UNIVERSIDADE FEDERAL DE SÃO CARLOS – UFSCAR
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE – CCBS
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

PHYSIOLOGICAL RESPONSES OF CHLOROPHYTA MICROALGAE UNDER ENVIRONMENTALLY RELEVANT COPPER CONCENTRATIONS: BIOMOLECULES, OXIDATIVE STRESS AND PHOTOSYNTHESIS

SULEIMAN DAUDA

São Carlos – SP, 2022

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PPGERN

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

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DEDICATION

I dedicate this work to my parents, Alhaji Dauda Adamu and Hajiya Fatima Dauda. Thank you for your unending care, love, support, and prayers.

ACKNOWLEDGEMENTS

RESUMO

As microalgas requerem cobre (Cu) em níveis traço para seu crescimento e metabolismo. É um componente vital para certas metaloproteínas, participa do processo fotossintético e catalisa várias reações redox. Embora este elemento tenha sido amplamente estudado na fisiologia de microalgas, os efeitos de níveis ambientalmente relevantes foram ainda pouco investigados. O objetivo deste estudo foi verificar o efeito das concentrações ambientais de cobre em alguns aspectos da fisiologia de microalgas, como taxas de crescimento, produção de biomoléculas (carboidratos, lipídios, proteínas e pigmentos), resposta antioxidante e fotossíntese. Para isso, *Ankistrodesmus flexuosus*, *Curvastrum pantanale*, *Chlamydomonas chlorastera* e *Monoraphidium* sp. foram os organismos-teste. Eles foram mantidos sob condições controladas em laboratório com concentrações de cobre livre (Cu^{2+}) variando de baixa (~0,1 nM) a alta (5480,0 nM). As culturas foram mantidas por 96 horas e todas as análises foram feitas na fase de crescimento exponencial. As densidades de celulares e as taxas de crescimento não foram afetadas em baixas concentrações de Cu até 9,1 nM Cu^{2+} em *C. chlorastera*, e 7,4 nM Cu^{2+} nas outras espécies. Em relação aos pigmentos, β-caroteno e luteína aumentaram em *C. chlorastera* (1,2 mg g^{-1} β-caroteno; 6,14 mg g^{-1} luteína) em 0,3–0,4 nM Cu^{2+}. *C. chlorastera* apresentou os maiores, carboidratos (> 50 pg célula^{-1}) em todas as concentrações de Cu testadas, teor de proteínas (270,2 pg célula^{-1}; 0,3 nM Cu^{2+}) e lipídios (61,9% dw; 1,2 nM Cu^{2+}). As atividades do α, α-difenil-β-picrilhidrazil (DPPH), Glutationa S-transferase (GST), peroxidase (POD), superóxido dismutase (SOD), e teor de malondialdeído (MDA) não foram afetadas pela baixa exposição ao Cu, mas aumentou em alto Cu. Os rendimentos quânticos efetivos das microalgas ($\Delta F/F_m{'}$) foi mais sensíveis ao Cu do que o rendimentos quânticos máximos (F_v/F_m). Em *Monoraphdium* sp., o aumento de Cu (3,4–7,4 nM Cu^{2+}) aumentou a fotossíntese, conforme registrado no rendimento quântico efetivo ($\Delta F/F_m{'}$), taxa de transporte de elétrons máxima relativa (rETR$_m$), irradiância de saturação (E$_k$) e extinção fotoquímica (qP e qL). A extinção não fotoquímica (NPQ) e o tamanho da antena PSII de *Monoraphidium* sp. aumentou em alto Cu. *Monoraphidium* sp. também teve a menor fotoinibição (β) sob alta irradiância em todas as exposições de Cu. A produtividade primária de *Monoraphidium* sp. foi inalterado em baixo Cu (1,7-21,4 nM Cu^{2+}), mas reduziu em alto Cu (589,0 nM Cu^{2+}). Respostas horméticas induzidas por Cu foram observadas no conteúdo de lipídios em *C. chlorastera* e na fotossíntese em *Monoraphidium* sp. Esses resultados mostram que dependendo da espécie e concentração, o Cu pode estimular o acúmulo de biomoléculas e aumentar a fotossíntese em microalgas, sem reduzir o crescimento.

Palavars chave: Produção de biomassa, Fisiologia de microalgas, Antioxidantes, Fotossíntese

ABSTRACT

Microalgae require copper (Cu) in trace levels for their growth and metabolism. It is a vital component of certain metalloproteins, participates in the photosynthetic process, and catalyzes various redox reactions. Although this element has been widely studied concerning microalgae physiology, the effects of environmentally relevant levels have been less investigated. The aim of this study was to see the effect of environmental copper concentrations on some aspects of algae physiology, as growth rates, biomolecules (carbohydrates, lipids, proteins, and pigments) production, antioxidant response, and photosynthesis. For this, *Ankistrodesmus flexuosus*, *Curvastrum pantanale*, *Monoraphidium* sp., and *Chlamydomonas chlorastera* were the test organisms. They were kept under laboratory controlled conditions with Cu concentrations ranging from low (0.1 nM) to high (5480.0 nM) free Cu ions (Cu^{2+}). Cultures lasted 96 h and all analyses were done in exponential growth phase. Cell densities and growth rates were unaffected in low Cu concentrations up to, 9.1 nM Cu^{2+} in *C. chlorastera* and 7.4 nM Cu^{2+} in the other species. In relation to pigments, β-carotene and lutein increased in *C. chlorastera* (1.2 mg g^{-1} β-carotene; 6.14 mg g^{-1} lutein) in 0.3–0.4 nM Cu^{2+}. *C. chlorastera* had the highest, carbohydrates (> 50 pg $cell^{-1}$) across all Cu concentrations tested, proteins content (270.2 pg $cell^{-1}$; 0.3 nM Cu^{2+}), and lipids (61.9% dw; 1.2 nM Cu^{2+}). The activities of α, α-diphenyl-β-picrylhydrazyl (DPPH) radical, Glutathione S-transferase (GST), peroxidase (POD), superoxide dismutase (SOD) and malondialdehyde (MDA) content were not affected by low Cu exposure, but increased in high Cu. The microalgae effective quantum yields ($\Delta F/F_m'$) were more sensitive to Cu than their maximum quantum yields (F_v/F_m). In *Monoraphdium* sp., Cu increase (3.4–7.4 nM Cu^{2+}) increased photosynthesis, as recorded in the effective quantum yield ($\Delta F/F_m'$), relative maximum electron transport rate ($rETR_m$), saturation irradiance (E_k), and photochemical quenching (qP and qL). The non-photochemical quenching (NPQ) and PSII antenna size of *Monoraphidium* sp. increased in high Cu. *Monoraphidium* sp. also had the lowest photoinhibition (β) under high irradiance across all Cu exposures. The primary productivity of *Monoraphidium* sp. was unaltered in low Cu (1.7–21.4 nM Cu^{2+}), but reduced by high Cu (589.0 nM Cu^{2+}). Cu-elicited hormetic responses were seen in lipids content in *C. chlorastera*, and in photosynthesis in *Monoraphidium* sp. These findings show that depending on the species and concentration, Cu can stimulate biomolecules accumulation and increase photosynthesis in microalgae, without reducing growth.

Keywords: Biomass production, Microalgal physiology, Antioxidants, Photosynthesis

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LIST OF ABBREVIATIONS, ACRONYMS, AND SYMBOLS

α	Alpha
β	Beta
ANOVA	Analysis of variance
BG-11	Blue green medium 11
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Chl a	Chlorophyll a
Cu	Copper
Cu^{2+}	Cupric ions
DMSO	Dimethyl sulphoxide
DPPH	α,α-diphenyl-β-picrylhydrazyl
DW	Dry weight
EC 50	Effective concentration 50
EDTA	Ethylenediaminetetraacetic acid
E_k	Saturation irradiance
ETC	Electron transport chain
$ETR\ (II)$	Absolute electron transport rate
F_o	Minimum fluorescence level in dark-acclimated state
$F_o{}'$	Minimum fluorescence yield in light-acclimated state
F_m	Maximum fluorescence level in dark-acclimated state
$F_m{}'$	Maximum fluorescence level in light-acclimated state
F_s	Steady-state fluorescence in actinic light
F_v	Variable fluorescence in dark-acclimated state
GST	Glutathione S-transferase
L	Avogadro's number
LHC	Light harvesting complex
LN	Natural logarithm
MDA	Malondialdehyde
NPQ	Non photochemical quenching
P680	Photosystem II primary electron donor
P700	Photosystem I primary electron donor
PAM	Pulse amplitude modulation

PAR	Photosynthetically active radiation
P^B	Biomass-specific photosynthetic rate
pH	A measure of acidity or basicity
POD	Peroxidase
PQ	Plastoquinone quotient
PS I	Photosystem I
PS II	Photosystem II
PSU	Photosynthetic unit
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
QA	Primary quinone acceptor of PSII
Q_B	Secondary quinone acceptor of PSII
qL	Photochemical quenching (Lake model)
qP	Photochemical quenching (Puddle model)
RC	Reaction centre
RLC	Rapid light curve
rO_2	Oxygen evolution rate
SOD	Superoxide dismutase
WC	Wright's cryptophyte

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1.0 INTRODUCTION

1.1 Microalgae and copper ions

Microalgae can be defined as eukaryotic photosynthetic microorganisms that can have either a unicellular or simple multicellular body structure, or tallus (TOMASELLI, 2004; DOLGANYUK et al., 2020). They inhabit most, if not all, earth ecosystems, from aquatic (where they can be either benthic, pelagic, or planktonic) to terrestrial, representing a big variety of species living in a wide range of environmental conditions (LEVINE, 2018; MATA; MARTINS; CAETANO, 2010). Although they are generally free-living, some microalgae live in symbiotic associations with other organisms (TOMASELLI, 2004). In freshwater ecosystems, the most diverse group of microalgae are the green microalgae (NORTON; MELKONIAN; ANDERSEN, 1996), which taxonomically belong to different phyla (GRAHAM; WILCOX, 2000; GARCIA-PICHEL; BELNAP, 2021).

All microalgae used in this study are green and belong to the Chlorophyta which possess chlorophylls *a* and *b* in a single chloroplast surrounded by two envelope membranes (MCCOURT, 1995; SINGH; SAXENA, 2015). Their cell walls usually have cellulose as the main structural polysaccharide, and form starch as the storage product. They differ from the rest of the eukaryotic algae by forming the starch in the chloroplast instead of in the cytoplasm (LEE, 2008).

Microalgae require copper (Cu) as an essential element in trace levels, it is usually taken up by algae via the formation of coordination complexes with specialized transport ligands in their outer membranes (SUNDA, 1988). Cu occurs in biological systems in two stable oxidation states: Cu^+ and Cu^{2+} (LIPPARD; BERG, 1994), and this enables its use as a catalyst in various redox reactions, including electron transfer and oxygen chemistry (KROPAT et al., 2015). Cu participates in numerous physiological processes and is an essential cofactor for many metalloproteins. However, problems arise when excess Cu is present in cells. In excess, copper can inhibit plant growth and impair important cellular processes, such as photosynthetic electron transport (YRUELA, 2005).

All the components of the photosynthetic electron transport chain (ETC) require different metal atoms for their regular operation, except plastoquinone (DROPPA; HORVÁTH, 1990). Cu is needed for the synthesis and proper functioning of plastocyanin, a protein of the photosystem II (PSII) ETC found in photosynthetic organisms such as algae (BOWYER; LEEGOOD, 1997; PEERS; PRICE, 2006). However, not all microalgae need

plastocyanin for the transportation of electrons in the PSII because it can be substituted by cytochrome c_6 (HOWE et al., 2006; KATOH, 2003).

Microalgae mostly grow in water bodies containing natural concentrations of Cu that can be referred to as environmentally relevant or realistic concentrations if the environment is not polluted (WELTJE; SUMPTER, 2017). These are usually non-toxic concentrations of Cu that range from 1–5 µg/L ($\sim10^{-8}$ - 10^{-7} molL^{-1}) (FERREIRA et al., 2008; PRASAD, 2004), but can reach up to 30 µg/L (US ENVIRONMENTAL PROTECTION AGENCY, 2007). Cu concentrations within these ranges are also used in common algal culture media recipes like BG-11 (RIPPKA et al., 1979), and WC medium (Guillard and Lorenzen 1972).

When considering the amount of Cu in a solution that can used by microalgae, working with the total amount of Cu only does not suffice. As not all the Cu in the solution will be available for uptake by the cells, some of it would be bound by ligands (both organic and inorganic) (ALLEN; HANSEN, 1996; BUCKLEY, 1994), and react with colloids or particulate matter (FLORENCE; BATLEY; BENES, 1980). The unbound and unreacted amount is known as free Cu, the hydrated form. This is the quantity of Cu that is bioavailable or that can cause toxicity. Cu, as other metals, has to be in an available form for algae and plants to be able to take up or they must have mechanisms to make the metals available (PRASAD, 2004). Therefore, Cu internalization by the cell is largely dependent on its free ion concentration (ALLEN; BRISBIN; HALL, 1980; LAGE et al., 1996; STAUBER; DAVIES, 2000). Growth rate inhibition and Cu content in algal cells were proportional to the cupric ion activity and not to the total dissolved concentration (SUNDA; GUILLARD, 1976).

There has been a gradual increase in Cu loading into aquatic environments due to industrial activities (HUBENY et al., 2021; MASINDI; MUEDI, 2018). Usually, high metal loading cause toxicity and has received much attention, but a subtle rise in Cu which does not cause pollution is poorly studied. Such small increases in Cu concentrations are normally considered to cause no effect, or are harmless to aquatic life. However, many natural and anthropogenic environmental factors including metals like Cu, can induce hormesis (AGATHOKLEOUS; CALABRESE, 2020).

The word hormesis comes from Greek meaning "setting into motion" or "to boost something" (POSCHENRIEDER et al., 2013). This is a dose–response concept that is characterized by a low-dose stimulation and a high-dose inhibition (AGATHOKLEOUS, 2018; CALABRESE, 2008; CEDERGREEN et al., 2006). An illustration of the hormetic dose-response relationship is given in Fig. 1. Kendig et al. (2010) defined hormesis as a dose-

response relationship for a single endpoint that is characterized by reversal of response between low and high doses of chemicals, biological molecules, physical stressors or any other initiators of a response. The low-dose stimulation represents an "over-compensation" response to a disruption in homeostasis (CALABRESE, 1999, 2008) caused by the stressor. This modest overcompensation response has been highly conserved in an evolutionary sense, and it may be hard to detect (CALABRESE, 2008).
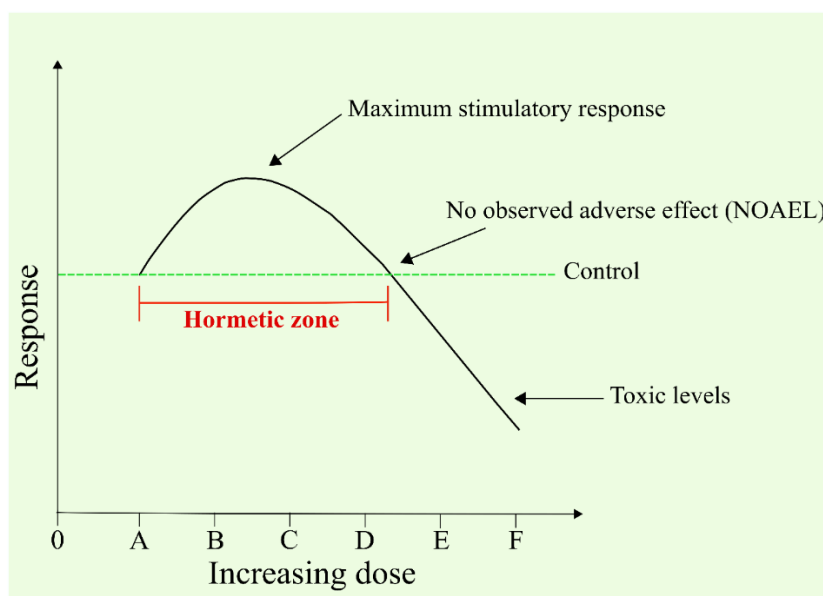
Fig. 1 Hormetic dose-time response relationship

1.2 Microalgal biomolecules/bioproducts

Microalgae are a rich source of high valuable compounds, like carbohydrates, proteins, lipids and different types of pigments (chlorophylls, carotenoids and phycobiliproteins) (BENAVENTE-VALDÉS et al., 2016; BHALAMURUGAN; VALERIE; MARK, 2018; YAAKOB et al., 2014). Algal bioproducts provide environmentally friendly alternatives to synthetic sources of such products. The production of these bioproducts varies from species to species and even in the same species of algae (MOBIN; CHOWDHURY; ALAM, 2019). So, to achieve optimum biocompounds production, microalgae species/strain selection is a key decision that influences the choice of culture location, culture conditions, and bioproduct production (Pulz and Gross 2004). The characteristics most desired for strain selection include rapid growth rate, high biomass, high lipids productivity, tolerance to variable environmental conditions, rapid CO_2 uptake, and ease of harvest and extraction (Griffith and Harrison 2009).

The cells of microalgae are composed of carbohydrates, proteins, and lipids, as major biomolecules (BARSANTI; GUALTEIRI, 2006), which may have commercial interest due to their potential applications. Among the various algal carbohydrates, sulfated polysaccharides are an important source of bioactive natural compounds exhibiting anti-inflammatory, antimicrobial, antitumor, antimutagenic, antiviral activities (MIŠURCOVÁ; ORSAVOVÁ; AMBROŽOVÁ, 2015). The protein content in green microalgae can vary from 6 to 56% of their dry weight (BECKER, 2007; BROWN et al., 1997). However, these levels vary based on species/strains, growth phase, nutrients, and other growth conditions. The quality of food and feed protein depends on the composition of its amino acids, and microalgae can accumulate high concentration of essential amino acids (MOBIN; CHOWDHURY; ALAM, 2019). There are two main groups of lipids in microalgae: polar lipids and neutral lipids (MIMOUNI et al., 2018). In most microalgal species, 41-92% of the total lipid is made up of polar lipids, while 5-51% is neutral glycerolipids (MIMOUNI et al., 2018). Triacylglycerols (TAG) are neutral lipids. Many microalgae have the ability to produce substantial amounts of triacylglycerols (TAG) (20–50% of their dry weight) under stress, and store them in the cytosol as lipid droplets (HU et al., 2008).

Microalgae are also important pigment producing organisms, such as chlorophylls and carotenoids. Carotenoids consist of 40 carbon atoms composed of eight isoprene units (GUEDES; AMARO; MALCATA, 2011), they are broadly divided into two groups: carotenes and xanthophyll. The former are tetra-terpenoid hydrocarbons that are made only of C and H, while the latter are oxygenated derivatives of carotenes (ELLISON, 2015; MACHMUDAH; GOTO, 2013). Microalgae synthesize both groups of carotenoids to different degrees (EGELAND, 2016; JIN; POLLE, 2009), for example, β-carotene belongs to the carotene group, while lutein belongs to the xanthophyll group. Microalgae carotenoids are used in food and pharmaceutical, cosmetics and textile industries (MOBIN; CHOWDHURY; ALAM, 2019). β- carotene and lutein (Fig. 2) among other carotenoids, function in a range of activities like, antioxidant, anticoagulant, antitumoral, anti-inflammatory, antibacterial, antiviral, anticancer, and these gives them the ability to prevent and reduce diseases (DE MORAIS et al., 2015; PLAZA et al., 2010). Most of the lutein produced commercially is extracted from the petals of the marigold flower, but microalgae show higher lutein productivities when compared to the marigold cultivars (BHALAMURUGAN; VALERIE; MARK, 2018). Carotenoids are excellent scavengers of singlet oxygen due to their conjugated double bond system, so they protect against oxidative

damage (MACHMUDAH; GOTO, 2013), and improve the function of the immune system, skin texture (WANG et al., 2018).
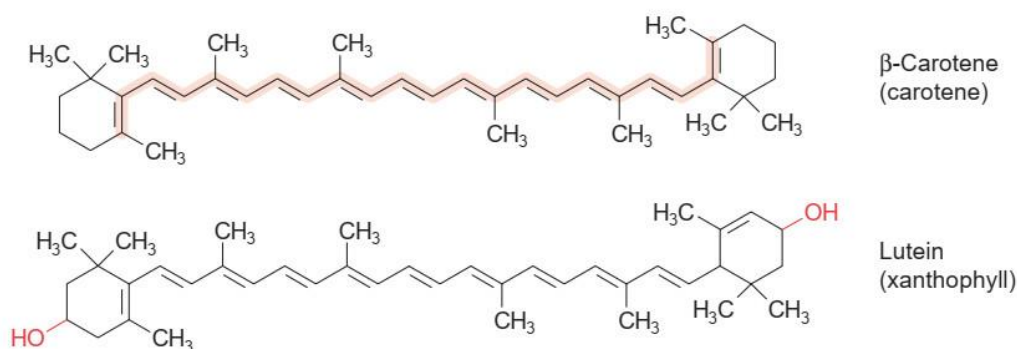
Fig. 2 Structure of β–carotene and lutein. Adapted from Heldt (2005)

Literature show that the manipulation or improvement of bioproduct in microalgae is possible and several techniques have been employed to induce altered microalgae composition, most of them related to stress. Stress strategies used in microalgae culturing can be classified into two groups: nutrimental and physical (BENAVENTE-VALDÉS et al., 2016). The nutrimental approach involves the manipulation of culture media composition (macro- and micro-nutrients). The physical approach has to do with the manipulation in operation conditions and external factors that affect the microalgae growth (light intensities, temperature, pH, etc.) (BENAVENTE-VALDÉS et al., 2016). Thus, the present study, in which we altered Cu concentrations in culture media, falls under the nutrimental approach. Different from most other studies, we search for stimulating the cells by finding the hormetic response. It finds applications in many environmental studies, since we evaluate the effect of environmentally significant Cu values on microalgae physiology and, in addition it has a potential for use in biotechnological applications, such as the improvement of bioproduct synthesis in microalgae.

