiO_2 in three species of green algae reported reduction in the primary production in the microalgae *Chlamydomonas moewussi* and *Scenedesmus quadricauda*, but increase in *Chlorella vulgaris*, while respiration rate reduced in *C. moewussi*, increased in *C. vulgaris*, and remained constant in *S. quadricauda*.

However, many phytoplanktonic microorganisms can assimilate organic matter from the environment at the same time as they perform photosynthesis (Baldisserotto et al., 2014). A metabolic strategy which organisms can use autotrophy and heterotrophy concomitantly is called mixotrophy (Juntila et al., 2015). During mixotrophy, microalgae can assimilate organic matter by osmotrophy (for example glucose, glycerol, and organic acids) or phagotrophy (predation of bacteria). Up to now, we did not find any study in the literature that takes into account the effects of NPs during mixotrophic growth of phytoplankton. However, considering that aquatic environments that receive NP-TiO_2 contain a myriad of dissolved organic materials, and probably mixotrophy occurs within phytoplankton cells, investigations onto the toxicity of NPs under such metabolic pathway can furnish important information regarding the environmental reality.

Considering the strategies of monitoring the toxic effects in microalgae, the majority of toxicity tests focused in the photosynthetic activity and growth parameters, generally showing low toxicity, but assigning this to a probable failure on the response detection and suggest the use of other, more specific parameters, such as cell viability and biochemical composition (Tang & Dobbs, 2007; Barreto & Lombardi, 2016).

This work aimed at studying the physiology of *Chlorella sorokiniana* under mixotrophic and photoautotrophic conditions in cells exposed to NP-TiO_2. As physiological parameters, we monitored its growth, photosynthetic activity, cell viability and biochemical composition. This study is a contribution to the understanding of the effects of NPs on phytoplankton in a condition that can resemble what happens in the environment.

MATERIAL AND METHODS

Characterization of Titanium Dioxide Nanoparticles

The nano-TiO_2 used in this work was acquired from Sigma-Aldrich (CAS No. 13463-67-7) for commercial use and with pre-informed characteristics. However because these characteristics vary widely, we evaluated some of them (average particle diameter, crystallinity, morphology, specific surface area and zeta potential) and are reported in Barreto & Lombardi (2016). In synthesis, they reported that the characterization of the NPs-TiO_2 showed crystallinity of 92% anatase and 18% rutile, specific surface area of 45.60 $m^2.g^{-1}$ and a zeta potential of |25| mV.

Experimental Design

The freshwater microalga *Chlorella sorokiniana* was cultured in 500 mL Erlenmeyer flasks previously coated with a silanizing solution to reduce the adsorption of NPs onto the flasks walls. A volume of 250 mL of AAP Medium (EPA, 2012), with no EDTA, was used for all treatments at initial pH 7.00. The cultures were maintained in controlled conditions of temperature (24 ± 1 °C), light intensity (130 µmol photons m^{-2} s^{-1}) and photoperiod (12 h light/12 h dark). Exponentially growing cells (10^5 $cell.mL^{-1}$ initial inoculum) were exposed for 72 h to the nominal (added) NPs-TiO_2 concentrations: 7.9 x 10^{-4} (log -3.10), 7.9 x 10^{-3} (log -2.10), 7.9 x 10^{-2} (log -1.10), 7.9 x 10^{-1} (log -0.10), and 7.9 $mg.L^{-1}$ (log 0.89), with 5 x 10^{-3} $mol.L^{-1}$ glucose to stimulate mixotrophy, and without glucose (photoautotrophic condition). The concentration range

was based in Mueller & Nowack (2008) that estimated nanoparticles concentrations in lakes, reporting what is referred to as environmental concentration. Reference cultures (without the addition of NPs) were performed, one without glucose, and the other with glucose. Reference cultures had a natural Ti concentration of 2.64 x 10^{-4} mg.L^{-1} (log -3.58). Following the procedure described in Aruoja et al. (2009), NPs-TiO_2 suspensions were sonicated (Ultrasonic Sonicator, DES500, Brazil) during 30 min prior to use in order to reduce agglomeration and sedimentation of the particles.

After 72 h of exposure to NP-TiO_2, the experimental parameters were determined. Hydrogen ion concentration was determined with a pH-meter (Gehaka, PG1800, Brazil), chlorophyll *a* concentration (mg.L^{-1}) by *in vivo* fluorescence using a fluorimeter (Turner Designs, Model Trylogy, U.S.A.), cell density (cell.μL^{-1}) and viability (% of viable cells) were determined in a cytometer Muse® Cell Analyzer (U.S.A.). Specific growth rates (μ) were calculated through graphic representation of the natural logarithm of chlorophyll *a* concentration per mL as function of time. The linear regression from the straight line (exponential growth phase) was calculated and the angular coefficient represents the specific growth rate.

The maximum fluorescence of photosystem II (Fv/Fm) was obtained in 20-min dark-adapted cells using a pulse amplitude modulated fluorimeter, Phyto-PAM (Heinz Walz Effeltrich, Germany). This parameter can be used to infer about the physiological status of photosynthetic microalgae (Lombardi & Maldonado, 2011).