Still related to the manipulation of microalgae biomass, Chisti (2007) highlighted the need to identify possible biochemical triggers and environmental factors that can favor, for example, the accumulation of oils in microalgae as a strategy to boosting biodiesel production. Lustigman (1986) reported an increase in carotenoids per cell in *Dunaliella tertiolecta* upon treatment with Cu, but total yield/mL did not increase because of a decrease in the number of cells/mL, thus decreasing growth rates. Cu ions was reported to have

increased the lipids content in *Chlorella protothecoides* (LI et al., 2013), and in *Chlorella minutissima* UTEX 2341 by 21.1% (YANG et al., 2015).

1.3 Effect of Cu on algae: oxidative stress and their antioxidant mechanisms

Cells live in a dynamic redox environment, whose status is determined by a complex balance between pro-oxidant and antioxidant molecules. It is this balance that determines the level of oxidative stress that a cell is exposed to (BIRBEN et al., 2012; COSTANTINI, 2014). The different oxidation states in which Cu occur in living organisms (mostly Cu^+ and Cu^{2+}) (LIPPARD; BERG, 1994), allows it to promote radical reactions that lead to the formation of ROS (HALLIWELL; GUTTERIDGE, 2015; PINTO et al., 2003). Cu toxicity generates ROS by catalysing reactions like the Fenton (RIJSTENBIL; GERRINGA, 2002) and Haber-Weiss (LIOCHEV; FRIDOVICH, 2002) reactions. Increased levels of ROS causes oxidative damage to macromolecules and the eventual damage of cellular organelles (HALLIWELL; GUTTERIDGE, 2015).

Algae have developed several adaptive/defence mechanisms to oxidative stress, acting to remove ROS before they can produce an irreversible damage to the cell (PINTO et al., 2003). Antioxidant defence mechanisms include endogenous enzymes that catalyse reactions that scavenge ROS, such as catalase, peroxidase, glutathione S-transferase, and superoxide dismutase (HALLIWELL; GUTTERIDGE, 2015; PINTO et al., 2003; SHERRATT; HAYES, 2002), and non-enzymes, such as the scavenging of the free radical α,α-diphenyl-β-picrylhydrazyl (DPPH) (KEDARE; SINGH, 2011). Antioxidant enzymes are prominent biomarkers of defense against oxidative stress (EL-NAGGAR; MOHAMMED, 2014) and they catalyze free radical quenching reactions in cells. These enzymes act on specific ROS after they are formed and degrade them to less harmful products (CUI et al., 2004).

Antioxidants or antioxidant-enriched extracts have important applications in the food industry. They are commercially used in preserving flavour, texture, and colour of food during storage, and also to prevent oxidative processes (ROY; NIELSEN; MILLEDGE, 2021). Although with restrictions because of its toxicity and carcinogenic properties, synthetic antioxidants such as butylated hydroxylanisole (BHA), butylated hydroxyltoluene (BHT), and propyl gallate have been used in the food and pharmaceutical industries (SHEBIS et al., 2013). Therefore, an interest in natural antioxidants is continuously increasing (KUMAR; GANESAN; RAO, 2008) and natural sources of antioxidants such as

microalgae, are increasingly being sought as safer alternatives (CHOOCHOTE; SUKLAMPOO; OCHAIKUL, 2014; GOIRIS; MUYLAERT; DE COOMAN, 2015; MTAKI; KYEWALYANGA; MTOLERA, 2020).

1.4 Photosynthesis and chlorophyll *a* fluorescence

The photosystem II (PSII) is a multi-subunit protein complex embedded in the thylakoid membranes of oxygenic photosynthetic organisms. By using chlorophyll and carotenoid molecules and several redox active cofactors, the PSII captures and uses solar energy to split water into molecular oxygen and reducing equivalents (BARBER, 2002; BARBER et al., 1997). The P680 is a chlorophyll *a* pigment which is the primary electron donor of the PSII, while the P700 chlorophyll is the primary electron donor of the PSI (BARBER, 2002) in microalgae.

Chlorophyll *a* (Chl *a*) fluorescence chiefly originates from the PSII, essentially from the P680 (BAKER, 2008; BARBER, 2003). The light energy absorbed by chlorophyll molecules can undergo one of three fates (Fig. 3): (a) it can be used to drive photosynthesis (photochemistry), (b) excess energy can be dissipated as heat, or (c) it can be re-emitted as light (chlorophyll fluorescence). These three processes occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of the other two (MAXWELL; JOHNSON, 2000; SCHREIBER, 2004). So, *in vivo* Chl *a* fluorescence is an intrinsic property of all photosynthetic organisms, providing information on the efficiency of primary energy conversion and making it useful as a probe of photosynthetic reactions (BAKER, 2008; OWENS, 1994; SCHREIBER, 2004).

Chl *a* fluorescence is often measured using the principle of Pulse-Amplitude-Modulation (PAM) (SCHREIBER, 2004). The non-invasiveness, ease of use, and high sensitivity of Chl fluorescence for photosynthetic activity have made it a preferred tool for characterizing the photophysiology of photosynthetic samples (BLACHE et al., 2011; SERÔDIO; SCHMIDT; FRANKENBACH, 2017). At ambient temperature, most (~ 90–95%) of the total Chl fluorescence emission originates from PS II in algae and higher plants (NEDBAL; KOBLÍŽEK, 2007), and this represents 0.6% to ~10% of the absorbed light (COSGROVE; BOROWITZKA, 2010). It is generally assumed that below 700 nm, the contribution of PS I to fluorescence is negligible (SCHREIBER, 2004).
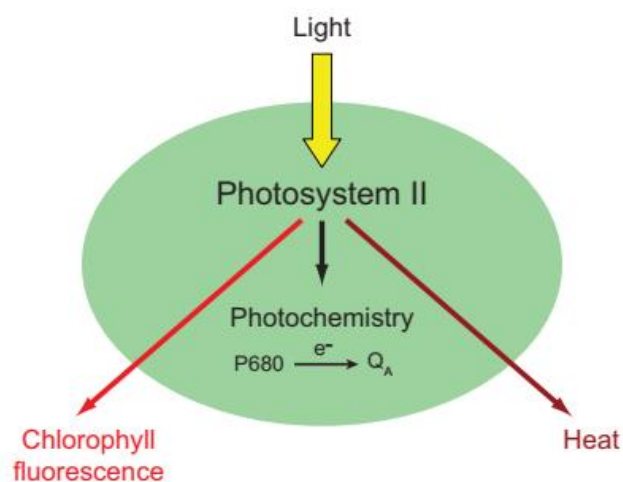
Fig. 3 Simple model of the possible fate of light energy absorbed by photosystem II (PSII). Adapted from Baker (2008)

Probing the PSII, which is the most sensitive of the two photosystems to metal (ANDRESEN; PEITER; KUPPER, 2018), provides valuable information about the response of microalgae to metal variation in the environment. Many studies have been conducted on the effect of high/lethal/toxic Cu levels on the photosynthesis of microalgae (CID et al., 1995; DENG et al., 2014b; ROCCHETTA; KÜPPER, 2009), which has been shown to have a detrimental effect especially on PSII. Other studies have reported the stimulation of oxygen evolution by low Cu in tobacco (BURDA et al., 2002), and *Scenedesmus incrassatulus* (PERALES-VELA et al., 2007). Chen et al. (2016) also recorded the stimulation of some photosynthesis parameters in *Chlorella vulgaris* under Cu concentration they termed as "low". But how much Cu stimulates or inhibits photosynthesis in microalgae remains unclear. These inconsistencies may be due to the choice of toxic Cu levels, culturing techniques, growth phase of cultures (LOMBARDI; MALDONADO, 2011), and perhaps differences in species Cu tolerance (NAKAJIMA; HORIKOSHI; SAKAGUCHI, 1979), or requirements.

1.5 Justification of the study

In the present investigation, the Chlorophyta microalgae *Ankistrodesmus flexuosus*, *Chlamydomonas chlorastera*, *Curvastrum pantanale*, and *Monoraphidium* sp. were selected, because there is little to no information regarding their physiological response to Cu. High biomolecule producers may potentially be discovered among them, and algal bioproducts provide environmentally friendly alternatives to existing technologies. However, there are

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hurdles to overcome before these bioproducts can compete with existing technologies in terms of quantity, quality, cost of production, market, and deployment. Hence the need to identify new or best strains for bioproducts production.

It is well known that not all forms of Cu are available to microalgae, either in culture medium or water bodies. This is because dissolved organic materials, including those synthesized by algae (ABBASSE; OUDDANE; FISCHER, 2003; LOMBARDI; VIEIRA, 2000; SUNDA, 2012), and synthetic chelating agents like ethylenediaminetetraacetic acid (EDTA) reduce Cu availability through complexation (MA et al., 2003; STAUBER; FLORENCE, 1987; SUNDA; HUNTSMAN, 1998a, 1998b). Sometimes EDTA is omitted in the algal culture media (PERALES-VELA et al., 2007), especially in toxicity studies (FAWAZ; SALAM; KAMAREDDINE, 2018), but this practice has some drawbacks in cultures where optimum growth, biomass, and biomolecules are desired. This is because EDTA ensures the constant availability of culture media-supplied iron (GERRINGA; DE BAAR; TIMMERMANS, 2000; HASEGAWA et al., 2018), another essential element needed for growth, metabolic processes, and biomass yield of algae (GEIDER; ROCHE, 1994; LIU et al., 2018; LIU; WANG; ZHOU, 2008). Free copper ions have been shown to be one of the most bioavailable forms of copper to microalgae (SUNDA; HUNTSMAN, 1998a, 1998b) and, it is for this reason that in the present research we fundament our results in free copper ions, not the total metal added.

Studying the effect of environmentally relevant Cu levels on the response of microalgae, relating it to possible hermetic effects in biomolecules production, oxidative stress response, photosynthesis, and primary productivity, can provide information for understanding aquatic system dynamics. The concept of environmental hormesis provides information for biological plasticity and is relevant for ecological implications within the context of global change (AGATHOKLEOUS, 2018).

In recent years, the concept of environmental hormesis has been increasingly used to explain the adaptive responses of living organisms to low doses of both abiotic and biotic, as well as various anthropogenic impacts (EROFEEVA, 2022). Subtle rise in Cu can induce a stimulation in photosynthesis and primary productivity of microalgae, and the microalgae that exhibit this response can outcompete others. This behavior can eventually lead to a decrease in microalgae species diversity and the dominance of superior competitors (AGATHOKLEOUS; CALABRESE, 2020), a scenario that can reshape or alter aquatic food chains. For biotechnological application, low dose stimulation of photosynthesis and primary

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productivity of microalgae can be a helpful strategy, for optimizing microalgae culturing in industrial application.

Cu concentrations in the order of 0.063 mM–3.1mM have been reported to increase chlorophyll and carotenoid content, and induce β-carotene production in *Chlamydomonas acidophilla* (GARBAYO et al., 2008), lipids content in *Micratinium pusillum*(ABOU SHANAB et al., 2012), to modify fatty acid composition in *Chlorella* spp.(ZHU; LI; HILTUNEN, 2016), and other biomolecules. However, these boosts in biomolecules happen at the expense of reducing the algal cell densities (MIAZEK et al., 2015), and growth rates (BOSSUYT; JANSSEN, 2004). Therefore, the Cu concentrations used for this study were chosen such that they mostly did not reduce the growth of the microalgae. Perhaps Cu could cause a hormetic response in biomolecules production, antioxidant response, photosynthesis and primary productivity of microalgae.

1.6 Research hypothesis

The hypothesis of this study is that Cu concentration that is within the environmentally relevant levels (1.57×10^{-8}–4.72×10^{-7} mol L^{-1} total copper) and does not reduce the growth of microalgae, could cause higher biomolecules production, antioxidant response, photosynthesis and primary productivity. These concentrations are slightly higher than the control values.

1.9 Objectives

The objectives of this study were to determine the effects of environmentally relevant Cu concentrations on four Chlorophyta microalgae (*Ankistrodesmus flexuosus*, *Chlamydomonas chlorastera*, *Curvastrum pantanale*, and *Monoraphidium* sp.). The objectives are presented in three chapters under Results and Discussion section, and listed with their respective chapter numbers below.

3.1 Biomass, growth, and biomolecules composition of the microalgae under different cu concentrations

3.2 Oxidative stress response of the microalgae under different Cu concentrations

3.3 Photosynthesis of the microalgae under different Cu concentrations

2.0 MATERIALS AND METHODS

2.1 Species Cultured

All microalga strains (Plate I) used for this study were axenic and were obtained from the freshwater microalgae culture collection of the Federal University of São Carlos, Brazil. Species used include: *Ankistrodesmus flexuosus* (CCMA-UFSCar 083)*, Chlamydomonas chlorastera* (CCMA-UFSCar 009)*, Curvastrum pantanale* (CCMA-UFSCar 350)*, Monoraphidium* sp (CCMA-UFSCar 606). These strains are registered in SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) under the number A5EF193.

2.2 Culture Condition

A. flexuosus, C. pantanale, and Monoraphium sp. were cultured in a modified BG-11 medium (RIPPKA et al., 1979). This is a modification of the original medium No. 11 of where there is a threefold increase in $NaNO_3$ and the replacement of Gaffron's minor trace elements with a modified A5 trace elements solution (ANDERSEN et al., 2005). While *C. chlorastera* was cultured in WC medium (GUILLARD; LORENZEN, 1972), because it grew poorly in BG-11 medium. Both media were prepared according to the recipes given in Andersen et al. (2005). Before culturing, the pH of all the media were adjusted to 7 using either 1N HCl or 1N NaOH as required, then all media were sterilized by autoclaving at 121 °C for 20 minutes. Cultures were maintained at 25 ± 2 °C under a 12:12 h lighting regime with an intensity of 200 µmol photons $m^{-2} s^{-1}$ using white LEDs.

2.2.1 Copper Treatments

Environmentally relevant copper includes a range of copper concentration that usually lies within 1.57×10^{-8}–4.72×10^{-7} mol L^{-1} total copper (FERREIRA et al., 2008; PRASAD, 2004; US ENVIRONMENTAL PROTECTION AGENCY, 2007). For this research, we define as low copper (0.1 to 21.4 nM free Cu^{2+}) values that are within the environmentally relevant ones, and as high copper, the highest value we used (589.0 or 5480.0 nM free Cu^{2+}).

The culture media were initially prepared without any Cu element in it. To the media in 1L polystyrene culture flasks, 7 different nominal Cu concentrations in triplicates (0.08, 0.12, 0.16, 0.18, 0.35, 1.00, and 19.6 µM), were prepared using a Cu standard for AAS (CAS: 38996; Sigma-Aldrich). These flasks were allowed to sit for 24 h for a chemical equilibrium of the nutrients and copper before algal inoculation.

Free cupric ion, Cu^{2+} is known as the bioavailable form of Cu to microalgae (LOMBARDI et al., 2007; SUNDA, 1989; VERWEIJ; GLAZEWSKI; DE HAAN, 1992). The chemical equilibrium calculation software, Visual MINTEQ 3.1 (GUSTAFSSON, 2013) was used to determine the initial Cu^{2+} concentrations at 25 °C, which is the temperature at which the cultures were kept. Cu concentrations in this manuscript are expressed as free cupric ions in nanomolar (nM), present in the culture media at the beginning of culturing.
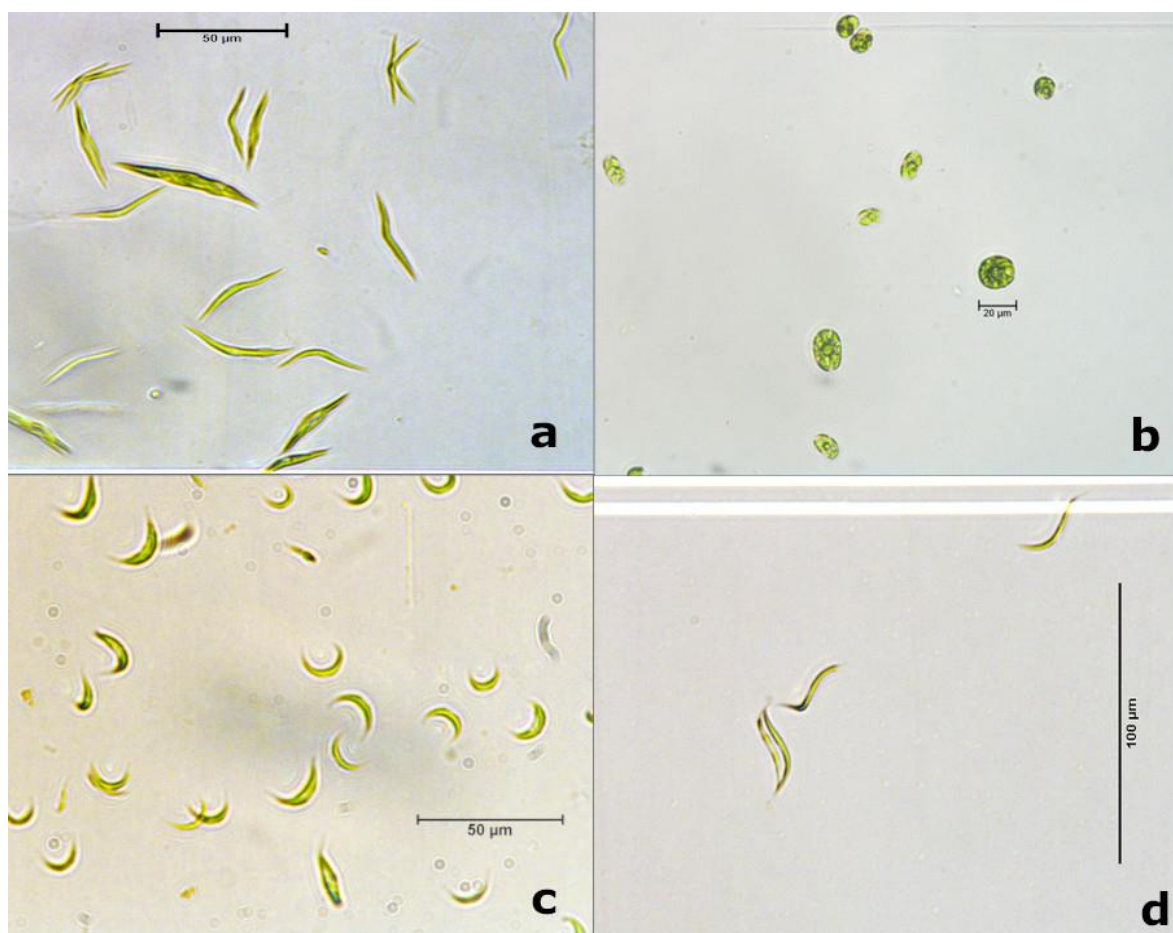
Plate. I *Ankistrodesmus flexuosus* (a), *Chlamydomonas chlorastera* (b), *Curvastrum pantanale* (c), *Monoraphidium* sp. (d). Mag = ×40.

Table 1 Nominal Cu concentrations (nM) and free Cu concentrations (Cu^{2+}, nM) for each species culture. Free Cu concentrations were calculated using the chemical equilibrium model, Visual MINTEQ 3.1. *C. chlorastera* was kept in WC culture medium, while the others in BG11.

Nominal Cu	*A. flexuosus*	*C. chlorastera*	*C. pantanale*	*Monoraphidium* sp.
80	1.7	0.1	1.7	1.7
120	2.5	0.2	2.5	2.5
160	3.4	0.3	3.4	3.4
180	3.8	0.4	3.8	3.8
350	7.4	1.2	7.4	7.4
1000	21.4	9.1	21.4	21.4
19600	589.0	5480.0	589.0	589.0

2.3.1 Biomass, Cell density, Growth, and Biomolecules Determination

2.3.1.2 Growth rate

The absorbance (684 nm) of an aliquot of the cultures were taken daily using a spectrophotomenter (Macherey-Nagel Nanocolor UV/VIS spectrophotometer). The growth rates (μ d^{-1}) were calculated from the growth curves, plotted with LN absorbance (684 nm) against time (days). At the exponential growth phase, a linear regression line was fitted to the curves, and the slopes of the regression lines was used as the growth rates.

2.3.1.3 Cell Counts

Cell counts were made daily during the culturing of each alga. This was made using the Muse Cell Analyzer (Millipore Corporation) for *C. chlorastera*. But for *A. flexuosus*, *C. pantanale*, and *Monoraphidium* sp., the Fuchs-Rosenthal chamber under a light microscope was used, because of the clumping of their cells.

2.3.1.4 Dry Weight

A 5 mL aliquot of algal culture was filtered on cellulose acetate filter. The cellulose acetate filters (0.45 μm pore size, 25 mm diameter; Sartorius-Stedim Biotech, Germany)

were pre-dried at 100 °C for 1 h in a muffle furnace, and kept in a desiccator before use. Before filtering the samples, the filters were weighed on a microbalance (XPE26, Mettler Toledo, Columbus, OH, USA) shortly before filtering the algal samples. After filtering the samples, the filters were dried at 38 °C for 24 h, kept in the desiccator overnight, and then weighed in gram to 6 decimal places. Dry weights were calculated using the according to eqn (1).

$$DW \ (mg/mL) = \frac{Weight \ of \ filters \ with \ dried \ biomass \ (mg) - Weight \ of \ dried \ empty \ filters \ (mg)}{Volume \ of \ filtered \ culture \ (mL)} \quad (1)$$

2.3.1.5 Total Chlorophylls and Total Carotenoids Quantification

An aliquot of 5 mL culture was filtered through a cellulose acetate filter (0.45 µm pore size, 25 mm diameter; Sartorius-Stedim Biotech, Germany), while an empty filter served as blank. The filters were extracted with 3 mL DMSO in glass test tubes, they were incubated in the dark for 10 min at room temperature, allowing the filters to completely dissolve. Subsequently, the extracts were read at 480, 649, and 665 nm, and chlorophylls a, b, and total carotenoids concentrations were calculated following the equations of Wellburn (1994):

$$C_a = 12.47A_{665} - 3.62A_{649} \quad (2)$$

$$C_b = 25.06A_{649} - 6.5A_{665} \quad (3)$$

$$Total \ carotenoids = (1000A_{480} - 1.29C_a - 53.78C_b)/220 \quad (4)$$

$$Total \ chlorophylls \ concentration = C_a + C_b \quad (5)$$

2.3.1.6 Carotenoids class quantification

Lyophilized biomass of 20 mg (*C. chlorastera*) and 10 mg (*Monoraphidium* sp.) were used for carotenoids extraction using a modified method of Soares et al. (2016). In a falcon tube wrapped with aluminium foil, 5 mL of hexane:ethanol (1:1) containing 1% BHT was added to the biomass, and sonicated continuously (65 kHz, 5 min) on ice. Then 2.5 mL of 30% KOH in ethanol was added to the solution and vortexed. The solution was kept overnight at room temperature to ensure saponification. Subsequently, 5 mL of hexane:ethanol (1:1) containing 1% BHT was added and vortexed. Then 3.75 mL 10%

Na$_2$SO$_4$ was added and vortexed. The solution was centrifuged (4221 g (4400 rpm), 0 °C, 5 min). The upper layer was collected, and the lower layer re-extracted, until the upper layer became colourless. The upper layer was pooled in a glass vial wrapped with aluminium foil and evaporated to dryness using nitrogen gas. This dried extract was frozen (-80 °C) until needed for carotenoid quantification.