Proteins were determined according to the methodology of Bradford (1976) with extraction based in Raush (1981), while carbohydrates followed the method of Albalasmeh et al. (2013). All biochemical composition data are reported in picograms per cubic micrometer (pg.μm^{-3}) instead of per cell because mixotrophic cells are at least twice the photoautotrophic ones.

Data Analysis

This study was run with three experimental replicates for each treatment. The results were tested for normality and homogeneity, and significant differences between means of each variable were tested by one-way ANOVA and Tukey's post-hoc analysis

using Assistat 7.7 beta software. Graphs were plotted using the software Origin Pro (version 8.5.0).

RESULTS

pH

The values of pH are shown in Table 4.1, among the photoautotrophic cultures, treatment without NPs-TiO_2 showed the highest pH value (7.57), whereas in the other treatments, the pH increased with the increase of o NPs-TiO_2, from 6.24 to 6.55. Comparing the two types of culture, with exception of the treatments without NPs, at each concentration, the pH was higher in the mixotrophic metabolism than in the photoautotrophic ($p > 0.05$).

Table 4.1 – Values of culture pH (72 h NPs-TiO_2 exposed cultures). Values are means ± standard deviation of three replicates (same letters indicate statistical similarity). Statistical analysis was performed separately for the treatments either photoautotrophy or mixotrophy. Ref = reference culture.

Treatment	**Photoautotrophy**	**Mixotrophy**
Ref (log -3.58)	7.57 ± 0.05^{a}	6.25 ± 0.03^{b}
7.9×10^{-4} $mg.L^{-1}$ (log -3.10)	6.24 ± 0.02^{a}	6.54 ± 0.03^{b}
7.9×10^{-3} $mg.L^{-1}$ (log -2.10)	6.24 ± 0.03^{a}	6.89 ± 0.05^{b}
7.9×10^{-2} $mg.L^{-1}$ (log -1.10)	6.39 ± 0.01^{a}	6.92 ± 0.02^{b}
7.9×10^{-1} $mg.L^{-1}$ (log -0.10)	6.42 ± 0.02^{a}	6.99 ± 0.03^{b}
7.9 $mg.L^{-1}$ (log 0.89)	6.55 ± 0.02^{a}	7.05 ± 0.04^{b}

Chlorophyll *a* and Photosynthetic Activity

Figure 4.1 reports the chlorophyll *a* concentration in the photoautotrophic cultures, which increased with the increase of nanoparticles concentration ($p > 0.001$), from 5.8×10^{-8} to 1.25×10^{-7} $\mu g.cell^{-1}$; however, in the highest NP-TiO_2 concentration tested (7.9 $mg.L^{-1}$; log 0.89), the chlorophyll *a* concentration decreased, reaching 4.24 x

10^{-8} µg.cell^{-1}. During mixotrophy, there was no change (p = 0.2047) in the concentration of chlorophyll *a*, all of which were lower in relation to photoautotrophic cultures.

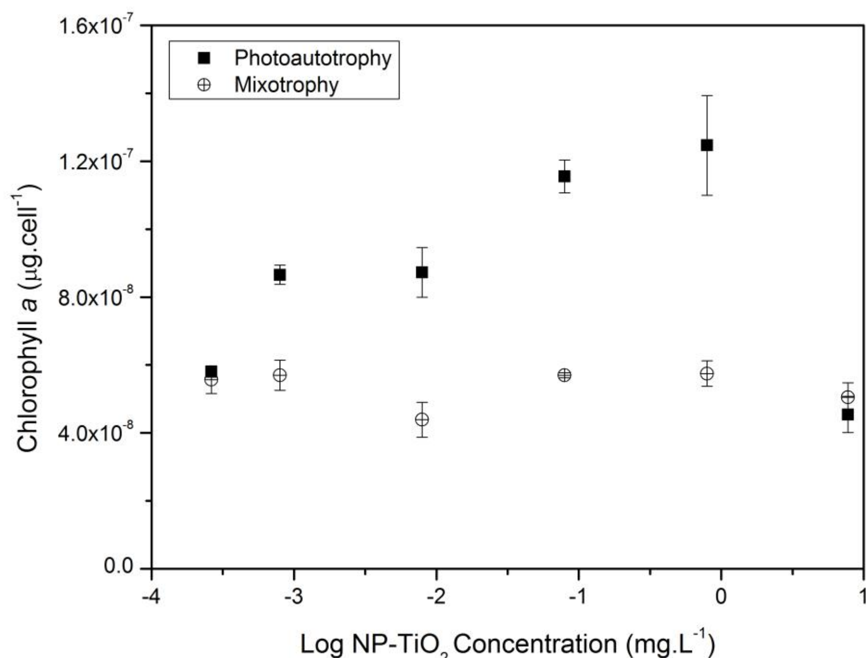

Figure 4.1 - Concentration of chlorophyll *a* (µg.cell^{-1}) in *C. sorokiniana* at 72 h of exposed to NP-TiO_2 in photoautotrophic and mixotrophic conditions. Error bars mean standard deviation from the mean (n = 3).