The quantification of carotenoids was done by dissolving a sample of the dried extract in 1mL methanol:dichloromethane (1:1) and filtered (PTFE, 0.22 μm pore size, 25 mm diameter) before quantifying the carotenoids. Quantification was according to the method described by Soares et al. (2016) using High Performance Liquid Chromatography (HPLC) with photo diode-array (PDA) detection (Waters Alliance e2695 Separations Module, Waters Corporation, Milford, USA). Twenty microliters of each extract was analyzed using a C30 (5 μm, 250 ×4.6 mm, Nano separation technologies, São Carlos, Brazil) column, and kept at a constant temperature of 25 °C during the analysis using a column heater (Waters Corporation, Milford, USA). The mobile phase consisted of two solvents, (A) methanol:acetonitrile:water (84:14:2; v/v/v) and (B) dichloromethane (100%), which were used to install the following gradient: 100% A at 0 min, 95% A for 25 min, 75% A for 30 min, 74% A for 35 min, 45% A for 50 min and 100% A for 55 min. The flow rate was 1 mL/min. Data were processed using Empower 2 Software (Waters, Milford, USA), recording the spectra from 250–750 nm and the detection wavelength set at 450 nm.

Calibration curves were made from two carotenoid standards, β-carotene (CAS: 7235-40-7; purity $\geq$ 95% HPLC), and Lutein (CAS: 127-40-2; purity $\geq$ 96% HPLC), all from Sigma-Aldrich (St. Louis, MO, USA). The quantities of these two carotenoids in the sample extracts were quantified from their respective calibrations curves. Carotenoids quantity in mg g^{-1} was calculated using the equation given by (ZEB; ULLAH, 2017), as follows.

$$Carotenoid\ (mg/g)\ = \frac{C_x(mg/mL)\ \times v\ (mL)\ \times D}{Wt\ (g)} \qquad (6)$$

Where:
C_x : the concentration of each carotenoid, calculated from the standard calibration curve
V: volume of the extraction
D: dilution factor
Wt: weight of the biomass

2.3.1.7 Total Carbohydrates Assay

Carbohydrates quantification was done according to the sulphuric acid method (ALBALASMEH; BERHE; GHEZZEHEI, 2013) using glucose as standard.

2.3.1.8 Total Proteins Assay

Protein extraction was done according to the method of Slocombe et al. (2013) with a modification, then extracted proteins were quantified using Lowry's assay as described by the previous authors. The modification was that humid rather instead of dried samples as described in the method, were used for the assay. The Slocombe et al. (2013) method uses TCA and heat to precipitate the protein, before proceeding to quantification using the Lowry's protein assay.

Briefly, in falcon tubes, 10 mL of algal sample was centrifuged at 4221 g (4400 rpm), 20°C, 15 min. The pellets were retained and frozen until extraction. To the frozen pellet, 200 µL of 24% (w/v) trichloroacetic acid (TCA) was added. The tubes were heated at 95 °C for 15 min and allowed to cool at room temperature, after which 600 µL milli-Q water was added. The tubes were centrifuged at 2597 g for 15 min, and the supernatant was discarded. To the tubes, 0.5 mL Lowry reagent D was added, the tubes vortexed, and kept at 55 °C for 3 h. Then the tubes were centrifuged at 2597 g for 15 min, and the supernatant was retained for the protein quantification. Samples and Bovine serum albumin (BSA) standards read at 600 nm in a microplate reader (Epoch, Bio Tek Instruments, Inc., USA). A protein calibration curve was generated from the absorbances the Bovine serum albumin (BSA) standards. And from the equation of the calibration curve fit, the concentrations of the samples were calculated.

2.3.1.9 Lipids Assay

The modified method of Folch as described by PARRISH, (1999) was used for lipid extraction. Algal sample of 100 mL was filtered through a GF/C glass micro-fibre filters (pre-baked at 400 °C, 8 h). The filters were transferred to glass vials (pre-combusted at 400°C, 8 h), 2 mL chloroform added to cover the filters, nitrogen gas was introduced into the vials, and then the filters were frozen (-22 °C) until extraction.
The samples were extracted by transferring all the contents of the stored vial (filter + solvent) into a Teflon tube, then 4 mL of chloroform and 2 mL methanol was added. The filters were

ground using glass rod, sonicated continuously (65 kHz, 5min) on ice, and centrifuged (4221 g (4400 rpm), 0 °C, 5 min). The supernatant was collected and the residue re-extracted. The whole supernatant was pooled and evaporated to about 1 mL using a rotary evaporator. The lipid extract was transferred to glass vials (pre-combusted at 400°C, 8 h), flushed with nitrogen gas, and frozen (-22 °C). Total lipids was quantified from the lipid extract using the gravimetric method, and presented in % biomass.

$$Lipids\ content\ (\%\ w/w) = \frac{Wl}{We} \cdot \frac{Vext}{Vweighed} \cdot 100 \qquad (7)$$

Where:

Wl: $Weight\ of\ lipids$

Vext: $Concentrated\ volume\ of\ extract$

We: $Weight\ of\ extracted\ biomass$

Vweighed: $Volume\ of\ extract\ weighed$

2.3.2 Oxidative Stress Assays

2.3.2.1 Extraction for Antioxidant assays

Algal sample of 10 mL was centrifuged at 2597 g for 15 minutes; the supernatant was then discarded and the pellet was used for antioxidants extraction. The extraction was done in glass test tubes using 3 mL ice-cold 0.05M phosphate buffer (pH 7.8) for all antioxidant assays (peroxidase, glutathione S-transferase, superoxide dismutase, and lipid peroxidation), and 70% ethanol for DPPH radical scavenging activity assay.

To the algal pellet in the glass test tube, 0.5 mL buffer/70% ethanol was added, then glass beads (3 mm in diameter) just enough to cover the buffer/70% ethanol were added. The test tube was vortexed for 3 minutes, pausing after the first and second minutes to add 1 mL buffer/70% ethanol, and 0.5 mL buffer/70% ethanol at the end of vortexing. The regular pausing and the addition of the buffer/70% ethanol was done in order to keep the extract cold. Each test tube was kept on ice before, in-between, and after vortexing. After vortexing, the test tubes were centrifuged at 2597 g, 4 °C for 15 minutes. The resulting supernatant was stored at -80 °C until needed for analysis. This method of enzyme extraction was adopted after it was found to be suitable for rupturing the cells of the microalgae used in this study.

2.3.4.2 Peroxidase Assay

Peroxidase (POD) was assayed according to the method of Reddy et al. (1985) with modifications. The reaction mixture consisted of 0.1 mL algal enzyme extract, 0.5 mL H_2O_2 (50 mM or 0.16 % (w/w)), and 2.4 mL pyrogallol solution (50 mM). The change in absorbance of the reaction mixture was monitored for 3 min, at 420 nm. POD activity was calculated from the equation given below.

$$Unit/mL\ enzyme\ = \frac{(\Delta A_{420\ nm}/min) \cdot Total\ volume\ of\ assay \cdot DF}{\varepsilon \cdot Volume\ of\ sample\ extract} \qquad (8)$$

$$Unit/mg\ protein\ = \frac{Eqn\ (8)}{Protein\ (mg/mL)} \qquad (9)$$

Where:

$\Delta A_{420\ nm}/min$ = Change in absorbance at 420 nm

DF = Dilution factor

ε = 12 (Extinction coefficient of 1.0 mg/mL purpurogallin at 420 nm)

2.3.2.3 Glutathione S-transferase Assay

Glutathione S-transferase (GST) activity was determined according to the method of Habig et al. (1974). The assay mixture contained 2.7 mL phosphate buffer (0.1 M, pH 6.5), 0.1 mL of reduced glutathione (GSH; 75 mM), and 0.1 mL of 1-chloro- 2, 4-dinitrobenzene (CDNB, 30 mM). The reaction was started by adding 0.1 mL of sample enzyme extract to the mixture, the change in absorbance was taken after every minute for five minutes at 340 nm. One unit of GST activity was defined as nanomoles of CDNB conjugated per minute. The change in absorbance was directly proportional to the GST activity. GST activity was calculated using the equations below.

$$Unit/mL\ enzyme\ = \frac{(\Delta A_{340\ nm}/min) \cdot Total\ volume\ of\ assay \cdot DF}{\varepsilon \cdot Volume\ of\ sample\ extract} \qquad (10)$$

$$Unit/mg\ protein\ = \frac{Eqn\ (10)}{Protein\ (mg/mL)} \qquad (11)$$

Where:

$\Delta A_{340\ nm}/min$ = Change in absorbance at 340 nm

DF = Dilution factor

$\varepsilon = 0.0096 \; \mu M^{-1} \; cm^{-1}$ (Extinction coefficient of 1.0 mg/mL GSH at 340 nm)

2.3.2.4 Superoxide dismutase assay

      Superoxide dismutase (SOD) was assayed according to the method of Beauchamp and Fridovich (1971) with modifications. The reaction mixture consisted of 0.1 mL of algal enzyme extract, 2.35 mL phosphate buffer (pH 7.8; 50 mM), 0.2 mL Na_2EDTA (10 mM), 0.1 mL nitroblue tetrazolium (NBT) (1mM), 0.3 mL methionine (100 mM), and 0.05 mL riboflavin (0.2 mM) in a test tube. The test tubes were incubated in light (~688 μmol photons $m^{-2} \; s^{-1}$), in an aluminum foil-lined box for 10 min. Then the absorbance was read at 560 nm in microplate reader (Epoch, Bio Tek Instruments, Inc., USA). One unit of SOD (U) activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction monitored at 560 nm (YILANCIOGLU et al., 2014). SOD activity was calculated using the equations below.

$$\% \; inhibition \; = (\frac{A_{sample} - A_{control}}{A_{control}}) \cdot 100 \tag{12}$$

$$SOD \; (Unit) \; = \frac{Eqn \; (12)}{50} \cdot \frac{Total \; volume \; of \; assay}{Volume \; of \; sample \; extract} \cdot DF \tag{13}$$

$$SOD \; (unit/cells) \; = \frac{Eqn \; (13)}{Cell \; density} \tag{14}$$

2.3.2.5 Lipid Peroxidation Assay

      The Malondialdehyde (MDA) concentration was measured using the method of Heath and Packer (1968). To 1 mL of the algal extract, 2 mL of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added. The mixture was then heated in a boiling water bath for 15 minutes and quickly cooled in an ice-bath. The mixture was then centrifuged at 3000 rpm for 5 minutes, and the absorbance of the supernatant read at 532 nm and 600 nm. The concentration of MDA was calculated using the following equations.

$$\text{MDA equivalents (nmol mL}^{-1}) = \frac{(A_{532} - A_{600} \cdot DF \cdot x \cdot 1000)}{\varepsilon \cdot 1 \cdot y} \tag{15}$$

$$\text{MDA equivalents (nmol } 10^6 \text{ cells }^{-1}) = \frac{\text{Eqn (15)}}{\text{cell density (cells mL}^{-1})} \tag{16}$$

Where:
DF = dilution factor
1000 = conversion factor (μmol to nmol)
x = volume of buffer used for extraction (mL)
y = volume of extracted algal sample (mL)
1 = cuvette light path length (cm)
ε = Extinction coefficient of 155 mM^{-1} cm^{-1}

2.3.2.6 DPPH Radical Scavenging Activity Assay

DPPH radical scavenging activity was quantified according to the method of Pires et al. (2017). Briefly, 20 µL of algal extract was mixed with 280 µL of DPPH (80 mM) in 70% ethanol solution. After 40 min of incubation at room temperature in the dark, the decrease in absorbance was monitored at 517 nm in a microplate reader (Epoch, Bio Tek Instruments, Inc., USA). DPPH radical scavenging activity was calculated according to eqn (17).

$$\text{Radical scavenging activity (\%/mg DW)} = \left[\frac{(A_0 - (A - A_b))}{A_0}\right] \cdot \frac{1}{DW} \cdot 100 \tag{17}$$

A_0 = Absorbance of control (DPPH solution only)
A = Absorbance of sample (sample + DPPH)
A_b = Absorbance of blank (Algal sample extract only)
DW = Extracted algal dry weight (mg)

2.3.3 Chlorophyll Fluorescence Measurements

All chlorophyll fluorescence measurements were made using the compact phytoplankton analyzer (Phyto-PAM II Compact, Heinz Walz). All chlorophyll fluorescence measurements were done when the algal cultures were in the exponential growth phase (48 h).

2.3.3.1 Chlorophyll Fluorescence Yields

The algal samples were dark acclimated for 20 min to ensure complete quinone A oxidation, before measuring the maximum quantum yield (F_v/F_m), and rapid light curves (RLC). The F_v/F_m was gotten after passing a high intensity saturation pulse. For the RLC, an increasing intensity of photosynthetically active radiation (PAR) was applied to the samples at a pulsed interval of 20 s. Subsequently, the minimum fluorescence (F_o), maximum fluorescence (F_m), and light-adapted maximum fluorescence (F_m') were measured. The relative electron transport rate (rETR) for each light intensity was calculated by multiplying the PAR with its F_v/F_m. The RLC was plotted as the rETR *vs* PAR, and then fitted using the model of Platt et al. (1980). From the model, the initial slope (α), photoinhibition parameter (β), saturation irradiance (E_k), and the maximum relative electron transport rate ($rETR_m$) were obtained. The algal samples were light-acclimated (180 µmol photons m^{-2} s^{-1}) before measuring the effective quantum yield ($\Delta F/F_m'$). The steady-state fluorescence (F_s) and F_m' were measured, and $\Delta F/F_m'$ for each irradiance was calculated. The minimum fluorescence in a light-acclimated state (F_o') was calculated according to Oxborough et al. (1997).

2.3.3.2 Fluorescence Quenchings

The parameters, F_v/F_m, $\Delta F/F_m'$, qP (photochemical quenching; an indicator of Q_A redox state), and NPQ (non-photochemical quenching mostly related to heat dissipation) were calculated according to Juneau et al. (2002). While qL (photochemical quenching; the fraction of open PS II centers based on the lake model) was calculated according to Kramer et al. (2004). Fluorescence quenchings (qL, qP, and NPQ) were calculated using a combination of dark-acclimated (F_m and F_o) and light-acclimated (F_m' and F_s) fluorescence yields (JUNEAU; EL BERDEY; POPOVIC, 2002; KRAMER et al., 2004). The equations used are as follows.

$$F_o' = \frac{F_o}{\dfrac{F_v}{F_m} + \dfrac{F_o}{F_m'}} \tag{18}$$

$$F_v/F_m = \frac{F_m - F_o}{F_m} \tag{19}$$

$$\Delta F/F_m{}' = \frac{F_m{}' - F_s}{F_m{}'} \qquad (20)$$

$$qP = \frac{F_m{}' - F_s}{F_m{}' - F_o{}'} \qquad (21)$$

$$qL = qP \cdot \frac{F_o{}'}{F_S} \qquad (22)$$

$$NPQ = \frac{F_m - F_m{}'}{F_m{}'} \qquad (23)$$

2.3.3.3 Quinone A reduction time, Quinone A re-oxidation time, and primary productivity

Exponentially growing cells containing < 300 µg/L Chl *a* were dark-adapted for 20 min, and illuminated with weak far-red light for 10 s, before the fast kinetics analyses at 440 nm (SCHREIBER; KLUGHAMMER; KOLBOWSKI, 2012). This was done using the fast kinetic mode in Phyto-PAM II Compact, which enables the analysis of the wavelength-dependent photochemical phase of the fast fluorescence rise (O-I_1 or O-J) kinetics, after passing a high actinic light pulse. The resultant O-I_1 curve gave the sample quinone A reduction time (Tau), quinone A re-oxidation time (Tau (reox)), and the wavelength-dependent functional absorption cross section of PS II (Sigma (II)$_{440}$). Sigma (II)$_{440}$ is defined for a specific reference state where Q_A and the plastoquinone (PQ) pool are oxidized, and NPQ (caused by membrane energization or state 2 formation) is absent. It is therefore different from σPSII (SCHREIBER; KLUGHAMMER; KOLBOWSKI, 2012).

From Sigma (II)$_{440}$, the absolute rate of PS II electron transport (ETR (II)) was computed and the absolute oxygen evolution rate (rO$_2$) was estimated using eqns (24 and 25)(SCHREIBER; KLUGHAMMER; KOLBOWSKI, 2011, 2012). Assuming each PSU contains 1000 molecules of Chl *a* with a molar mass of 900 g mol^{-1} per Chl, and 4 electrons are required for the evolution of 1 molecule of O$_2$ (SCHREIBER; KLUGHAMMER; KOLBOWSKI, 2011, 2012).

$$ETR \ (II)_{440} = Sigma \ (II)_{440} \cdot L \cdot PAR \cdot \frac{\Delta F/F_m{}'}{F_v/F_m} \qquad (24)$$

$$rO_2 = \frac{ETR \ (II)_{440}}{PSU \cdot ne \ (O_2) \cdot M \ (Chl)} \qquad (25)$$

Where:

PSU (Photosynthetic unit) is the number of Chl *a* molecule per photosynthetic unit

ne (O_2) is the number of electrons required for the evolution of 1 molecule of O_2

M (Chl) is the molecular weight of Chl *a.*

The ratio of oxygen produced per fixed carbon was calculated using eqn (**Error! Reference source not found.**), provided by FRANKENBACH et al. (2020), where a PQ (Plastoquinone quotient) value of 1.4 was used because nitrate was the primary source of nitrogen (LAWS, 1991) in our culture media. M (C) is the molar mass of carbon (12.01 g mol^{-1}) and 3600 is the conversion factor for hourly rates.

$$P^B = \frac{rO_2}{PQ} \cdot M\ (C) \cdot 3600 \tag{26}$$

2.4 Data analyses

One-way analysis of variance (ANOVA) was used to compare the difference between the means of the Cu treatments for each measured parameter. Where significant differences existed, Tukey's HSD test was used for multiple comparisons of the means. The assumptions of normality and homoscedasticity were verified before analysis using the Shapiro–Wilk test and Levene's test, respectively. Statistical analyses were done using R for Windows (R DEVELOPMENT CORE TEAM, 2020).