As expected, the maximum fluorescence of photosystem II, F*v*/F*m* (Figure 4.2) was higher in photoautotrophy than in mixotrophy, confirming a reduction in photosynthetic activity during mixotrophic growth conditions. NPs-TiO_2 does not appear to affect the photosynthesis both in photoautotrophy and mixotrophy cultures, since no statistically significant differences (p = 0.085 for photoautotrophy and p = 0.0608 for mixotrophy) were detected among the different concentrations tested in this study.

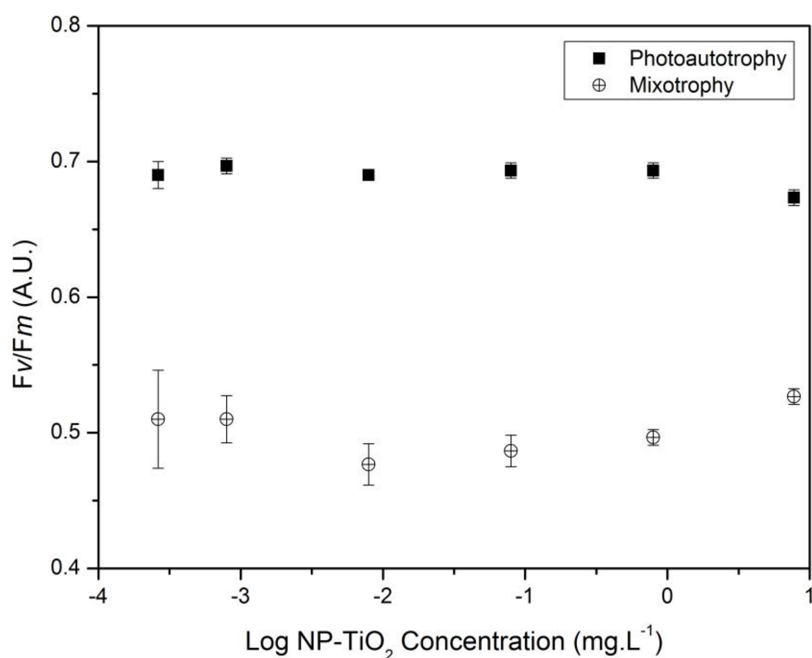

Figure 4.2 - Maximum fluorescence of photosystem II (F*v*/F*m*) of microalga *C. sorokiniana* at 72 h exposed to NP-TiO_2 in photoautotrophic and mixotrophic conditions. Error bars mean standard deviation from the mean (n = 3). A.U. means Arbitrary Units.

Specific Growth Rate

The specific growth rates are shown in Figure 4.3. No statistical difference ($p = 0.0079$) was obtained among the treatments during photoautotrophic growth, whereas during mixotrophy, the treatments with nanoparticles showed growth rates smaller than the control with glucose; this was higher than the photoautotrophic control (no NPs-TiO_2 addition).

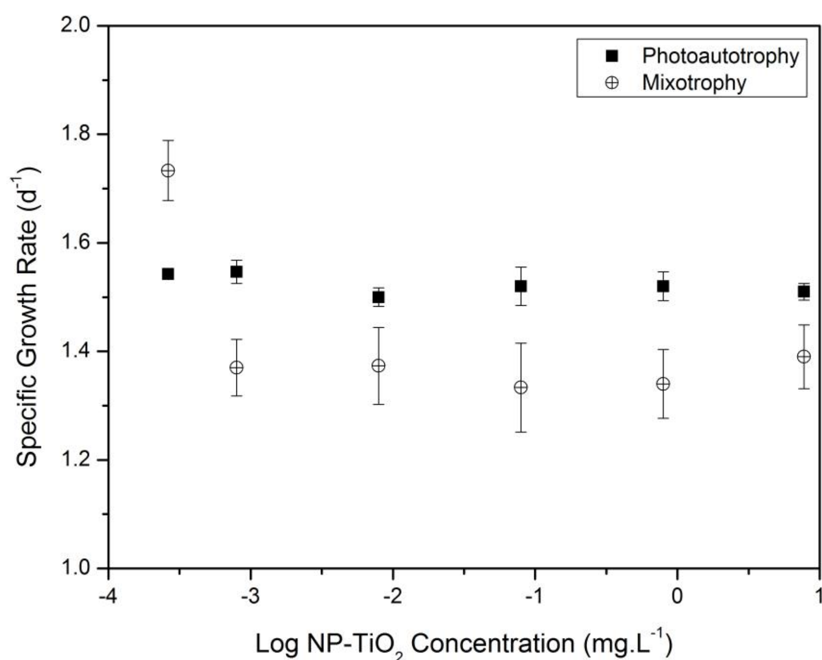

Figure 4.3 – Specific growth rates (d^{-1}) based in chlorophyll *a* concentration ($mg.L^{-1}$) of microalga *C. sorokiniana* exposed to NPs-TiO_2 in photoautotrophic and mixotrophic conditions. Error bars mean standard deviation from the mean (n = 3).