3.0 RESULTS AND DISCUSSION

3.1 Biomass, growth, and biomolecules composition of the microalgae under different cu concentrations

3.1.1 Cell density, dry weight, and growth

Cell densities of the algal species were unaltered by low Cu exposure, but decreased under 21.4 nM Cu^{2+} in *A. flexuosus* (Fig. 4a) and *Monoraphidium* sp. (Fig. 4d), 5480.0 nM Cu^{2+} in *C. chlorastera* (Fig. 4b), and 589.0 Cu^{2+} in *C. pantanale* (Fig. 4c).
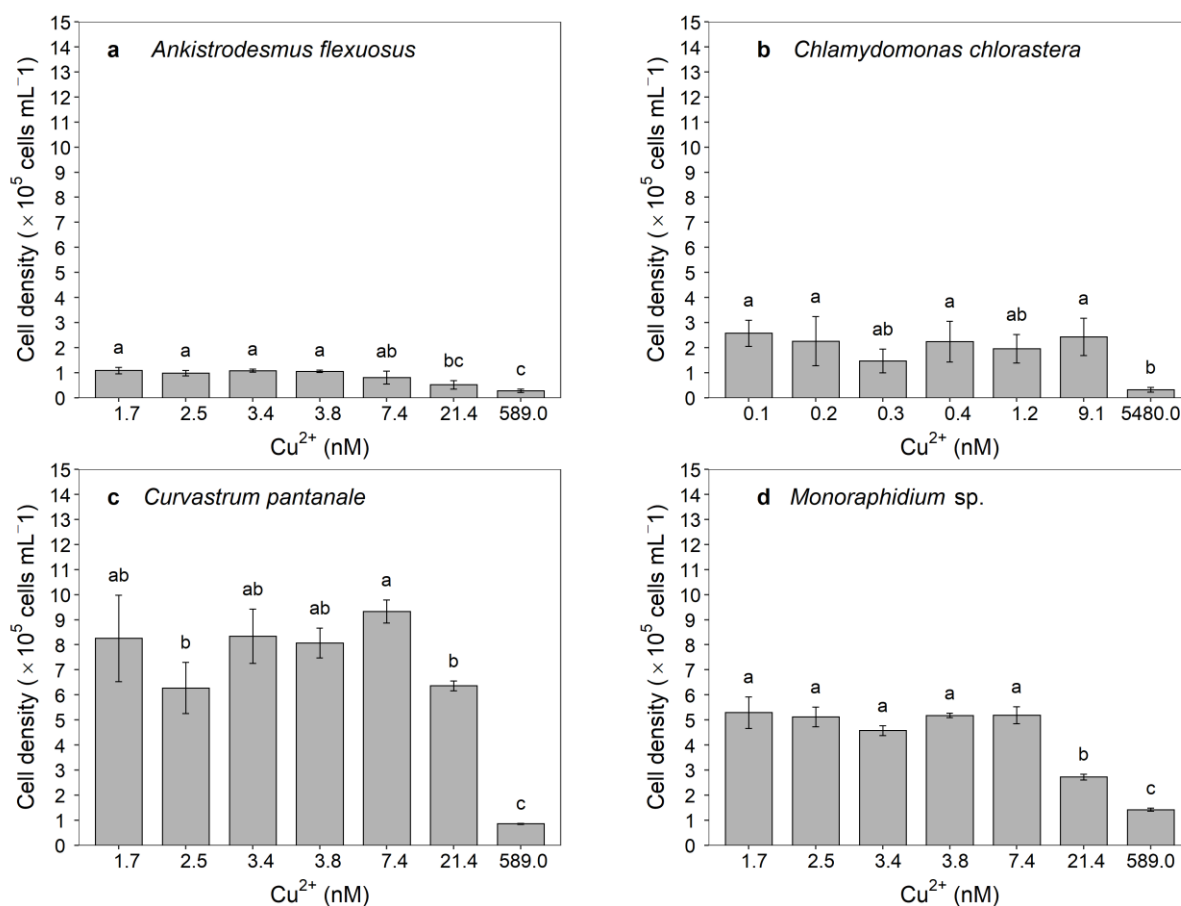
Fig. 4 Cell density for the Cu concentrations during the exponential growth phase (72 h), of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

The dry weight of *A. flexuosus* was not affected by all Cu exposures (Fig 5a). There was also no difference in the dry weights of *C. chlorastera*, under 0.1 to 9.1 nM Cu^{2+} (Fig 5b). Similar dry weights were recorded in *C. pantanale* under Cu exposure (Fig 5c), with the

lowest dry weight under 589.0 nM Cu^{2+}. The dry weights of *Monoraphidium* sp. in 3.8 nM Cu^{2+} (0.12 ± 0.004 mg mL^{-1}) and 7.4 nM Cu^{2+} (0.12 ± 0.012 mg mL^{-1}), were significantly higher than in 589.0 nM Cu^{2+}(0.09 ± 0.001 mg mL^{-1}), but similar to those of other Cu concentrations (Fig. 5d). Comparing the four species, *Monoraphidium* sp. produced the highest ($p < 0.05$, Two way ANOVA) dry weight. Followed in decreasing order by *A. flexuosus*, *C. pantanale*, and *C. chlorastera*.
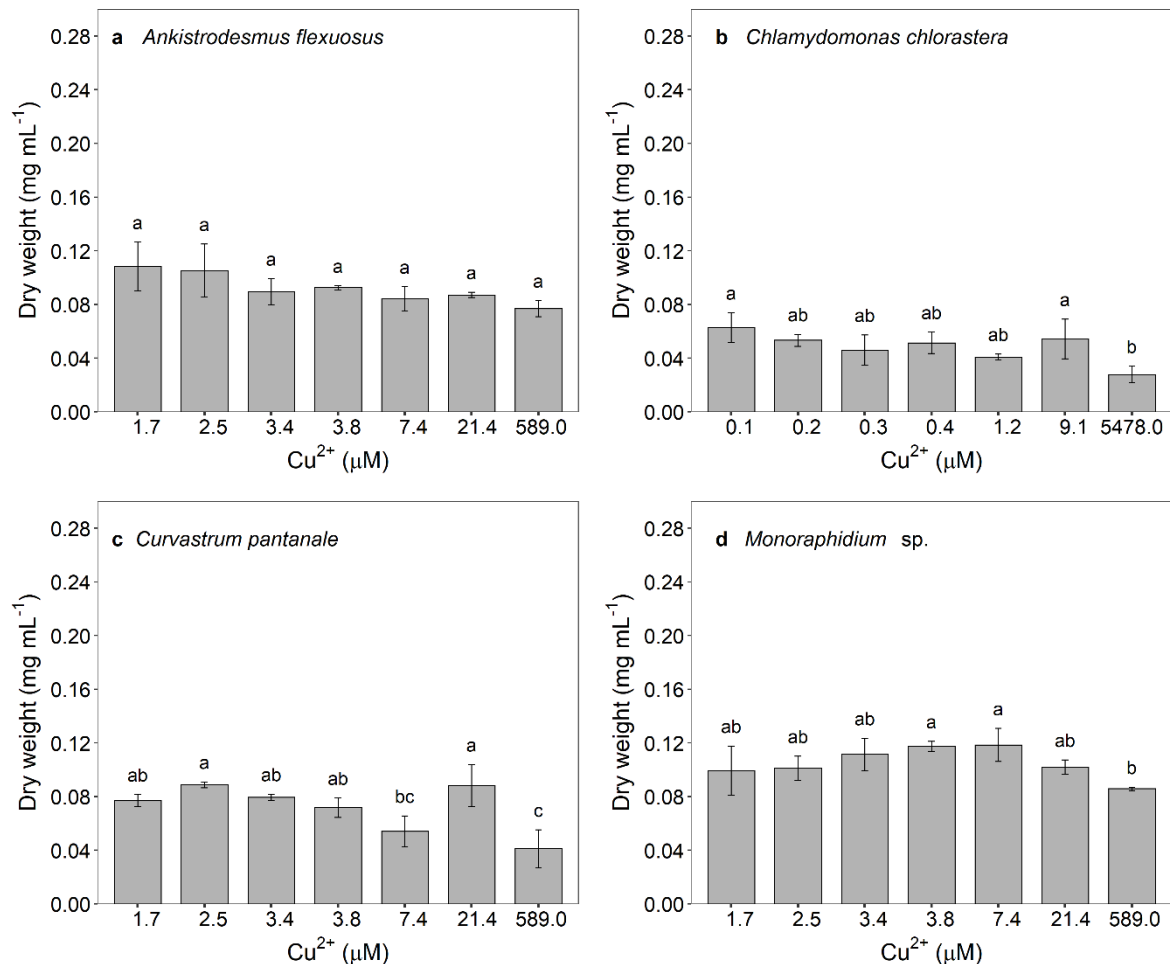
Fig. 5 Dry weight for the Cu concentrations during the exponential growth phase (72h), of (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

All the four cultured algal species were grown for a period of 96 h (Fig 6) under the different Cu concentrations. The growth rates gotten during the exponential phase of growth for each algal species under the different Cu concentrations are shown in Fig 7.

The growth rates of all the species were unaltered by Cu^{2+} below 7.4 nM. An increase in Cu concentration led to an increase in growth rate in *A. flexuosus* (Fig 7a), with the highest under 3.8 nM Cu^{2+} (0.64 ± 0.04 d-1) and 7.4 nM Cu^{2+} (0.61 ± 0.11 d^{-1}) respectively. Although these were not significantly ($p > 0.05$, One-way ANOVA) different from the growth rates under the 3 lowest concentrations. The growth rate of *C. chlorastera* (Fig 7b) was significantly ($p < 0.05$) reduced under the highest Cu concentration (5478.0 nM Cu^{2+}; 0.04 ± 0.03 d^{-1}). While the growth rates of *C. pantanale* (Fig 7c) and *Monoraphidium* sp. (Fig 7d) were reduced significantly ($p < 0.05$) in 589.0 nM Cu^{2+} and 21.4 nM Cu^{2+} respectively. Comparing the growth rates from the four species, *A. flexuosus*, *C. pantanale*, and *Monoraphidium* sp. had similar ($p > 0.05$, Two-way ANOVA) growth rates, while *C. chlorastera* had the lowest ($p < 0.05$) growth rate of the four species.
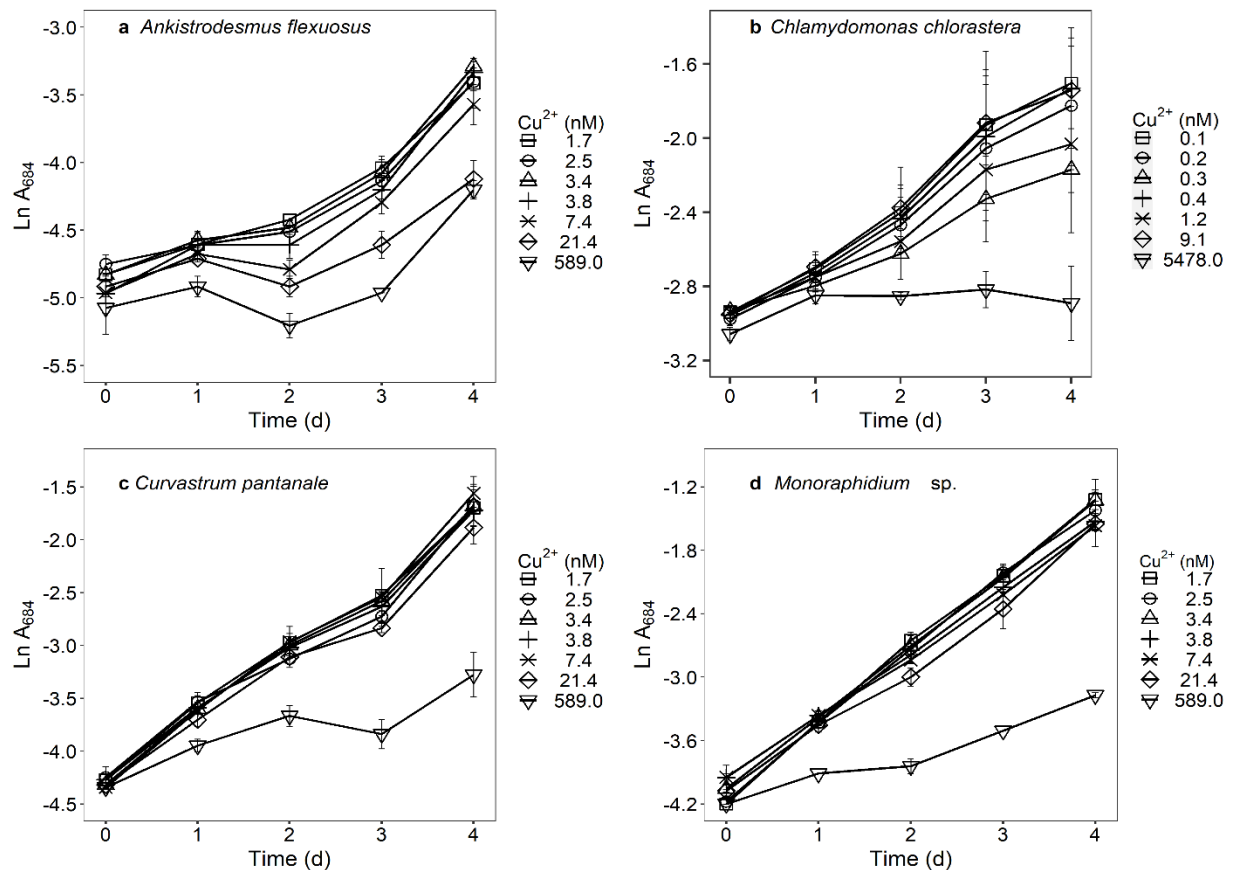
Fig. 6 Growth curves (Ln absorbance 684 nm versus time, days) under different Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3).
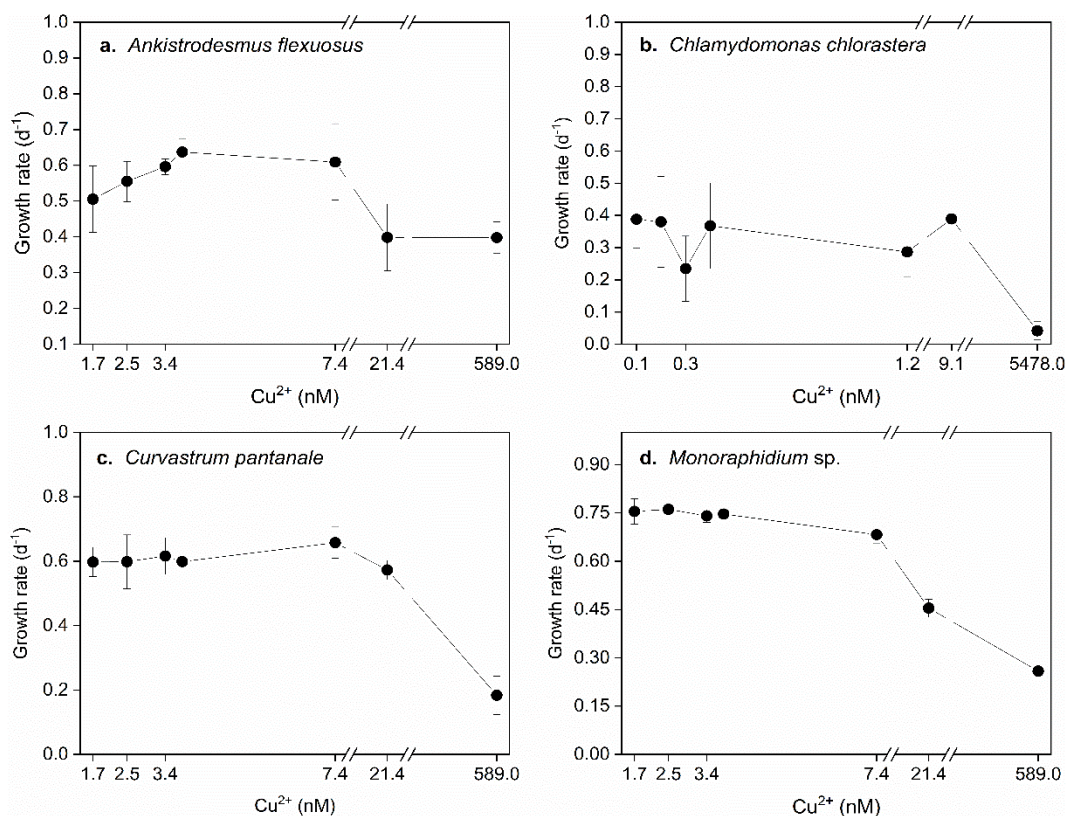
Fig. 7 Growth rates as functions of Cu concentrations during the exponential growth phase, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3).

These results show that low Cu exposure (1.7 nM to 9.1 nM Cu^{2+}) did not alter cell densities, dry weights and growth rates of the algae. This Cu range signifies the optimum Cu for the growth of these algal species, under the environmental conditions used in this study. Different microalgal species and strains have been reported to grow optimally under a varying range of Cu^{2+}. For example, *Scenedesmus subspicatus* grew optimally between 10^{-6} nM to 100 nM Cu^{2+} (KNAUER; BEHRA; SIGG, 1997), *Scenedesmus acuminatus* between 0.01 nM to 30 nM Cu^{2+} (LOMBARDI et al., 2007), which shows that these species share a similar Cu need with the algae used in this study. However, the growths of *Chlamydomonas reinhardtii*, *Chlorella fusca* (KNAUER; BEHRA; SIGG, 1997), and *Pseudokirchneriella subcapitata* (BOSSUYT; JANSSEN, 2004) were more sensitive to Cu, with all having an optimum growth in 0.1 nM Cu^{2+} and less.

The cell densities and growth rates were more sensitive to Cu in comparison to the dry weights. Cell density is usually the biomass parameter first affected by Cu, because when it enters the cytosol, it may react with free thiols and thiol enzyme groups, thereby disrupting enzyme-active sites and cell division (STAUBER; FLORENCE, 1987). Additionally, Lupi

et al. (1998) showed that algal cells are capable of gathering autospores inside their cells, even though the cells did not divide. Possibly, due to this, their dry weights were less affected by Cu. Similar to these results, Wang et al. (2017) reported the non-inhibition of growth in *Phaeodactylum tricornutum* in Cu concentrations up to 26 nM Cu^{2+}. Also, Cu^{2+} concentrations higher than 790 nM were required to give a 50% reduction (EC 50) in cell division rate in *Chlorella pyrenoidosa* (STAUBER; FLORENCE, 1987), while in the diatom *Phaeodactylum tricornutum,* 1270 nM Cu^{2+} was required to cause a 50% reduction in growth (CID et al., 1995).

3.1.2 Pigments

3.1.2.1 Chlorophyll a and Total carotenoids

Pigments (Chl *a* and total carotenoids) contents were mostly unaltered by Cu exposure in the four microalgae species (Fig. 8), but decreased in the highest Cu exposure, 589.0 nM in *A. flexuosus* (Fig. 8a), 5478.0 nM in *C. chlorastera* (Fig. 8b), 589.0 nM in *C. pantanale* (Fig. 8c), and *Monoraphidium* sp. (Fig. 8d). *Monoraphidium* sp. produced the highest ($p < 0.01$; Two way ANOVA) amount of pigments, followed by *C. chlorastera* that was similar to *C. pantanale*, then *A. flexuosus* with the lowest pigments quantity.
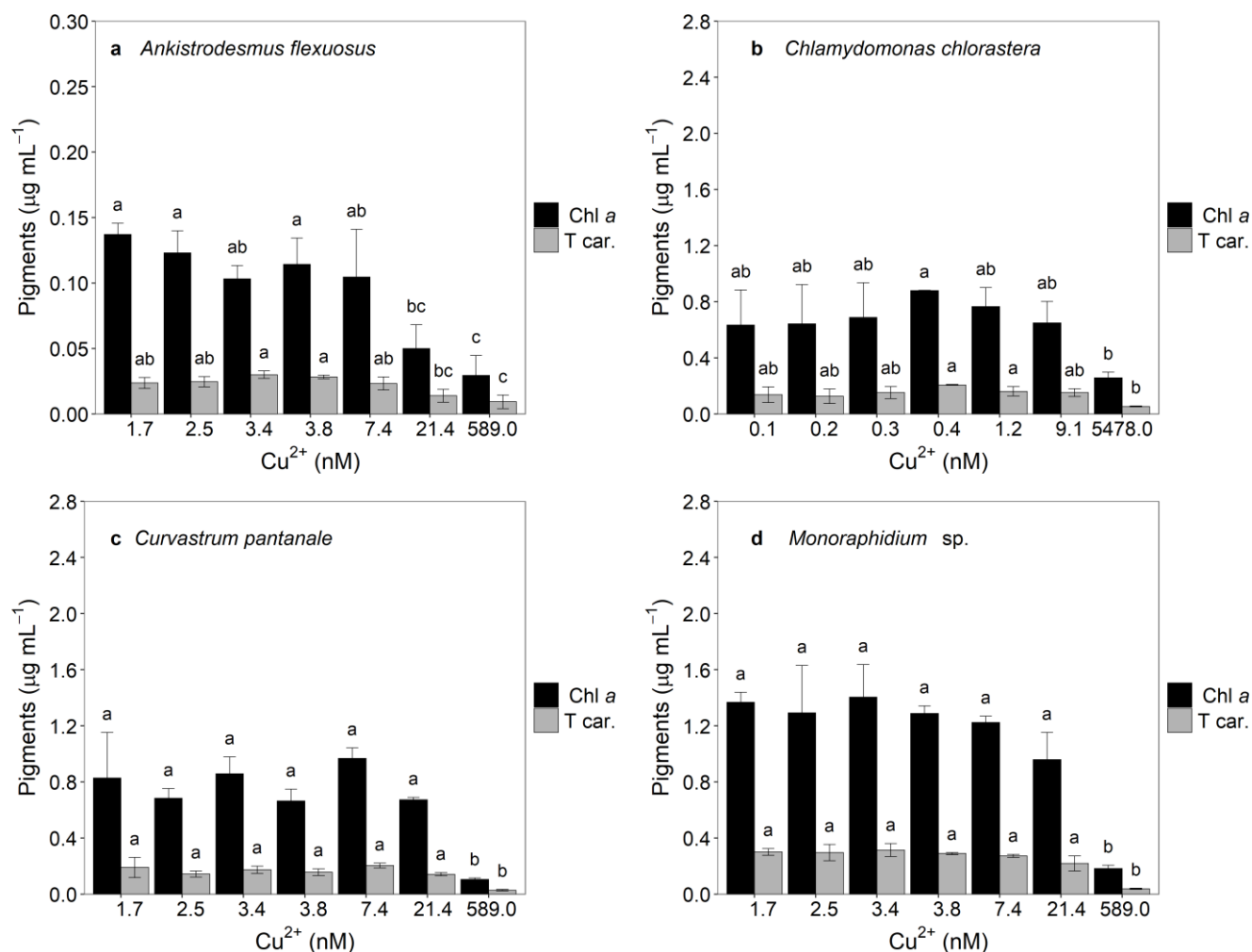
Fig. 8 Chlorophyll *a* (Chl *a*) and total carotenoids (T car.) in different Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA) per parameter, for each species.

The unaltered pigment concentration recorded under low Cu shows that Cu did not affect the chloroplast integrity and pigment synthesis in the algal species tested. In contrast to these results, Chl *a* and carotenoids increased under 15.7 nM and 1570 nM Cu^{2+} respectively, in *Pseudokirchneriella subcapitata* (BOSSUYT; JANSSEN, 2004). However, the decrease in pigments we observed under high Cu is related to the decrease in cell density per mL of culture recorded under this condition. Decrease in chlorophylls has been reported in algae exposed to high copper concentrations (SCHIARITI; JUÁREZ; BEATRIZ RODRÍGUEZ, 2004). Such decrease in pigments content in high Cu exposures can be due to the oxidation of chloroplast membranes, because metals can breakdown the membrane through their free radical formation, which oxidizes the chloroplast membrane (DIETZ; BAIER; KRÄMER, 1999; SOTO; GAETE; HIDALGO, 2011). The chloroplast has been

reported as a primary target for metal toxicity in *Chlorella fusca* (WONG; NAKAMOTO; WAINWRIGHT, 1994).

3.1.2.2 Carotenoids composition of C. chlorastera and Monoraphidium sp. as a function of Cu concentrations

The β-carotene content of *C. chlorastera,* and *Monoraphidium* sp. are shown in Fig. 9. In *C. chlorastera* β-carotene of (Fig. 9a) increased with an increase in Cu, from 0.82 ± 0.11 mg g^{-1} under 0.1 nM Cu^{2+}, to 1.15 ± 0.17 mg g^{-1} and 1.09 ± 0.34 mg g^{-1} under 0.3 and 0.4 nM Cu^{2+} respectively. However, there was no significant ($p > 0.05$) difference in β-carotene between these Cu concentrations. Similarly, the β-carotene of *Monoraphidium* sp. (Fig. 9b) was not significantly ($p > 0.05$) affected by Cu exposure.

The lutein content of *C. chlorastera,* and *Monoraphidium* sp. are shown in Fig. 10. Their lutein contents were not significantly ($p > 0.05$) affected by Cu exposure. However, *C. chlorastera* (Fig. 10a) produced more lutein across all Cu exposure in comparison to *Monoraphidium* sp. (Fig. 10b).
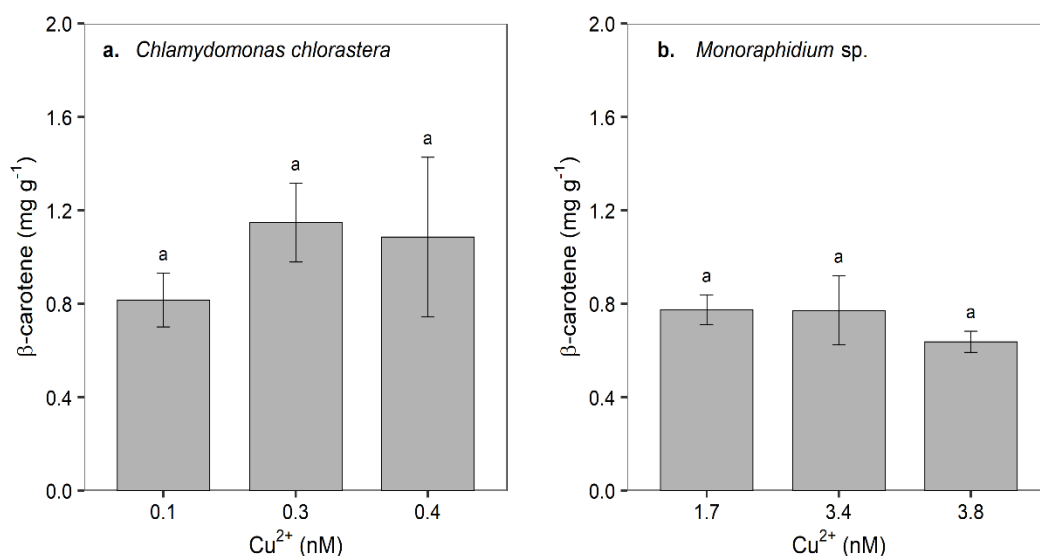
Fig. 9 β-carotene contents in different Cu concentrations of, (a) *Chlamydomonas chlorastera*, (b) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

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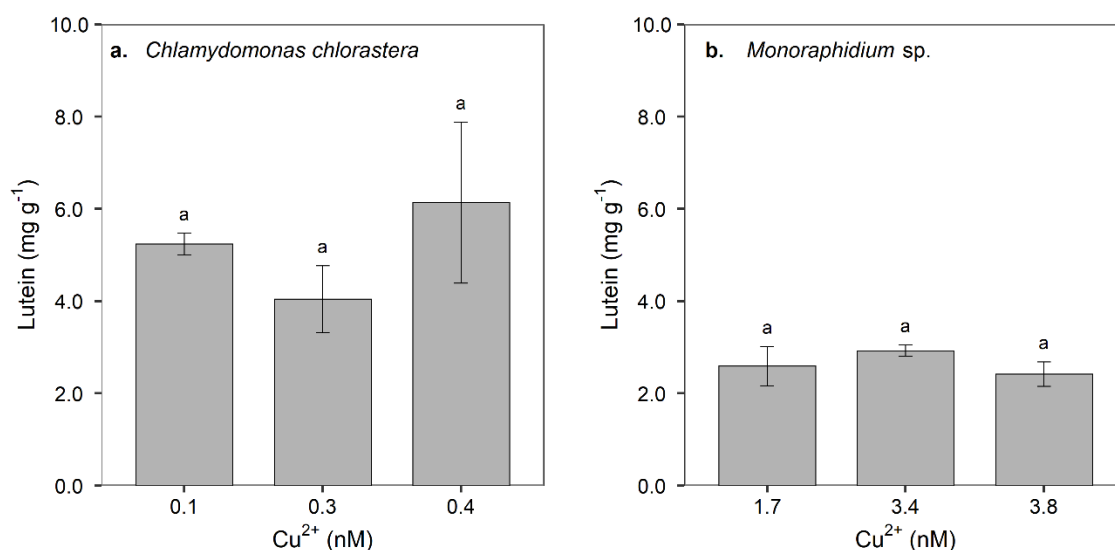
Fig. 10 Lutein contents in different Cu concentrations of, (a) *Chlamydomonas chlorastera*, (b) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

The Cu concentrations used in this study did not significantly alter the cellular production of β-carotene and lutein for neither species studied. Nonetheless, an increase in β-carotene and lutein with Cu increase was seen in *C. chlorastera*. This can be attributed to an increase in antioxidant response induced by Cu (VAQUERO et al., 2012). Such slight increase in antioxidant response was reflected in the GST activity (see, oxidative stress results, page 42). Lutein and β-carotene themselves also act as antioxidants, they exhibited a significant antioxidant activity and potent inhibitory effects against several metabolically important enzymes (WANG et al., 2018).

Cu has been reported to alter the cellular concentrations of carotenoids, for example, 790 µM Cu^{2+} (total Cu) increased the carotenoids in *Dunaliella tertiolecta* after 14 days of culturing (LUSTIGMAN, 1986). Similarly, lutein content in *Coccomyxa onubensis* grown in 0.2 mM Cu^{2+} (total Cu), was roughly 50% higher than that of control cultures during the exponential growth (VAQUERO et al., 2012). A Cu concentration of 4 mM (total Cu) increased the synthesis of β-carotene and lutein in *Chlamydomonas acidophila* (GARBAYO et al., 2008), but this species produced more β-carotene than lutein, contrary to our results. Comparing these mentioned studies with our results, far more Cu and/or a longer culture duration was required to achieve the said carotenoids amounts.

The lutein production in *C. chlorastera* (6.14 ± 1.7 mg g^{-1}) in 0.4 nM Cu^{2+} during the exponential growth (72 h) from this study, exceeds the reported lutein production in many

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algae species (ALUÇ; BAŞARAN KANKILIÇ; TÜZÜN, 2018; MINHAS et al., 2016a; SAFAFAR et al., 2015; SHI; JIANG; CHEN, 2002). Our results are comparable to a high lutein producing strain of *Coelastrella* sp. (6.49 mg g^{-1}) after culturing for 9 days (MINHAS et al., 2016a), and *Coccomyxa onubensis* (6.48 mg g^{-1}), which the authors described as an amount never reported previously for other commercial microalgae (VAQUERO et al., 2012). Soares et al. (2019) recorded higher lutein production than ours in *Desmodesmus protuberans*, *Desmodesmus denticulatus* var. *linearis*, and *Chlamydomonas planctogloea.* However, it is not clear for how long the microalgae were cultured before lutein quantification.

As found in some plants (ESTEBAN; GARCÍA-PLAZAOLA, 2014), some mutants of the green algae *Chlamydomonas* have also shown that lutein, like the de-epoxidation products of violaxanthin or diatoxanthin found in diatoms, has a significant role in energy dissipation through the xanthophyll cycle (LATOWSKI; KUCZYNSKA; STRZALKA, 2011; LAVAUD; ROUSSEAU; ETIENNE, 2002). In fact, our NPQ results confirms activation of the xantophyll cycle (see Photosynthesis results, page56).

Cu has been shown to increase the transcripts encoding the enzyme phytoene synthase in *Ulva compresa* (RODRÍGUEZ et al., 2018), an enzyme that catalyzes the synthesis of phytoene, which is the precursor molecule for all the carotenoids synthesis (JIN; POLLE, 2009). With such increase in gene expression, Cu increased the synthesis of β-carotene in *Ulva compresa* (RODRÍGUEZ et al., 2018).

3.1.3 Carbohydrates

Cu exposure did not affect carbohydrate content in *A. flexuosus* (Fig. 11a), *C. chlorastera* (Fig. 11b), and *Monoraphidium* sp. (Fig. 11d). Similarly, carbohydrate content was unaltered in *C. pantanale* (Fig. 11c) in 1.7–21.4 nM Cu^{2+}, but it significantly ($p < 0.05$) increased by ~ 400% in 589.0 nM Cu^{2+}. The four species produced significantly different ($p < 0.01$; Two-way ANOVA) quantities of carbohydrates. With *C. chlorastera* having the highest, followed in decreasing order by *A. flexuosus*, *C. pantanale*, and *Monoraphidium* sp.

Fig. 11 Carbohydrates contents in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, and (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

The lack of variation in carbohydrates content per cell for most Cu concentrations means that the cells maintained or even increased their carbohydrate synthesis despite the increase in Cu concentration. Carbohydrates are synthesized in the Calvin cycle, using the energy derived from NADPH and ATP from the light reaction of photosynthesis (MASOJÍDEK; TORZILLO; KOBLÍŽEK, 2013; RAVEN; BEARDALL, 2003). With an increase in Cu, we recorded a decrease in $rETR_m$, which implies a decrease in NADPH and ATP being produced during the light dependent reactions of photosynthesis. However, this did not reduce carbohydrate content, meaning that carbohydrate metabolism would just be affected at the highest copper (*C. pantanale*). It has been reported that the accumulation of carbohydrates in phytoplankton is a common physiological response to stress (CHIA et al., 2015; MIAZEK et al., 2015; VALENZUELA-ESPINOZA; MILLÁN-NÚÑEZ; NÚÑEZ-CEBRERO, 2002), where in place of growth, the cells invest in the accumulation of

carbohydrates to store excess fixed carbon produced by unbalanced carbon metabolism (Chia et al. 2015). This was not confirmed in the present research, except for *C. pantanale*, even though all four species had decreased growth rates at the highest Cu.

An increase in carbohydrates under high metal has been linked to its protective effects against oxidative stress, where at high amounts, carbohydrates can also act as direct ROS scavengers (LIANG et al., 2020). Similar to our results, Rocha et al. (2016) also recorded no effect of Cu^{2+} up to 130 nM on carbohydrates in *Selenastrum gracile*. As we recorded in *C. pantanale*, an increase in carbohydrates under high Cu was also recorded in *Chlorolobion braunii* (BARACHO; SILVA; LOMBARDI, 2019), and other algal species (LIANG et al., 2020; MARKOU; ANGELIDAKI; GEORGAKAKIS, 2012).

The higher carbohydrate content in *C. chlorastera* irrespective of Cu concentration, shows that this species synthesizes more carbohydrates in comparison to the others. It has been shown that different microalgae species synthesize different carbohydrate quantity, due to difference in cell size and growth condition (PRISCILLA DE SOUZA et al., 2020).

3.1.4 Proteins

Proteins content was mostly unaffected by Cu exposure in *A. flexuosus* (Fig. 12a), *C. chlorastera* (Fig. 12b), and *Monoraphidium* sp. (Fig. 12d). Similarly, the proteins content of *C. pantanale* was uniform under 1.7 to 21.4 nM Cu^{2+}, but was significantly ($p > 0.05$) increased, under 589.0 nM Cu^{2+} (Fig. 12c). Comparing the four species, *C. chlorastera* produced the highest proteins content. Followed in decreasing order by *A. flexuosus*, *C. pantanale*, and *Monoraphidium* sp.

Fig. 12 Proteins contents in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, and (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different (p ≤ 0.05, one-way ANOVA), for each species.

The generally unaltered protein content per cell under Cu exposure recorded in *A. fusiformis*, *C. chlorastera*, and *Monroraphidium* sp., suggest that their protein production was unaffected by Cu. Whereas, high Cu stimulated protein synthesis in *C. pantanale*, a different response from the other microalgae species. Such behavior agrees with that observed under Cu stress in *Scenedesmus quadricauda* (CHIA; GALADIMA; JAPHET, 2015), *Chlorolobion braunii* (BARACHO; SILVA; LOMBARDI, 2019), and *Dunaliella tertiolecta* (EL AGAWANY et al., 2021). The increase in protein content may have been directed towards increasing the biosynthesis of the antioxidant enzymes induced by Cu stress (CHIA; GALADIMA; JAPHET, 2015; JAMERS et al., 2006), or metal binding molecules, such as phytochelatin (MARTÍNEZ-MACIAS et al., 2019), since it was not accompanied by growth rate increase. Proteins identified in microalgae under Cu exposure include: heat-shock proteins, rubisco, α-and β-tubulins, and ATP synthase (β subunit), which reflected

changes in protein expression, conformation or metal complexation within the cell. (SMITH et al., 2014).

Even though more protein production was stimulated under high Cu in *C. pantanale*, *C. chlorastera* was a higher producer of proteins than all the microalgae species studied. The highest protein produced by *C. chlorastera* was obtained under 0.3 nM Cu^{2+}, about 4 times that of *A. flexuosus*, ~3 times that of the stimulated amount in *C. pantanale*, and ~19 times that of *Monoraphidium* sp.

3.1.5 Lipids

We recorded different lipids responses to Cu exposure, as shown in figure 13. Some algae had lipids content increased (*C. chlorastera*), whereas for others it decreased or did not alter at all.
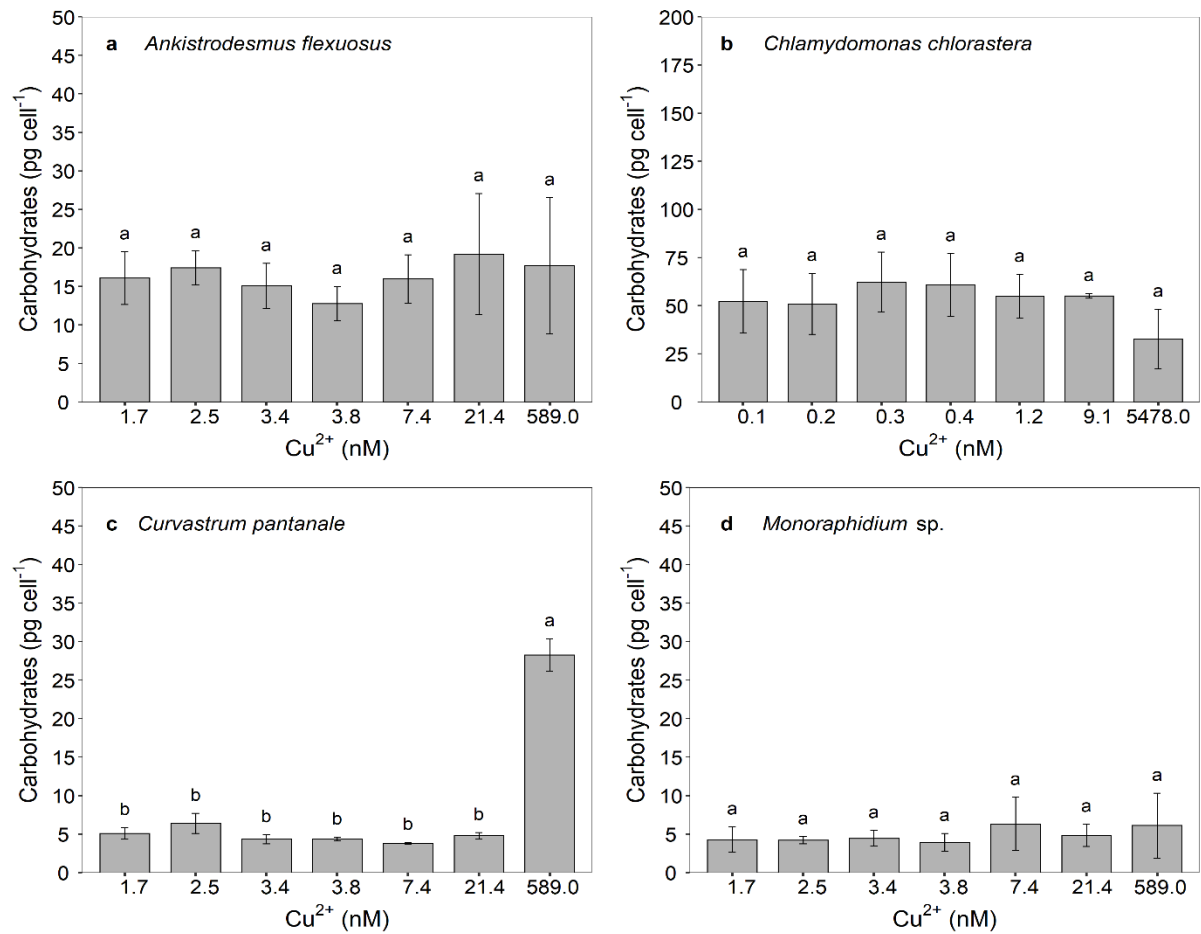

Fig. 13 Lipids contents in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

In *A. flexuosus* (Fig. 13a) and *C. pantanale* (Fig. 13c) no significant variation in lipids were observed for the Cu treatments, but a progressive increase was obtained in *C. chlorastera* (Fig. 13b) in intermediate Cu levels, from 0.1 nM (25.6 ± 7.5 % dw) to 1.2 nM (61.9 ± 14.3 % dw). An inverse behavior was obtained for *Monoraphidium* sp., in which Cu increase led to a decrease in lipids (Fig 13d). These results confirms that the production of lipids by algae is species-specific and can be enhanced under different environmental stresses (MINHAS et al., 2016b; ZIENKIEWICZ et al., 2016). Lipid peroxidation can affect lipids content in microalgae under stress. Despite the increase in lipid peroxidation with an increase in Cu seen in *A. flexuosus* and *C. pantanale*, their unaltered lipids content suggests that they can be more resistant to copper. On the other hand, the decrease in lipids content of *Monoraphidium* sp. with Cu increase, suggests that lipid peroxidation in the presence of excess Cu surpassed the algal lipids synthesis. Therefore, demonstrating the susceptibility of *Monoraphidium* sp.'s lipid synthesis to Cu, unlike in *A. flexuosus* and *C. pantanale*. As recorded in *A. flexuosus* and *C. pantanale* in this study, *Scenedesmus* sp. was also resistant to an increase in Cu concentration (TRIPATHI; GAUR, 2006). Similar to our results, an increase in lipid with an increase in Cu was also recorded in other microalgae (GUO et al., 2021; NEETHU et al., 2021; ROCHA et al., 2016; TRIPATHI; GAUR, 2006).

The stimulus in lipids content of *C. chlorastera* by Cu increase happened without a decrease in cell density or growth rate. This behavior can be considered as a hormetic response of the lipid biosynthesis apparatus of this alga to Cu. Lipids and carbohydrates biosynthesis occur through competing metabolic pathways in microalgal cells (Tan et al., 2016). This is because the intermediate, 3-phosphoglycerate (3-PG) is a necessary substance for the fatty acid biosynthesis (GRIEHL et al., 2012; GUSCHINA; HARWOOD, 2013), and carbohydrates synthesis. We recorded no change in the carbohydrates content of *C. chlorastera* under Cu exposure. This, therefor, suggests that in response to Cu increase, the 3-PG produced in the Calvin cycle was preferentially used for the synthesis of acetyl co-enzyme A, the precursor for fatty acids synthesis in the chloroplast of algae and plants (GRIEHL et al., 2012), and not carbohydrates. Triacylglycerols are accumulated in algae cells during stress (CHIA et al., 2013; GRIEHL et al., 2012; ZIENKIEWICZ et al., 2016), and they are the main lipid components used to produce liquid biofuels (CHEN; WANG, 2021). So, with the quest towards having higher algal lipids (GRIFFITHS; HARRISON, 2009; GUSCHINA; HARWOOD, 2006), this stimulation in lipids synthesis in *C. chlorastera* by Cu increase could be used in applications such as biofuels production.

3.2 Oxidative stress response of the microalgae under different Cu concentrations

3.2.1 DPPH

Cu exposure did not affect the DPPH activity (Figure 14) of the microalgae, since no statistical significant difference was obtained among most copper treatments.
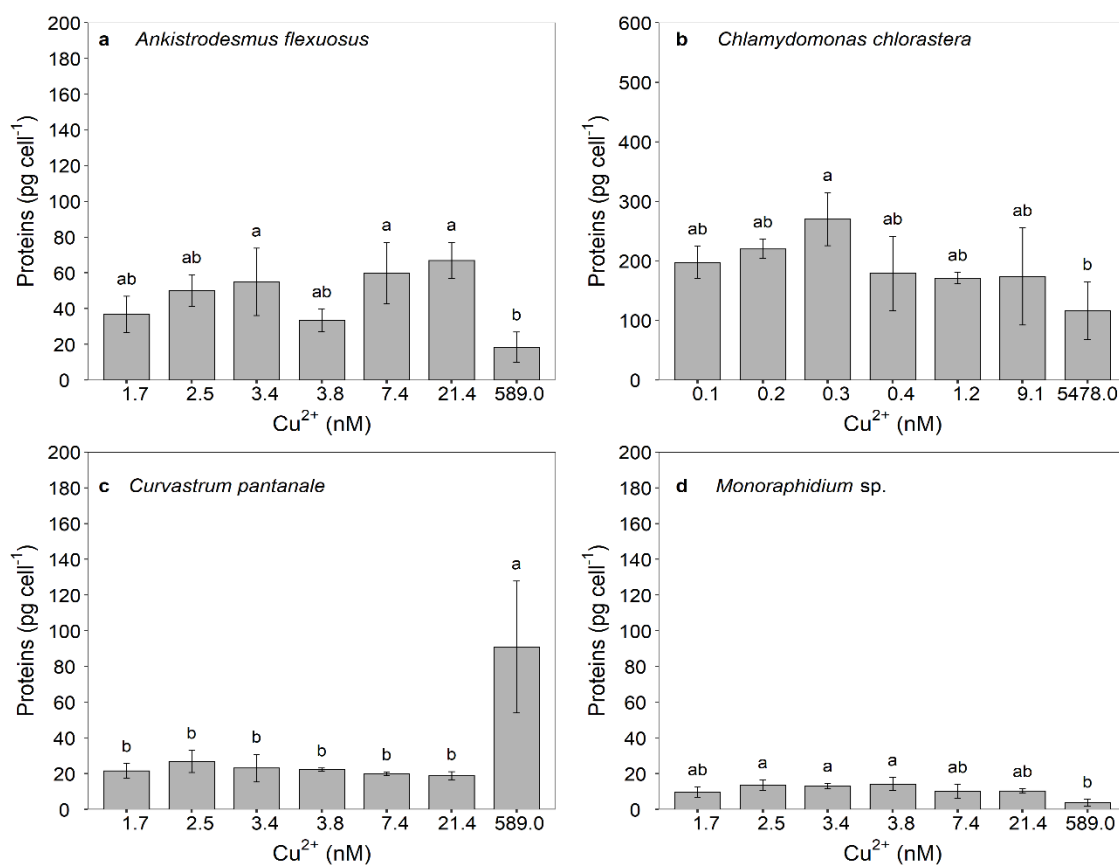

Fig. 14. DPPH radical scavenging activity in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different (p ≤ 0.05, one-way ANOVA), for each species.