Cell Density and Viability

In photoautotrophic cultures, the cell density increased in the first two NPs-TiO_2 concentrations (Figure 4.4), reaching the maximum value of 5.42 x 10^6 cell.mL^{-1}; decreasing thereafter. This suggest a toxic potential of the NPs-TiO_2 to *C. sorokiniana*. Cell density in the mixotrophic cultures decreased with the increase of NPs concentrations with the highest density in the control (with glucose, but without NPs).

Cell viability describes the percentage of live cells in the sample, and as Figure 5 shows, in the mixotrophic condition, cell viability was not affected by the NP-TiO_2, with 97 to 99% of the cells remaining alive. However, in the photoautotrophic cultures, cell viability decreased with the increase in the NPs-TiO_2 concentration ($p < 0.001$), exhibiting 39% of viable cells in the highest concentration tested.

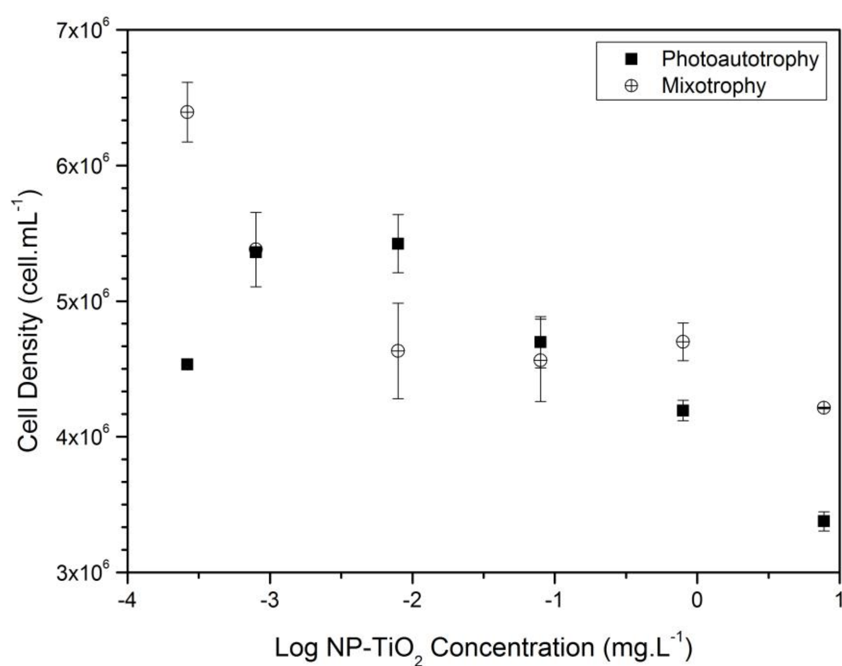

Figure 4.4 – Cell density (cell.mL^{-1}) at 72 h of exposed to NPs-TiO_2 in photoautotrophic and mixotrophic conditions. Error bars mean standard deviation from the mean (n = 3).

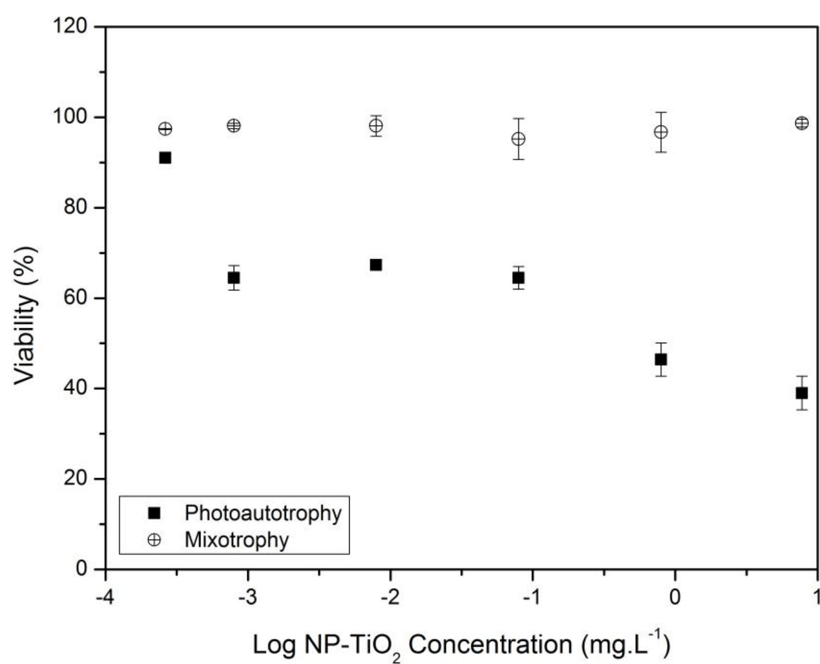

Figure 4.5 – The percentage of viable cell at 72 hours of exposed to NPs-TiO_2 in photoautotrophic and mixotrophic conditions. Error bars mean standard deviation from the mean (n = 3).

Biochemical Composition

Figure 4.6 shows the concentration of carbohydrates (Fig. 4.6A) and proteins (Fig. 4.6B) in *C. sorokiniana* after 72 h of NPs exposure, which are reported per unit cell volume. Considering the carbohydrates, no significant differences ($p < 0.0001$) among the treatments were obtained for the photoautotrophic conditions, except in the higher concentration of NPs (7.9×10^{-4} mol.L^{-1}; log 0.89), doubling its value in relation to the control. However, under mixotrophy, a decrease in carbohydrates concentrations were obtained for the three higher concentrations of NPs (7.9×10^{-2}, log -1.10; 7.9×10^{-1}, log -0.10; and 7.9 mg.L^{-1}, log 0.89). For proteins, the photoautotrophic cultures always had higher values than the mixotrophic condition. In addition, this figure shows that protein synthesis was affected by the NP-TiO_2 concentration, being approximately two times higher in both photoautotrophic and mixotrophic cultures at 7.9×10^{-2} mg.L^{-1} (log -1.10) of NP-TiO_2 and above. The P:C ratios (Fig. 4.6C) were higher in the photoautotrophic cultures, once again the highest values were present in the three highest NP concentrations (7.9×10^{-2} and above).

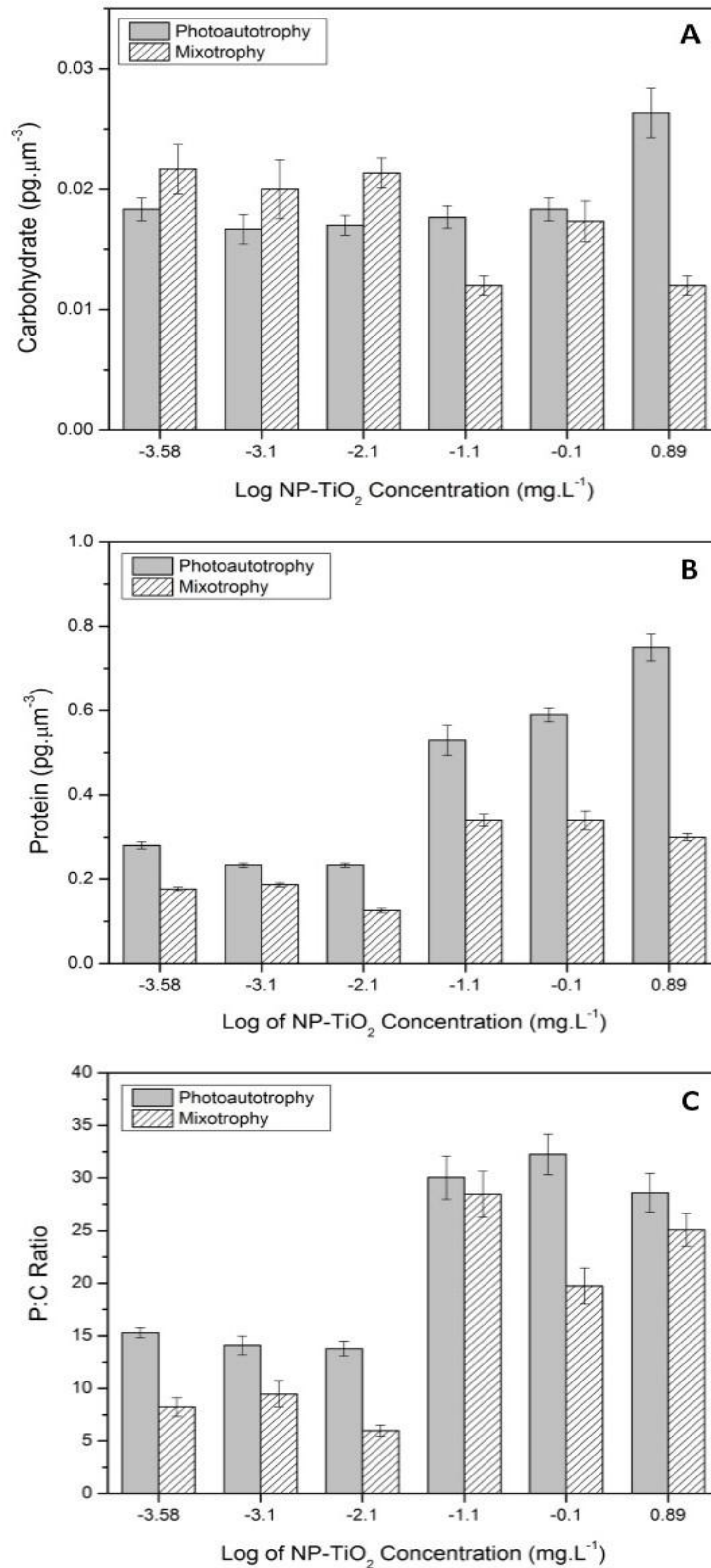

Figure 6 - Biochemical composition of microalga *C. sorokiniana* at 72 h exposed to NP-TiO_2 in photoautotrophic and mixotrophic conditions. (A) Carbohydrate content (pg.μ^{-3}). (B) Protein content (pg.μ^{-3}). (C) P:C ratio. Error bars mean standard deviation from the mean (n = 3).