DPPH is a compound that has been used extensively as a free radical to evaluate the radical scavenging antioxidant abilities in organisms such as algae (BONDET; BRAND-WILLIAMS; BERSET, 1997; KEDARE; SINGH, 2011). Algal polyphenol content determines the antioxidant activity of their extracts. DPPH scavenging activity is positively correlated with the content of carotenoid and phenolic compounds (SAFAFAR et al., 2015). The quantity of three phenolic compounds in *Dunaliella tertiolecta*: gentisic acid, (+) catechin and (−) epicatechin were 1.4 times higher under 790 nM Cu^{2+} (total Cu) (LÓPEZ

et al., 2015). The DPPH scavenging activities recorded in this study are comparable to that recorded in *Dunaliella tertiolecta* (14%) (LÓPEZ et al., 2015). Contrary to our results, RICO et al. (2013) recorded an increase in DPPH scavenging activity in *Phaeodactylum tricornutum* with an increase in total Cu from 315 nM to 790 nM. Another factor that also affects the phenolic contents synthesized by algae, is the type of culture media in which they grow (MTAKI; KYEWALYANGA; MTOLERA, 2020). The latter authors recorded higher DPPH activity in *Chlorella vulgaris* grown in BCM media, in comparison to BBM and ANM.

3.2.2 Lipid peroxidation

In general, we observed that an increase in Cu concentration caused an increase in lipid peroxidation (Figure 15), but this varied among the algae. In *A. flexuosus*, higher values were observed under 21.4 and 589.0 nM Cu^{2+} (Fig. 15a). In *C. chlorastera* it was significantly higher under 5478.0 nM Cu^{2+} in comparison to 0.3 nM Cu^{2+}. In *C. pantanale* lipid peroxidation was low except under 589.0 nM Cu^{2+} (the highest Cu). In *Monoraphidium* sp. (Fig. 15d), lipid peroxidation also increased with Cu increase, the highest value at 589.0 nM Cu^{2+} exposure.

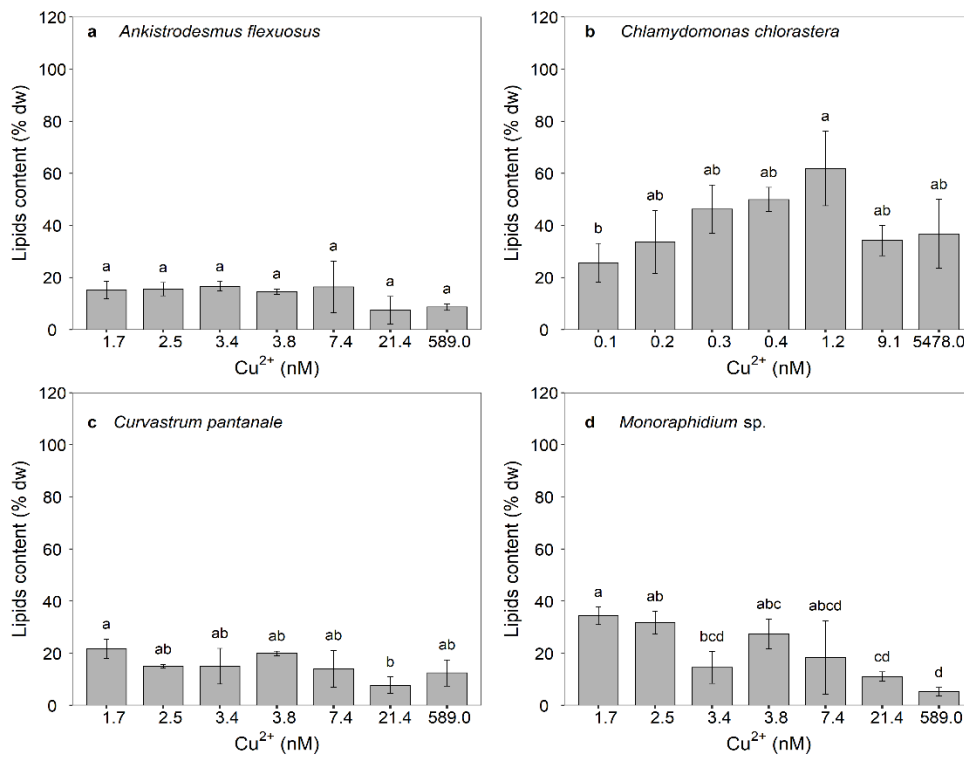Fig. 15 Lipid peroxidation (Malondialdehyde, MDA, activity) in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

The increase in MDA shown in the algal species (except *C. chlorastera*), indicates the deterioration of the lipids under high in these species. This usually includes the deterioration of both membrane and storage lipids. Lipid peroxidation shows the oxidative deterioration of lipids, especially PUFAs (AYALA; MUÑOZ; ARGÜELLES, 2014; HALLIWELL; GUTTERIDGE, 2015), as a result of aldehydes formation (GOIRIS; MUYLAERT; DE COOMAN, 2015). It is an indicator of oxidative stress in organisms, and is monitored using changing concentrations of the by-product MDA (HALLIWELL; GUTTERIDGE, 2015; MOORE; ROBERTS, 1998). Transition metals like Cu^{2+} catalyze the production of $^{\bullet}OH$ (hydroxyl radical) from H_2O_2 through the Haber-Weiss cycle (LIOCHEV; FRIDOVICH, 2002; PINTO et al., 2003); $^{\bullet}OH$ is a chief initiator of lipid peroxidation (HALLIWELL; GUTTERIDGE, 2015). Similar to this result, an increase in lipid peroxidation was recorded with an increase in Cu in *Scenedesmus vacuolatus* (SABATINI et al., 2009), *Chlorella vulgaris* (MALLICK, 2004), and *Pseudokirchneriella subcapitata* (SOTO; GAETE; HIDALGO, 2011). However, the unaltered MDA levels seen in

C. chlorastera could be due to the increase in lipid content with an increase in Cu concentration. This boost could mean that this species is less prone to lipid peroxidation, within the range of Cu tested, in comparison to the other species.

3.2.3 Glutathione S-transferase

GST activity is shown in Figure 16. In general, at the highest copper the highest activity was obtained. In *A. flexuosus* (Fig. 16a) a gradual increase with Cu increase was observed, with 589.0 nM Cu^{2+} having the highest activity, and significantly higher than 1.7–7.4 nM Cu^{2+}. The GST activity in *C. chlorastera* (Fig. 16b) was significantly higher under 5478.0 nM Cu^{2+} in comparison to the other copper levels, except 0.3 nM Cu^{2+}.
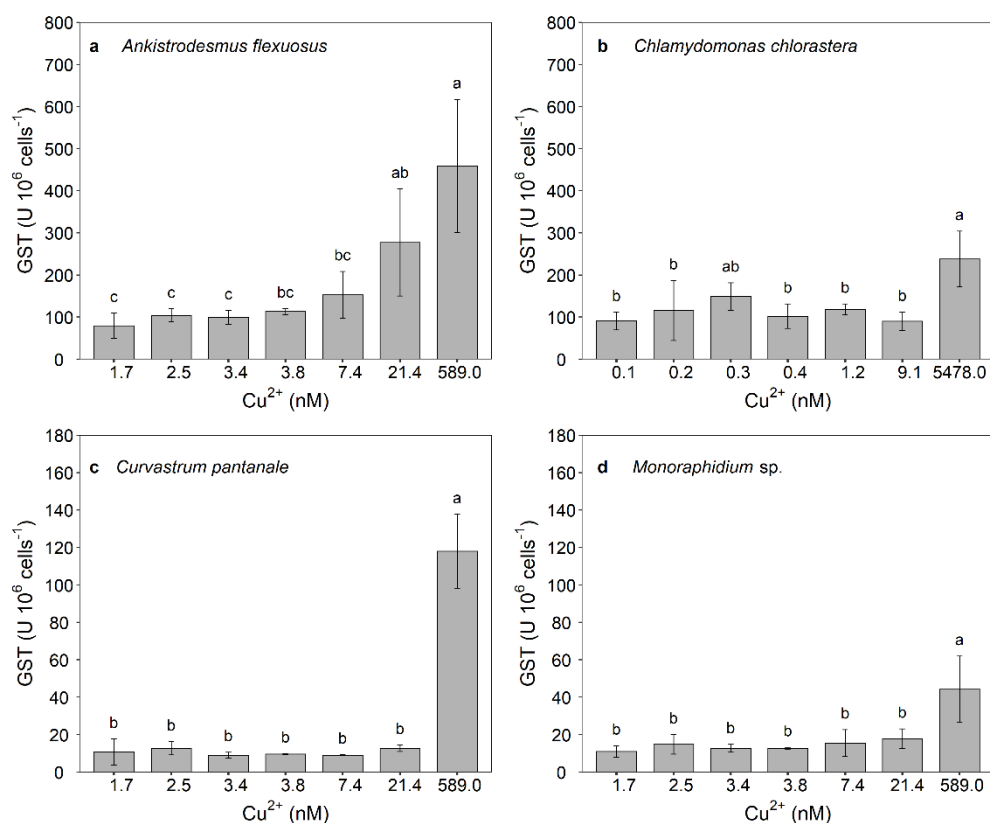
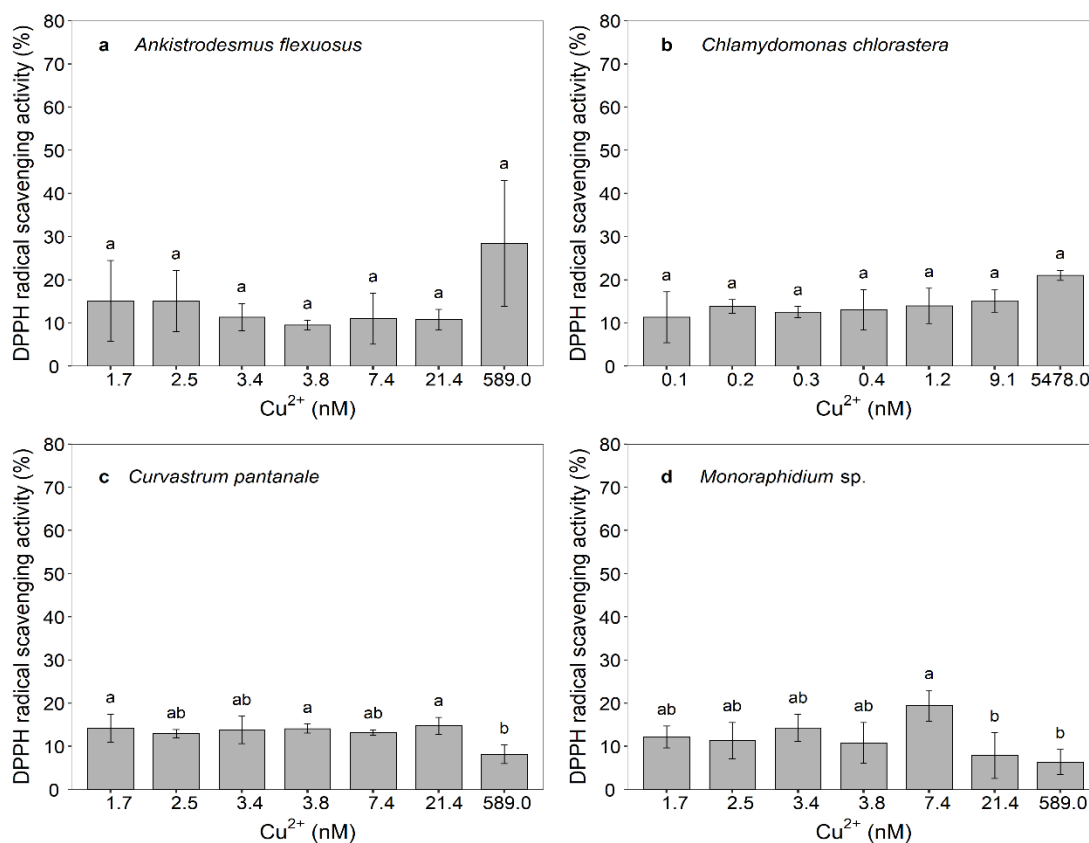Fig. 16 Gluthathione-S-transferase (GST) activity in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \le 0.05$, one-way ANOVA), for each species.

The increased GST activity in the algal cells exposed to the highest copper concentration can result from the increase in GST scavenging of ROS, which are known to accumulate in algae under metal stress (SABATINI et al., 2009; TRIPATHI et al., 2006). The binding of Cu^{2+} to thiols disrupts the redox status of cells, thereby enhancing the production of ROS (HULTBERG; ANDERSSON; ISAKSSON, 1997; PINTO et al., 2003). GST is an enzyme which catalyzes the conjugation of reduced glutathione with metabolic disturbing compounds in the cells. This conjugation is usually the first step in detoxifying an unwanted molecule in cells (HALLIWELL; GUTTERIDGE, 2015; STRANGE et al., 2001). Similar to our results, HAMED et al. (2017) showed increase in GST activity in *Chlorella sorokiniana* exposed to Cu. The conditions that promote growth were also found to stimulate glutathione biosynthesis (CAMERON; PAKRASI, 2010). Higher GST activity (*A. flexuosus* and *C. chlorastera* in this study) means a higher glutathione content in their cells, suggesting a higher cellular metabolic activity, what differed among the microalgae we investigated. Glutathione serves many functions in cells, such as cell protection, folding of proteins (HALLIWELL; GUTTERIDGE, 2015), and also the regulation of the cell cycle (FORMAN; ZHANG; RINNA, 2009).

3.2.4 Peroxidase

Peroxidase (POD) activity was uniform under all Cu exposures but significantly increased, in the highest Cu concentrations in all the four Chlorophyta microalgae investigated in the present research (Figure 17). Similar to our results, the POD activity in *Selenastrum capricornutum* was increased under high Cu concentrations (SAUSER; LIU; WONG, 1997)

Peroxidase (POD) activity was stimulated only under the highest Cu treatments in all the algal species studied. POD scavenges H_2O_2, a ROS that is toxic to cells (BANCI, 1997; MARINHO et al., 2014). The recording of low POD activity under most Cu concentrations suggest that less H_2O_2 was formed in the cells. Several reasons can support this result. For example, it is known that most of the H_2O_2 formed can be used in other reactions, like the Fenton, Haber-Weiss (LIOCHEV; FRIDOVICH, 2002), and Fenton-like (PHAM et al., 2013) reactions, where it serves as a substrate. Also, another antioxidant enzyme called catalase (CAT), which also catalyzes the direct decomposition of H_2O_2 (WEYDERT; CULLEN, 2010), could have competed with POD for the scavenging of H_2O_2 in the cells.
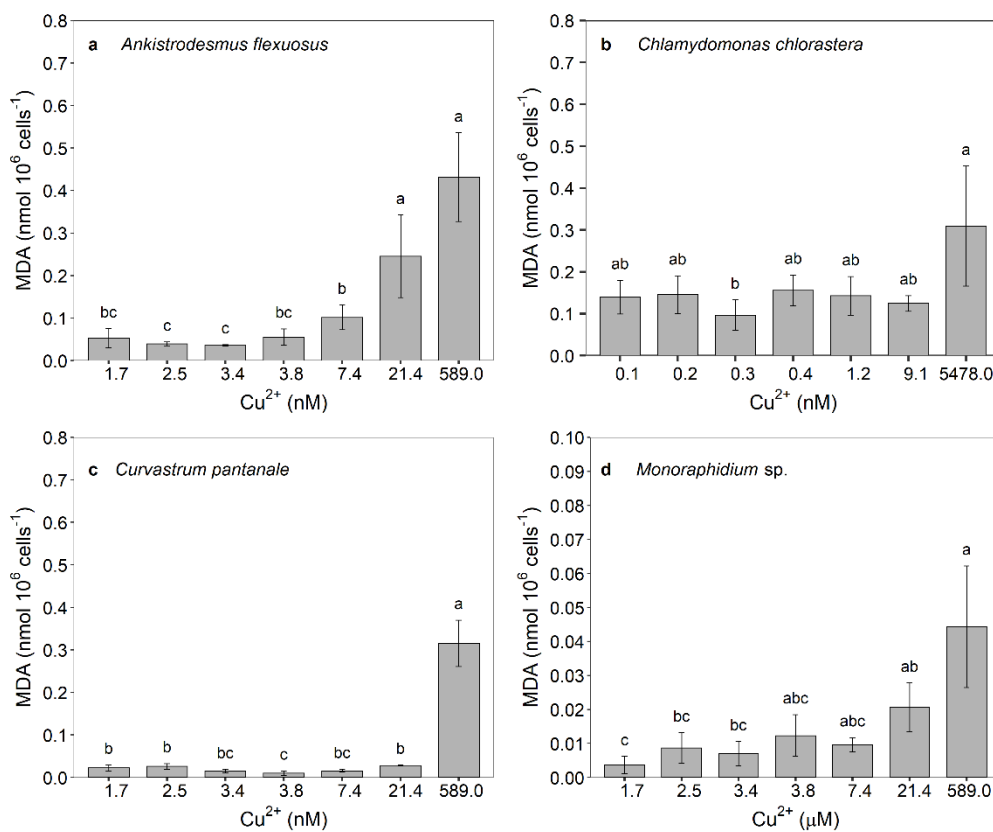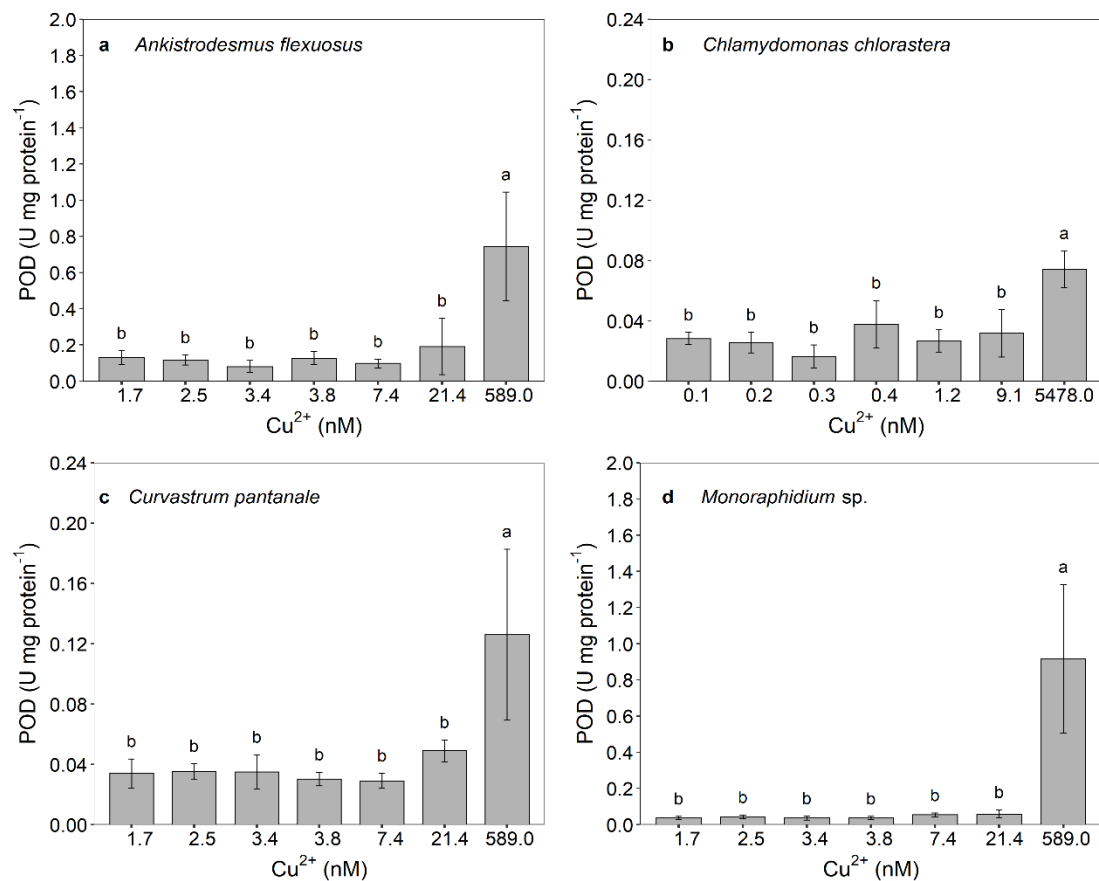
Fig. 17 Peroxidase activity in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

In addition to these, we can add the ameliorative effect of carbon monoxide (endogenously formed in algae), which has been shown to reduce metal induced activity of POD (WEI et al., 2011; ZHENG et al., 2011). Similarly, ZHENG et al. (2011) showed that under low Cu, the activity of ascorbate peroxidase did not increase in Cu exposed *Chlamydomonas reinhardtii*, and TRIPATHI et al. (2006) showed it in < 2500 nM Cu (total Cu) in *Scenedesmus* sp. The increase in POD activity we obtained under high Cu means that H_2O_2 increased in the cells, and considering that it is a by-product of oxygen metabolism in its presence, the redox cycling of Cu may result in the generation of highly reactive and damaging intermediate(s) (PHAM et al., 2013).