DISCUSSION

In microalgae cultures usually pH above 7.00 indicates photosynthetic activity by the cells, which remove carbon dioxide, so reducing the carbonic acid content and increasing pH (Reynolds, 2006), but in mixotrophy, the pH increases due to the symport uptake of protons and glucose (Komor & Tanner, 1974; Juntila et al., 2015). Considering that our initial culture pH was 7.00, values below this in the photoautotrophic cultures indicates that the NP-TiO_2 affected the pH of the culture medium, since the cells were doing photosynthesis (Fig. 4.2).

It is known that the physicochemical surface properties of nanoparticles are dependent on environmental factors, including pH (Navarro et al., 2008; Sharma, 2009). Nanoparticles of TiO_2 are expected to have negative surface charge at pH > 7.00, but positive surface charge at pH < 6.00 (Ridley et al., 2006). Considering that cell wall exhibits negative charge and the pH in this study was around 6.00, we expected that most NPs must be adhered to the cell wall, as described by Navarro et al. (2008).

The increase in chlorophyll *a* concentration in the photoautotrophic cultures may have been due to a shading effect caused by the nanoparticles that adhered electrically to the cell wall of the microalgae. According to Kulacki & Cardinale (2012), the adhesion of NPs to microalgae surface reduces the availability of light for each cell in the culture, and this stimulates chlorophyll production by the cell as an attempt to overcome the shading effect (Navarro et al., 2008; Sharma, 2009; Cardinale et al., 2012; Melegari et al., 2013). Recalling the photoautotrophic culture pH (6.24 – 6.55) and the consequent positive charge of the NPs in these cultures together with negative charge of the cell wall, we can rationale that in the photoautotrophic growth, the cells would probably be more coated with NPs-TiO_2 than in the mixotrophic condition, whose pH varied between 6.54 – 7.05. In more neutral pH, the mixotrophic cells would inversely be less coated with NPs and chlorophyll would not increase, what in fact was detected. Even with the nanoparticles reducing light availability to the cell, the photosynthetic activity (Fv/Fm) was not affected, remaining with values close to 0.7, indicating that the algae were healthy, possibly with no stress or nutrient limitation (Kumar et al., 2014). However, shading seems to have been excessive in the concentration of 7.9 $mg.L^{-1}$ (log

-0.10) of NPs-TiO_2 and, consequently, a decline of cell density and chlorophyll *a* was observed even though this did not interfere with the photosynthetic activity.

Differently, in the mixotrophic cultures the chlorophyll *a* concentration and the photosynthetic activity around 0.5 allied to the cell growth confirmed that microalgae were using another source of energy and carbon (Giovanardi et al., 2014; Juntila et al., 2015). Consequently, microalgae reduced their need for light to be used in photosynthesis, and produced less chlorophyll *a* than in photoautotrophy (Perez-Garcia et al., 2011; Alkhamis & Qin, 2016).

Cardinale et al. (2012) reported increase in the photosynthetic activity in *Chlorella vulgaris*, and decrease in *Chlamydomonas moewusii* and *Scenedesmus quadricauda* cultivated photoautotrophically with NPs-TiO_2, while Baldisserotto et al. (2014) and Giovanardi et al. (2014) found increase in *Neochloris oleoabundans* during mixotrophic conditions without nanoparticles. According to Giovanardi et al. (2014), the pigment content and photosynthetic activity vary depending on algal species, organic carbon source and chemical composition of the culture medium.

In photoautotrophic cultures, the specific growth rate was not affected by presence of NPs-TiO_2. This result is in agreement with others in literature. Kulacki & Cardinale (2012) also observed no significant effects of NPs-TiO_2 in 10 phytoplanktonic species of belonging to Cyanobacteria, Bacillariophyta, Chlorophyta and Charophyta. According to Barreto & Lombardi (2016), specific growth rates reflect the general microalgae metabolism and it is not a parameter as sensitive as cell viability to detect the effects of nanoparticles.

In contrast to what occurs in mixotrophic cultures with no NPs, where the addition of an organic carbon source stimulates the growth of microalgae and higher growth rates are obtained in comparison with photoautotrophic conditions (Li et al., 2014; Rosemberg et al., 2014; Juntila et al., 2015). In the presence of nanoparticles, the growth rates in the mixotrophic cultures were lower than in the photoautotrophic ones. This difference is an evidence that somehow the nanoparticles affected the rate of cell division of this species of microalgae, as has also been shown in Linkous et al. (2000) and Cardinale et al. (2012).

As discussed by Tang & Dobbs (2007), total cell density does not express the real effects of a compound on the microalgae's physiology, but a total effect on the population, not discriminating living and dead cells. As an alternative, cell viability has been proposed as a measure closer to the reality of possible toxic effects on cellular metabolism, since it considers the percent of living organisms in a population sample (Barreto & Lombardi, 2016).

In the present work, the microalgae cultured in mixotrophy were more resistant to NPs-TiO_2 than the photoautotrophic cells. In the mixotrophic situation, no changes in the viability occurred and it was kept always close to 100%, albeit the reduced values of specific growth rates under NPs exposure. This indicates that cell division was affected, but not the algal health. Whereas in photoautotrophy, as the concentration of NP increased, the percentage of living cells decreased, showing the negative effects of NPs-TiO_2 in the microalgal population. Barreto & Lombardi (2016) observed similar results related to the viability of *Scenedesmus bijugus* exposed to NPs-TiO_2, and Melegari et al. (2013) related decrease in cell viability of *C. reinhardtii* cultivated with cooper oxide nanoparticles.

The present results showed that cell viability, considered an expression of algal health was impaired in photoautotrophic cell in NP-TiO_2 concentration as low as 7.9 x 10^{-4} mg.L^{-1} (log -3.10) while cell density was only affected in the NP-TiO_2 concentration of 7.9 x 10^{-2} mg.L^{-1} (log -1.10), emphasizing the need to use the cell viability as a more sensitive parameter to identify the toxicity of nanoparticles.

The differences observed in the content of total intracellular carbohydrates and proteins at concentrations of NPs-TiO_2 from 7.9 x 10^{-2} to 7.9 mg.L^{-1} (log -1.10 to log 0.89) indicate that the particles affected the microalga *C. sorokiniana*. The increase in carbohydrates in the photoautotrophic cultures at the highest NP-TiO_2 concentration tested (~ 1.4 times higher at 7.9 mg.L^{-1}; log 0.89) can be considered a signal of cellular stress. As reported in literature, the accumulation of storage compounds in microalgae indicates they are facing a physiologically problematic situation, as commonly reported for trace metal stress. Chia et al. (2013) reported that *Chlorella vulgaris* exposed to Cd (1.12 x 10^{-2} mg.L^{-1}; log -1.95) had its carbohydrate content increased ~ 3 times in comparison with the controls. Miao et al. (2009) observed increase in the production of polymeric substances in *Thalassiosira weissflogii*, exposed to silver engineered

nanoparticles at concentrations between 1.08 x 10^{-10} to 1.08 x 10^{-4} $mg.L^{-1}$ (log -9.96 to log -3.96). Granum et al. (2002) found an increase higher than 4 times in the carbohydrates content in *Skeletonema costatum* cultured in media with nitrogen depletion; Yang et al. (2012) reported that light intensity and nitrogen concentration had significant effects on polysaccharide production in *Microcystis aeruginosa*. The opposite effect observed for the mixotrophic growth (~ 1.6 times lower carbohydrates at 7.9 x 10^{-2} to 7.9 $mg.L^{-1}$; log -1.10 to log 0.89) should also be related to a stressing condition. Cardinale et al. (2012) reported that *Chlorella* sp. (photoautotrophic growth) exposed to NPs-TiO_2 (50 to 300 $mg.L^{-1}$; log 1.69 to log 2.47) showed increased respiration rate. It is known that mitochondrial respiration consumes carbohydrates and this can end up resulting in decreased intracellular carbohydrates, as we observed. Nevertheless, we should mention that respiration rate increase would be expected to occur in both photoautotrophic and mixotrophic metabolisms, thus further research is needed to understand the differences in carbohydrates metabolic routes under the different metabolisms.

The increase in intracellular protein concentration for both mixotrophic and photoautotrophic metabolisms at NP-TiO_2 of 7.9 x 10^{-2} $mol.L^{-1}$ (log -1.10) and above can be related to a detoxification mechanism, as reported in Miao et al. (2009). These authors hypothesized that microalgae also synthesize proteins as a way of protecting them from the action of nanoparticles, such as phytochelatins for metal ion (Kaplan et al., 1995; Perales-Vela et al., 2006). However, more research is needed to verify this hypothesis. It is know that plants and microalgae can produce peptides that bind metal ions so decreasing its internal availability for the cell.

The P:C ratio reports on the physiological status of the cell, and values lower than one indicate nutrient-limited environment according to Ganf et al. (1986), Kilham et al. (1997) and Rocha et al. (2014). Therefore, based on the P:C ratios obtained in this research, microalgae were not suffering from nutrient limitation, evidencing that NPs does not affect the uptake of nutrients by the cells. In addition, the doubled P:C ratio at NP-TiO_2 at 7.9 x 10^{-2} $mg.L^{-1}$ (log -1.10) and above found in both photoautotrophy and mixotrophy is an indication that the NPs induced the synthesis of proteins in these cultures and, if this is a matter of detoxification or other cellular compensation process still needs further investigation. It is becoming clear from literature (Barreto &

Lombardi, 2016) that the effects of the NPs on microalgae vary according to the species and to the NP tested. For example, Barreto & Lombardi (2016) did not find differences in the composition of carbohydrates and proteins or in P:C ratio in cultures of *S. bijugus* treated with NPs-TiO_2; Cherchi et al. (2015) reported that NPs-TiO_2 induced changes in intracellular composition and nutrient stoichiometry in cultures of Cyanobacteria *Anabaena variabilis*.