3.2.5 Superoxide dismutase

Generally, no significant variation was obtained for SOD activity for the Cu concentrations and species, except for the highest copper, whose SOD activity increased (Figure 18).
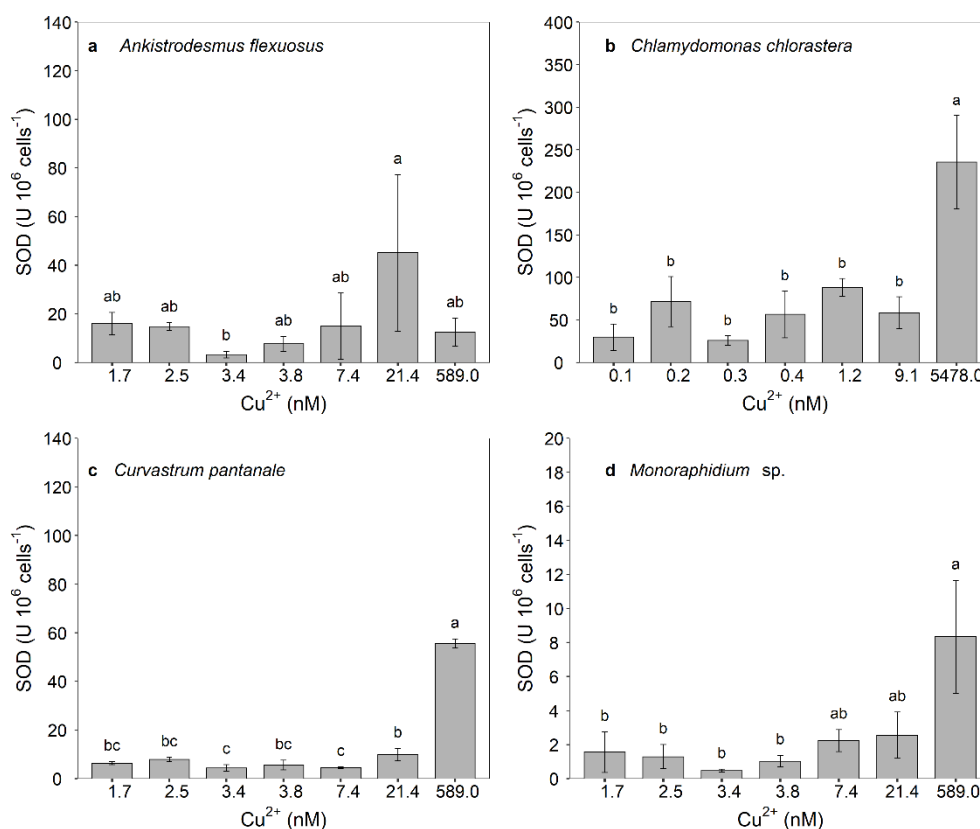
Fig. 18 Superoxide dismutase (SOD) in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different (p ≤ 0.05, one-way ANOVA), for each species.

Similar to our results, the SOD activity in *Scenedesmus* sp. (TRIPATHI et al., 2006), and *Scenedesmus vacuolatus* (SABATINI et al., 2009) were increased under high Cu. The SOD increase under high Cu concentrations in this study signifies an increase in cytotoxic superoxide free radical, $O_2 \cdot^-$ in the algal cells, because Cu^{2+} reacts with H_2O_2 through a Fenton-like reaction to produce $O_2 \cdot^-$ (PHAM et al., 2013). Cu^{2+} primarily triggers oxidative stress in the chloroplast through the formation of $O_2^{\bullet-}$, which can be further reduced by the activity of SOD (PINTO et al., 2003). Cellular oxygen metabolism also generates $O_2 \cdot^-$ (MCCORD, 2008; PHAM et al., 2013) and SOD uses $O_2^{\bullet-}$ as its unique substrate (HALLIWELL; GUTTERIDGE, 2015) by catalyzing its conversion to O_2 and H_2O_2.

(MCCORD, 2008). When not degraded, $O_2^{\cdot -}$ forms $^\cdot OH$ (PINTO et al., 2003), another ROS harmful to cells.

3.3 Photosynthesis of the microalgae under different Cu concentrations

3.3.1 Maximum and Effective Quantum Yields

The F_v/F_m of *A. flexuosus* was unaffected by Cu exposure (Fig. 19a), while its $\Delta F/F_m'$ decreased with an increase in Cu. The F_v/F_m and $\Delta F/F_m'$ of *C. chlorastera* (Fig. 19b) gradually decreased with increase in Cu concentration, both parameters having the lowest values under the highest Cu (589.0 nM Cu^{2+}). The F_v/F_m of *C. pantanale* significantly increased in 21.4 nM Cu^{2+} (0.78 ± 0.01) and was lowest in 589.0 nM Cu^{2+} (0.57 ± 0.00) (Fig. 19c). On the other hand, the $\Delta F/F_m'$ was mostly about 0.4, and significantly decreased under 589.0 nM Cu^{2+} in *C. pantanale*. The F_v/F_m of *Monoraphidium* sp. was generally unaltered, except for the lowest value in the highest Cu, however, its $\Delta F/F_m'$ increased under 2.5 to 7.4 nM Cu^{2+} (Fig. 19d). *Monoraphidium* sp. and *C. pantanale* had the highest $\Delta F/F_m'$, followed in decreasing order, by *C. chlorastera*, and *A. flexuosus*.
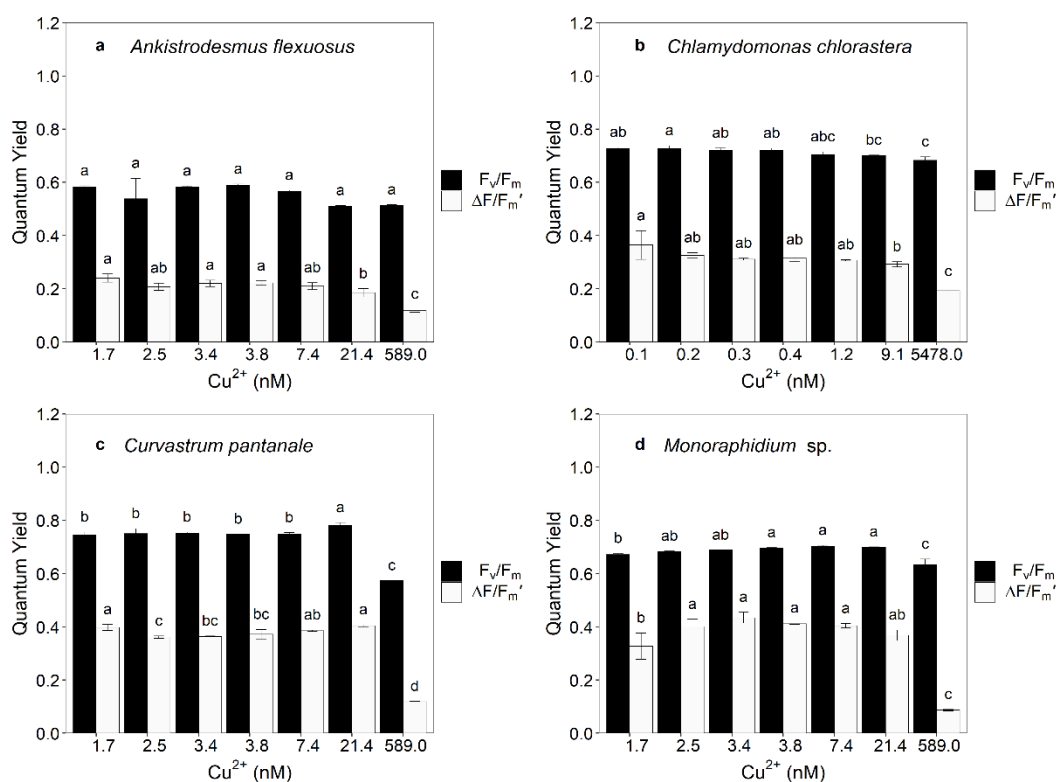
Fig. 19 Maximum quantum yield (F_v/F_m) and effective quantum yield ($\Delta F/F_m'$) in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. Error bars represents standard deviation of the mean (n = 3). Means with different letters are significantly different ($p \leq 0.05$, one-way ANOVA), for each species.

The F_v/F_m represents the maximum efficiency with which the light absorbed by PSII in a dark-adapted sample is used for the reduction of Q_A (BAKER, 2008). It remained largely

unaltered by low Cu exposure, which implies that upon acclimating samples in the dark, the maximum quantum efficiency is unaffected by Cu fluctuation. Similar to our findings, the F_v/F_m was unaltered under low Cu in *Scenedesmus quadriacauda* (SILVA; ECHEVESTE; LOMBARDI, 2018). In *Chlorella pyrenoidosa* it decreased only in Cu^{2+} concentrations higher than 700 nM (VAVILIN et al., 1995). This demonstrates the poor sensitivity of F_v/F_m to low changes in Cu. Interestingly, F_v/F_m was also found to be a poor indicator of nutrient status (KRUSKOPF; FLYNN, 2006), and temperature effect (FIGUEROA et al., 2019) in algae.

The $\Delta F/F_m'$ however showed a more sensitive response, and provided a clearer picture of the microalgae quantum yield as affected by Cu. Where F_v/F_m decreased in the highest Cu by 12%, 6%, 23%, and 6% in *A. flexuosus*, *C. chlorastera*, *C. pantanale*, and *Monoraphidium* sp. respectively, in comparison to the lowest Cu, their $\Delta F/F_m'$ decreased by 51%, 47%, 70%, and 74%, respectively, between those Cu concentrations. The $\Delta F/F_m'$ estimates the efficiency with which light absorbed by PSII is effectively used for Q_A reduction at a given PAR (BAKER, 2008), so it indicates the acclimation of the PSII (FIGUEROA et al., 2014) under light. From 2.5 to 7.4 nM Cu^{2+}, the PSII of *Monoraphidium* sp. is better acclimated for higher efficiency to reduce Q_A, as signified by the increased $\Delta F/F_m'$. However, this efficiency decreased by Cu concentration below and above this range. As recorded in the present study, Juneau et al. (2002) also found the $\Delta F/F_m'$ to be more sensitive to Cu effect than the F_v/F_m in *Chlamydomonas reinhardtii*. Similarly, 1.57 μM Cu^{2+} concentration caused a 25% decrease in F_v/F_m and 29% decrease in $\Delta F/F_m'$ in *Microcystis aeruginosa* (DENG et al., 2014a).

3.3.2 Rapid light curves

Rapid light curves (RLC) of the microalgae treated with the different Cu concentrations are shown in Fig. 20. *C. chlorastera* (Fig. 20b) and *C. pantanale* (Fig. 20c) were the species most adapted to increasing light intensity under low Cu concentrations, as shown by the high amplitudes of their curves. While *A. flexuosus* (Fig. 20a) and *Monoraphdium* sp. (Fig. 20d) were less adapted to increasing light intensities under low Cu concentrations. All the microalgae species however, had their least adaptation to light under their respective high Cu exposures. The parameters obtained from the RLC – the initial slope of the RLC (α), photoinhibition parameter (β), maximum relative electron transport rate

(rETR$_m$), and saturation irradiance (E$_k$) are each presented in their respective subsections below.

3.3.2.1 Alpha

The initial slope of the RLC (α) gives an indication of how effectively light energy is absorbed and used for photochemistry (HENLEY, 1993). In this research, the alpha values for the microalgae were generally similar under the Cu concentrations, except for the highest Cu (Fig. 21). *Monoraphidium* sp. (Fig. 21d) and *C. chlorastera* (Fig. 21b) had the highest alpha values, followed in decreasing order, by *C. pantanale* (Fig. 21c), and *A. flexuosus* (Fig. 21a). This signifies that light energy absorption and usage by the photosynthetic apparatus of the algae were preserved in environmentally relevant Cu values, but negatively impacted at high Cu. Similar to our results and also investigating environmentally important and higher Cu concentrations to microalgae, BARACHO et al. (2019) showed that α values decreased just at high copper.
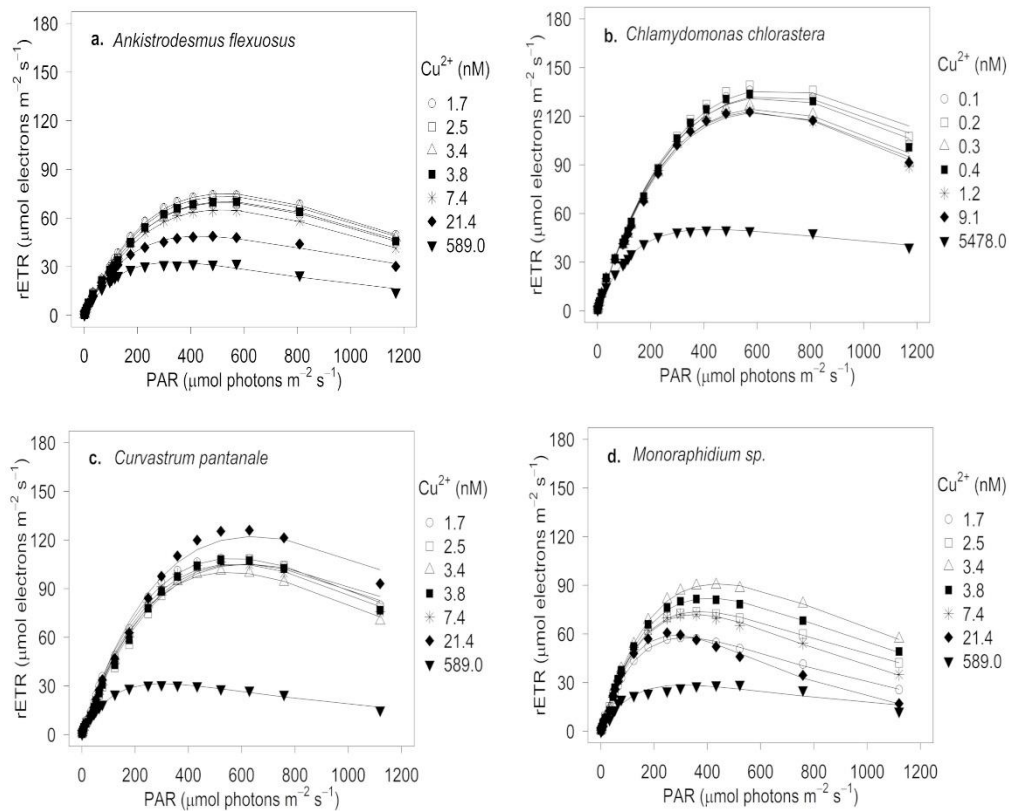
Fig. 20 Rapid light curves; relative electron transport rate (rETR) as a function of PAR for the different Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, (d) *Monoraphidium* sp.

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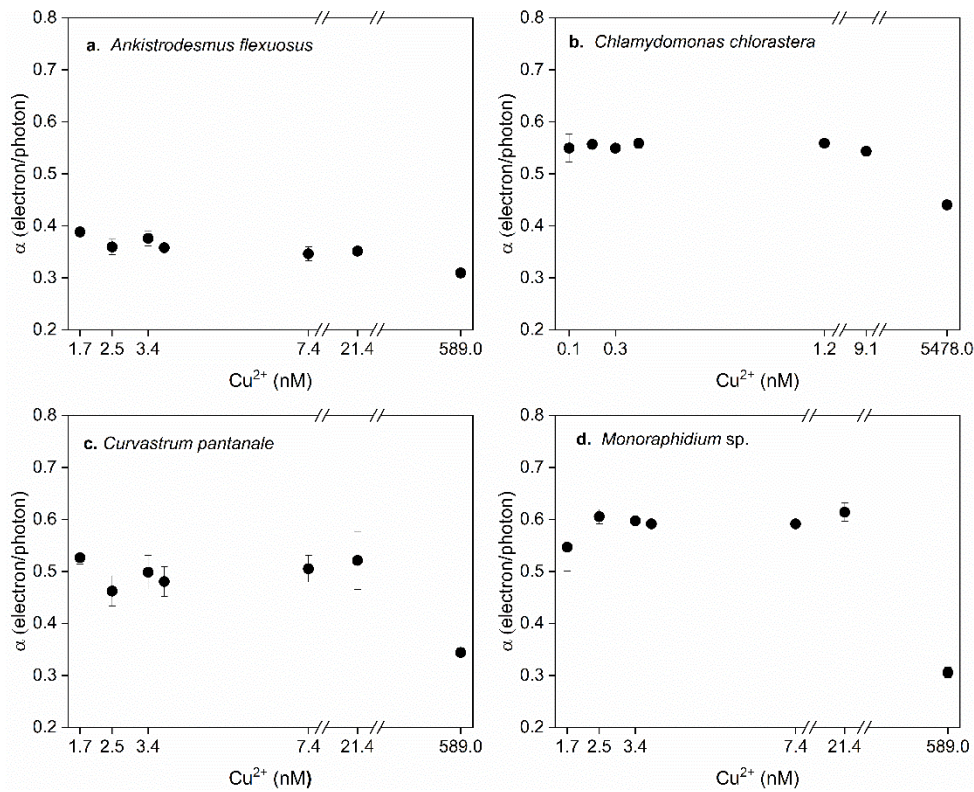
Fig. 21. α $_{rETR}$ (Alpha $_{rETR}$) as a function of Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, and (d) *Monoraphidium* sp.

Comparing the α values between the algal species, *Monoraphidium* sp. and *C. chlorastera* had the highest values. This means that they have better light absorption and usage efficiency under environmentally relevant Cu concentrations, when compared to *C. pantanale* and *A. flexuosus*. By implication, this quality of better light absorption and usage of *Monoraphidium* sp. and *C. chlorastera* may confer a better competitive advantage in the environment, through their ability to use low light.

3.3.2.2 Beta

The value of beta (photoinhibition) was unaltered up to 7.4 nM Cu^{2+} (Fig. 22) for most species tested, but decreased beyond this Cu concentration. *Monoraphidium* sp. was and exception, without (or very low) photoinhibition. Followed in increasing photoinhibition order, by *C. chlorastera*, then *A. flexuosus* and *C. pantanale*
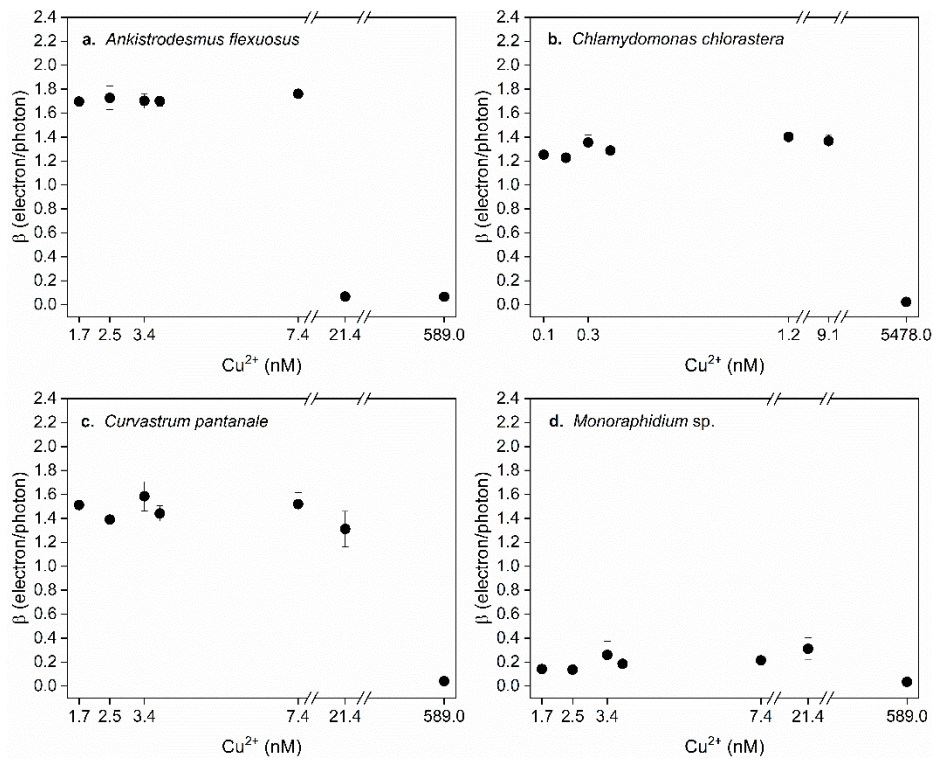
50

Fig. 22 β $_{rETR}$ (Beta $_{rETR}$) as a function of Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, and (d) *Monoraphidium* sp.

In this study, the microalgae cultures were grown between 180 to 250 μmol photons m^{-2} s^{-1}, and the results showed that photoinhibition was not influenced at the low Cu treatments. It occurred under irradiances above 600 μmol photons m^{-2} s^{-1}, which is higher than those with which the cultures were grown. This is the dynamic kind of photoinhibition, which is reversible, and involves the down-regulation of the PSII to protect the photosynthetic apparatus by rendering excess absorbed energy harmless through thermal dissipation, NPQ (FIGUEROA et al., 2003). With a photoinhibition coefficient of 0.2 electron/photon, which is about 7 times lower than the other species, *Monoraphidium* sp. is likely to be more tolerant to high irradiance under environmental Cu concentrations. This shows that *Monoraphidium* sp. is the best adapted of the four microalgae species to high irradiance. This information makes *Monoraphidium* sp. a good candidate for outdoor culturing, where light intensity is mostly high during the day, and difficult to control.

On the other hand, beginning from 21.4 nM Cu^{2+} for *A. flexuosus* and above for the other species, there was practically no photosynthesis going on as supported by the reduced α, low rETR$_m$, and all other photosynthetic parameters. Altogether these indicate the loss of the photo-protection offered by the photoinhibition mechanism under high irradiance, due to the damage of the PSII RC. Cu affects photoinhibition in algae indirectly, by facilitating the

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inhibition of PSII damage repair (MURATA et al., 2007; NISHIYAMA; MURATA, 2014). High Cu also causes the increased formation of singlet oxygen in algae (PINTO et al., 2003), and in turn, singlet oxygen degrades the D1 protein in the RC (HAN et al., 2000). In line with our results, Vavilin et al. (1995) reported the complete suppression of the synthesis of the PS II D1 protein in *Chlorella* cells in 0.7 µM Cu^{2+}, thus causing the inactivation of the PS II and inhibiting photosynthesis.

3.3.2.3 Saturation irradiance

The E_k was generally uniform under Cu concentrations (Figure 23), and decreased under high Cu for *A. flexuosus*, *C. chlorastera*, and *C. pantanale*, but for *Monoraphidium* sp., the increase in $rETR_m$ caused an increase in E_k (since α was constant) in the three lowest Cu concentrations, hence stressing the coupling of these parameters. The E_k of *Monoraphidium* sp. increased from 1.7 to 3.4 nM Cu^{2+}, and then decreased under higher Cu concentrations (Fig. 23d). *C. chlorastera* had the highest E_k, followed in decreasing order of E_k, by *C. pantanale*, *A. flexuosus*, and *Monoraphidium* sp.