CONCLUSION

It was verified through this research that NP-TiO_2 at concentrations from 7.9 x 10^{-2} to 7.9 mg.L^{-1} (log -1.10 to log 0.89) affected the physiology of the microalga *Chlorella sorokiniana*. Protein synthesis was the unique parameter that was equally affected independent of the metabolic pathway, e.g., if mixotrophic or photoautotrophic, its concentration doubled. However, usually depending on the growth metabolism different aspects of the cell physiology were affected.

Considering the effects individually, for the mixotrophic cultures and the photoautotrophic ones, the first had more conservative behavior for chlorophyll *a*, photosynthetic activity (F*v*/F*m*) and cell viability, parameters in which NPs-TiO_2 had no detectable effect, whereas the cell density, specific growth rate and concentration of carbohydrates were all negatively affected by the nanoparticles. In the case of photoautotrophic condition, the more sensitive parameters to NP-TiO_2 were chlorophyll *a*, cell viability and cell density.

In spite of the above mentioned differences, as a general view, our results support the higher resistance of *C. sorokiniana* to NP-TiO_2 under mixotrophy than photoautotrophy. This can implicate that under natural environmental conditions, where a myriad of organic substances occur, those species that can benefit from mixotrophy will possibly have higher survival than those that cannot. As time goes, a selection and possibly reduction of biodiversity can take place. In addition, energy imbalance throughout the aquatic food chain can become a problem since the intracellular biochemical composition was affected by the NP-TiO_2 at environmentally important concentrations.

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FINAL CONSIDERATIONS

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According to the results of this work, we can consider that mixotrophy is an important metabolic pathway and that it should be studied with physiological and ecological approaches and that the microalgae *Chlorella sorokiniana* proved to be effective in the use of organic carbon as source of structural carbon and energy via respiration.

During mixotrophy, the microalga *C. sorokiniana* presented higher population growth parameters (chlorophyll *a*, cell density and specific growth rate); the photosynthetic activity was affected, but it was more efficient in the use of light energy. It was also during the mixotrophy that the microalgae produced the largest quantity of lipids, increased their cellular volume and, consequently, the algal biomass. From the biotechnological point of view, the production of microalgae in mixotrophy represents a strategy to increase productivity and, consequently, profits. With the addition of organic carbon, *C. sorokiniana* cells grew rapidly, were efficient in the use of light (indicating that the cells were healthy) and stored high amounts of lipids per unit of volume in a short time (approximately 4 days of culture). It is also important to note that the contamination by bacteria in mixotrophic cultures was not a problem, but a benefit for the production of microalgae, considering that many bacteria produce growth stimulant factors to algal growth, while microalgae have mechanisms to control the bacterial densities in the cultures.

When we consider the action of contaminants in microalgae, the literature is clear that metals such as cadmium (Cd) are toxic, but it is still controversial in relation to titanium dioxide nanoparticles (NPs-TiO_2). In this research, we observed that in mixotrophy cultures, the microalgae presented lower probabilities of being affected by the contaminants tested than it did in photoautotrophic metabolism. These results are probably due to both Cd and NPs-TiO_2 affecting mainly the photosynthetic apparatus of the microalgae, reducing their photosynthetic activity. However, when microalgae do not rely exclusively on photosynthesis to survive (it is worth noting that ecotoxicological studies are only carried out under photoautotrophic conditions), they may be more resistant to the action of both Cd and NPs-TiO_2.

Extrapolating these results to contaminated aquatic environments, mixotrophy has great effects on the structure and functioning of aquatic ecosystems. The action of metals and nanoparticles on microalgae can reduce the photosynthesis and increase the respiration due to the increase in the consumption of organic carbon. The consequences of the interactions between mixotrophy and contaminants can be a reduction of the primary production, with reduction in the concentration of dissolved oxygen and increase of the acidity due to respiration increase and, consequently, the formation of carbonic acid in the water. Another important issue besides the higher resistance of the microalga to Cd and NPs-TiO_2, is that the biochemical composition of their cells was altered, and this can have consequences for the whole food chain.

We conclude that studies on mixotrophy should be carried out when dealing with phytoplankton organisms, since it is a metabolism that can occurs frequently in most water bodies due to the vast sources of organic carbon present. Studies relying just on photoautotrophy of microalgae may be over or underestimating the true role of these organisms in the ecosystems as well as of the contaminants on the organisms. Finally, the present study showed that mixotrophy or photoautotrophy imply in distinct biochemical composition of the microalga *C. sorokiniana* which, occurring in a large group of phytoplankton populations, can affect the energy balance, and together with contaminants, the biodiversity in aquatic environments.