Fig. 23 E_k (saturation irradiance) in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp.

This behavior shows that higher irradiance is required to accomplish higher electron transport through the PSII. However, algae adjust their E_k upwards if irradiance increases, and vice versa, as E_k is an indicator of the photoacclimation state of phytoplankton (SAKSHAUG et al., 1997). This result showed that the photoacclimation of the microalgae was altered by Cu concentrations. Because the value of α was similar for all Cu concentrations, except the highest one, E_k was directly coupled with the $rETR_m$. Our result demonstrates that the light intensity needed to maintain the balance between light absorption and photochemical energy conversion on one hand, and the reductant utilization on the other hand (SAKSHAUG et al., 1997) in the PSII of *Monoraphidium* sp. reached its peak in 3.4 nM Cu^{2+} and then decreased as Cu increased. Therefore, depending on Cu concentration, the microalga cells will require different light intensities to maintain optimum photosynthesis. Similar to our results, the E_k of *C. braunii* was unaffected at low copper but decreased at high Cu values (BARACHO; SILVA; LOMBARDI, 2019). Whereas, contrary to our results, the E_k of *Scenedesmus quadricauda* showed very high resilience to Cu ions, remaining unaltered in concentrations as high as 262.48 μM Cu^{2+} (YONG et al., 2018)

3.3.2.4 Relative maximum electron transport rate

The $rETR_m$ response of the microalgae to Cu exposure followed a similar trend to their E_k response, as shown in figure 24.
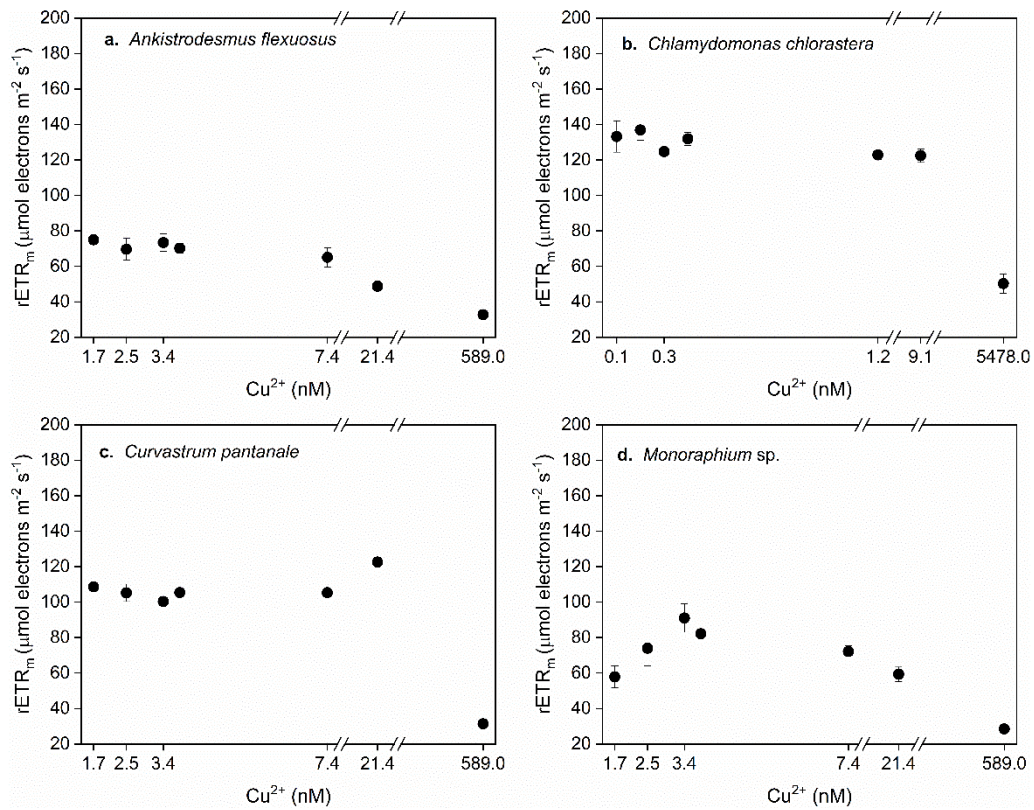
Fig. 24 rETR$_m$ (relative maximum electron transport rate) as a function of Cu concentrations, of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp.

The increase in the rETR$_m$ of *C. pantanale* (in 21.4 nM Cu^{2+}) and *Monoraphidium* sp. with a slight increase in Cu shows that, while maintaining the photosynthetic efficiency, Cu can increase the electron transport capacity of the PSII. This increase can be regarded as a hormetic response of the electron transport chain induced by Cu (Cedergreen et al. 2006; Calabrese 2008). High Cu has been shown to inhibit photosynthetic electron transport through damage to both the donor (SCHRODER et al., 1994) and acceptor sides (DEWEZ et al., 2005; YRUELA et al., 1993) of PS II (PÄTSIKKÄ; ARO; TYYSTJÄRVI, 1998). Following this, we attribute the increase in rETR$_m$ to a Cu-induced increase in electron transport down the pathway, from the acceptor side of the PSII through the plastocyanin to the PSI. This underscores the importance of Cu in the PSII electron transport chain, as a key component of plastocyanin (BURDA et al., 2003; DROPPA; HORVÁTH, 1990), and the availability of Cu regulates the plastocyanin expression in chlorophytes (HONG HUA LI; MERCHANT, 1995; RAVEN; EVANS; KORB, 1999). Eukaryotic algae and most cyanobacteria contain either plastocyanin, or cytochrome c$_6$ (cyt c$_6$), or both (DE LA ROSA et al., 2002, 2007; KATOH, 2003).

On the other hand, the low rETR$_m$ under low Cu in *Monoraphidium* sp. and its drastic reduction under high Cu in all the algal species, demonstrates that insufficient or excess Cu hinders the transport of electrons through the PSII electron transport chain. Plastocyanin performs the function of electron transport to the PSI in copper-sufficient cells, but it is replaced by cytochrome c$_6$ under copper deficiency (DE LA ROSA et al., 2007; WOOD, 1978). Insufficient Cu leads to the poor synthesis of plastocyanin, and consequently a decrease in the amount of this important electron transporter in the PSII (YRUELA, 2005). We rationalize that the low rETR$_m$ under the lowest Cu in *Monoraphidium* sp. to be due to low plastocyanin quantity, but not so low as to trigger the synthesis of cytochrome c$_6$, since if this had occurred, we would expect no difference in rETR$_m$ for the low copper concentrations. Similar to the result seen in *Monoraphidium* sp. in this study, the electron transport rate of *Chlorella vulgaris* was also shown to increase with an increase in Cu of up to 2 µM (CHEN et al., 2016), although it is not clear whether the free Cu ion or nominal Cu were reported. Nevertheless, this stimulation occurred in much higher Cu concentrations than seen in our study.

High Cu^{2+} directly influence the photosynthetic electron transport through the oxidative breakdown of chlorophylls and carotenoids, oxidative damage of proteins and membrane structures, and the substitution of metal cofactors. (DIETZ; BAIER; KRÄMER, 1999). Similar to our result, a reduction in rETR$_m$ was observed under high Cu, 1180 nM Cu^{2+} in *Microcystis aeroginusa* (DENG et al., 2014a) and 5000 nM Cu^{2+} in *Chlorolobion braunii* (BARACHO; SILVA; LOMBARDI, 2019).

3.3.3 Quenching parameters

The photochemical quenching parameters (qL and qP) of *A. flexuosus* (Fig. 25a), *C. chlorastera* (Fig. 25b), and *C. pantanale* (Fig. 25c), were mostly unaltered by Cu exposure, except in the highest Cu, where they decreased. But the qL and qP of *Monoraphidium* sp. was higher under 0.085 to 0.374 µM Cu^{2+} (Fig 25d), in comparison to Cu exposure below and beyond this range. The NPQ of *A. flexuosus* (Fig. 25a) and *Monoraphidium* sp. (Fig. 25d), progressively increased with Cu increase, while the NPQ of *C. chlorastera* (Fig. 25b) and *C. pantanale* (Fig. 25c) decreased under the highest Cu exposure.

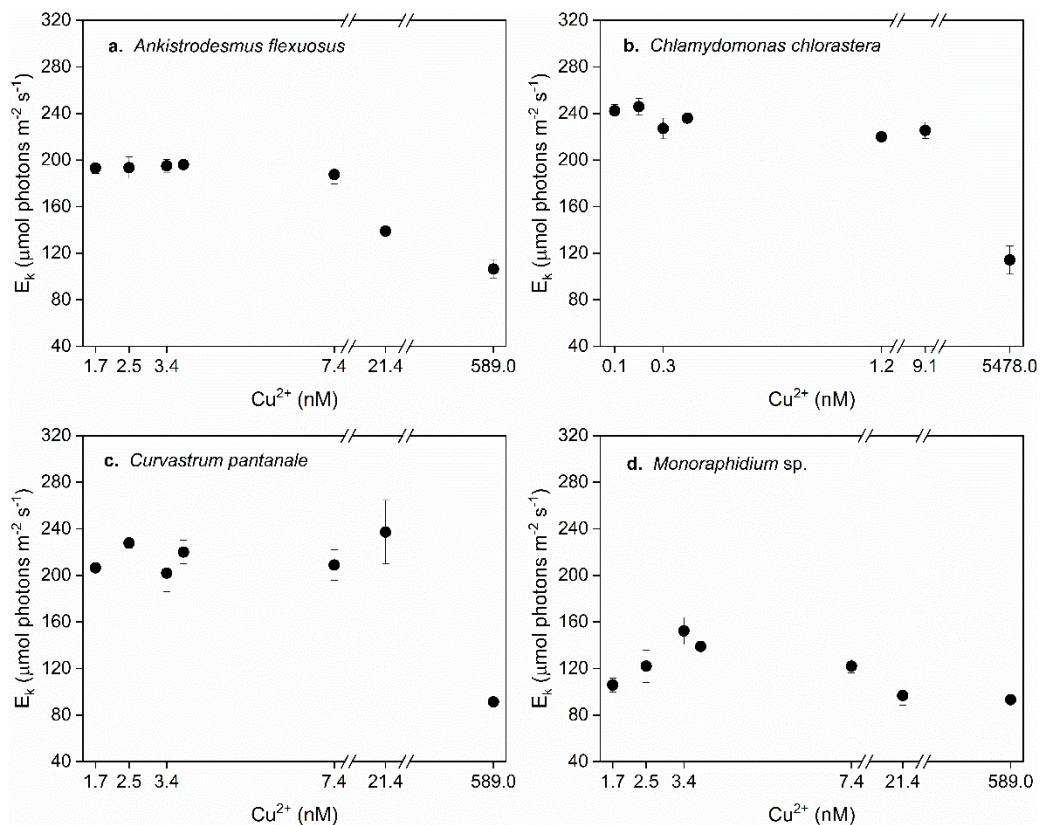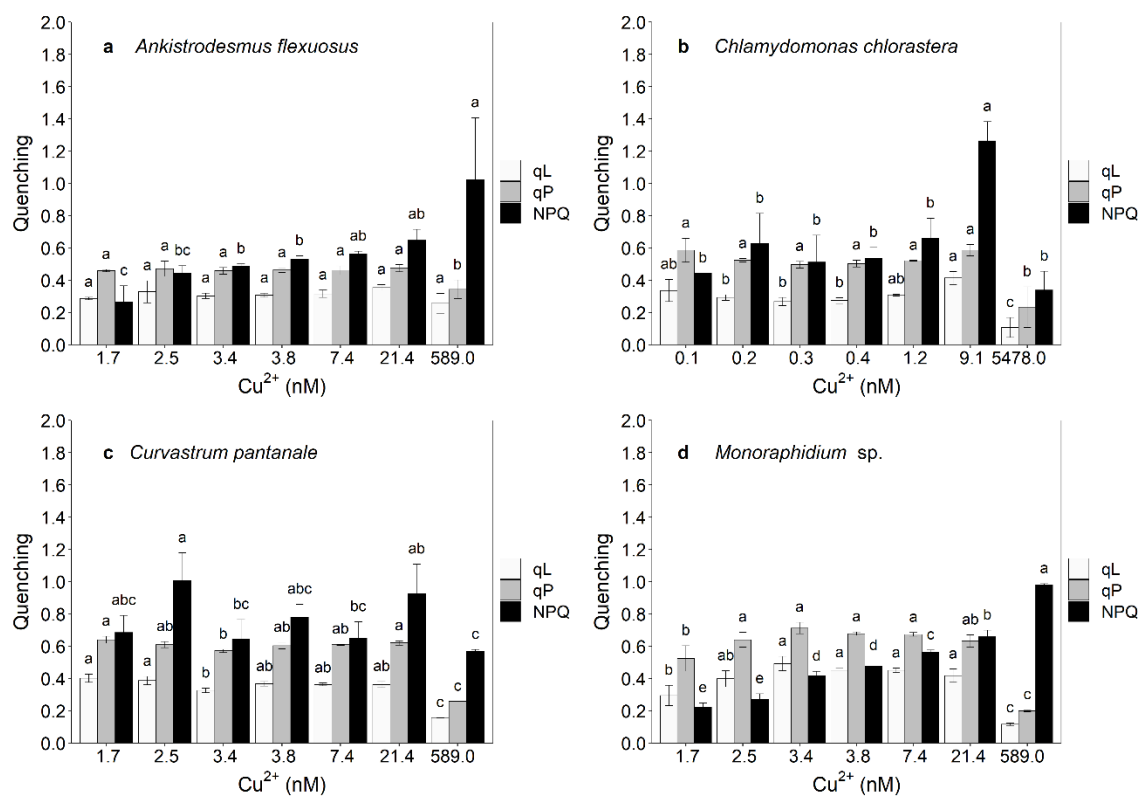Fig. 25 Chlorophyll fluorescence quenching parameters in different Cu concentrations of, (a) *Ankistrodesmus flexuosus*, (b) *Chlamydomonas chlorastera*, (c) *Curvastrum pantanale*, & (d) *Monoraphidium* sp. qL: photochemical quenching, lake model; qP: photochemical quenching, puddle model; NPQ: non-photochemical quenching.

Low and intermediate Cu concentrations did not affect the photochemical quenchings of the algae species, except *Monoraphdium* sp., where Cu concentrations from 2.5 to 21.4 nM Cu^{2+} favoured higher photochemical quenchings (qL and qP) in comparison to control and the highest Cu. Such increase in qL and qP signifies a more efficient electron trapping by the Q_A in PSII, e.g. the Q_A is better oxidized. It could also mean that more oxidized Q_A is available under these Cu concentrations. On the other hand, the reduction in qL and qP under high Cu in all species means a reduction in the quantity of open Q_A, which will consequently reduce the quantity of electron transport. This reduction is related to the reduction in Chl *a* recorded under high Cu. Although both the qL and qP represent the proportion of open PSII reaction centers used for electron transport, they differ in their premise (KRAMER et al., 2004). The qP model assumes that each PSII has its own LHC, while the qL model assumes that different PSII units share a common LHC (KRAMER et al., 2004). Due to the connectivity between PS II units as a result of energy transfer between PS II reaction centers via common antenna domains (SCHREIBER, 2004), qP is not strictly linearly related to the redox state of Q_A and plastoquinone (KRAUSE; JAHNS, 2004), which makes the qL model

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more accurate for estimating the photochemical quenching (Kramer et al. 2004). This reason explains why the qL is less than the qP gotten under all Cu concentrations, because in the qL model the reaction centers are connected and compete for the excitation energy from a common LHC (NIKOLAOU et al., 2014). An increase in the qP of *Chlorella vulgaris* with an increase in Cu concentration (CHEN et al., 2016) was shown, but this happened in much higher Cu than recorded in *Monoraphidium* sp. in this study. The decrease in qP under high Cu implies the closure of most of the available reaction centers. The qP of *Chlorella vulgaris* (CHEN et al., 2016), and *Chlorolobion braunii* (BARACHO; SILVA; LOMBARDI, 2019) also decreased under high Cu and were unaffected in low and intermediate Cu levels, as seen in this study.

The parameter NPQ is characterized by heat dissipation, it is considered more robust and is often used in preference to other non-photochemical quenching parameters, such as the qN (RALPH; GADEMANN, 2005). The progressive increase in NPQ from the lowest to the highest Cu concentration in *A. flexuosus* and *Monoraphidium* sp. signifies an increase in the protection of the PSII offered by the NPQ as Cu concentration rises. This protective mechanism was not compromised even under the highest Cu concentration. However, the protective mechanisms of the NPQ in *C. chlorastera* and *C. pantanale* were not able to withstand high Cu as seen in *A. flexuosus* and *Monoraphidium* sp. These results show that they used the NPQ as a protective mechanism under Cu stress, but to different degree of Cu exposure. It is known that not all microalgae use NPQ for protection, for example, *Desmodesmus armatus* uses the alternative electron transport (AET) in contrast to the NPQ for photoprotection (WARE et al., 2020). Similar to the NPQ response seen in *A. flexuosus* and *Monoraphidium* sp. in this research, an increase in NPQ under high Cu (50 μM Cu^{2+}) was reported for *Euglena gracilis* (ROCCHETTA; KÜPPER, 2009). Additionally, just as seen in *C. chlorastera* and *C. pantanale* in this study, the NPQ of *Selenastrum gracile* decreased beyond 24 nM Cu^{2+} (ROCHA; PARRISH; ESPÍNDOLA, 2021), in 0.079 nM Cu^{2+} and above in the Haptophyta *Phaeocystis cordata* (LOMBARDI; MALDONADO, 2011).

3.3.4 Functional absorption cross-section of *Monoraphidium* sp. under different cu concentrations

The functional absorption cross-section was uniform under low Cu concentrations (1.7 to 21.4 nM Cu^{2+}), which implies that low Cu did not alter the antenna size of the PSII LHC of *Monoraphidium* sp. (ANOVA, $p > 0.05$), while high Cu (589.0 nM Cu^{2+}) induced increase in the functional absorption cross-section (ANOVA, $p < 0.01$). These results are

shown in Figure 26 and the increased antenna size corresponds to a decrease in Chl *a*, and an increase in the Chl *b* per cell.
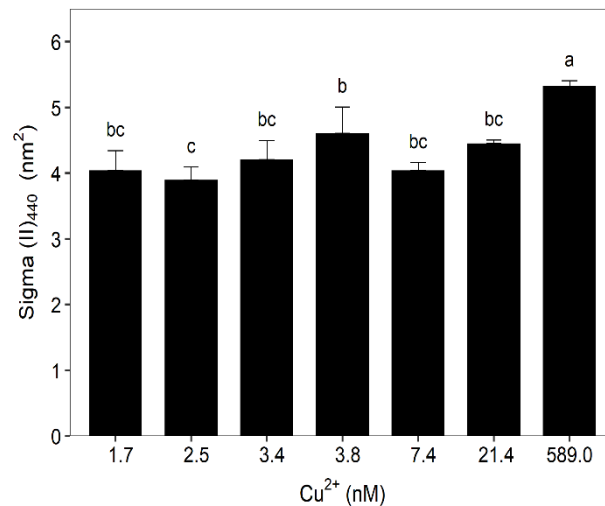
Fig. 26 Functional absorption cross-section (Sigma (II)$_{440}$) in different Cu concentrations of, *Monoraphidium* sp. Means with different alphabets are significantly (p < 0.01) different.

Chlorophyll *b* accounts for about half the total Chl in peripheral light-harvesting complexes (LHC) and is not present in the photosynthetic reaction centers (FRIEDLAND et al., 2019; LOKSTEIN; RENGER; GÖTZE, 2021). This, therefore, explains the reason for recording higher Chl *b* against Chl *a* under high Cu. However, the increase in Sigma (II) was negatively correlated with α, F_v/F_m, and $\Delta F/F_m{'}$, which means the increase in the number of the antenna pigments (or antenna size) under high Cu does not translate to either, an increase in α (light absorption efficiency), or an increase in the quantum efficiency (F_v/F_m and $\Delta F/F_m{'}$) of light usage in the PSII RC. Therefore, the larger antenna under high Cu was not effective in light absorption. In agreement with our findings, Polle et al. (2003) stated that the assembling of large arrays of light-absorbing Chl antenna molecules in algae photosystems is a survival strategy, but is detrimental to the yield and productivity of cultures. Plants with smaller antenna sizes have better photosynthetic performance and biomass yield (FRIEDLAND et al., 2019), and in fact, the tuning of the light-harvesting antenna to a smaller size has been a positive approach to increase the efficiency of photosynthesis (PERRINE; NEGI; SAYRE, 2012). The antenna enlargement under high Cu therefore, signifies a coping strategy of the algal photosynthetic apparatus, by attempting to increase their light harvesting capacity due to light stress. This is because high Cu reduced

cell density, as a result, fewer cells were exposed to more light. Another aspect to consider is the oxidative stress induced by high Cu, which makes the cells more sensitive towards light stress. Larger antenna sizes have been shown to assist in the tolerance to light stress (FRIEDLAND et al., 2019; LOKSTEIN; RENGER; GÖTZE, 2021). Interestingly, high Cu exposure also increased the antenna size of the bacteriochlorophyll containing bacterium, *Rhodospirillum rubrum* (JAIME-PÉREZ et al., 2019).

3.3.5 Oxidation reduction/re-oxidation times of *Monoraphidium* sp. under different Cu concentrations

The time constant for the reduction of quinone A (Q_A) (Tau) in the PSII reaction center significantly decreased ($p < 0.01$) with increasing Cu concentration (Fig. 27). Cu concentrations from 1.7 nMCu^{2+} to 21.4 nM Cu^{2+} produced similar Tau values. While 589.0 nM Cu^{2+} (the highest Cu concentration) produced the lowest Tau value (0.12 ± 0.01 ms). There was no significant difference ($p > 0.05$) in terms of the time constant for the reoxidation of reduced quinone A (Q_A^-) (Tau (reox)) between all the Cu concentrations tested.
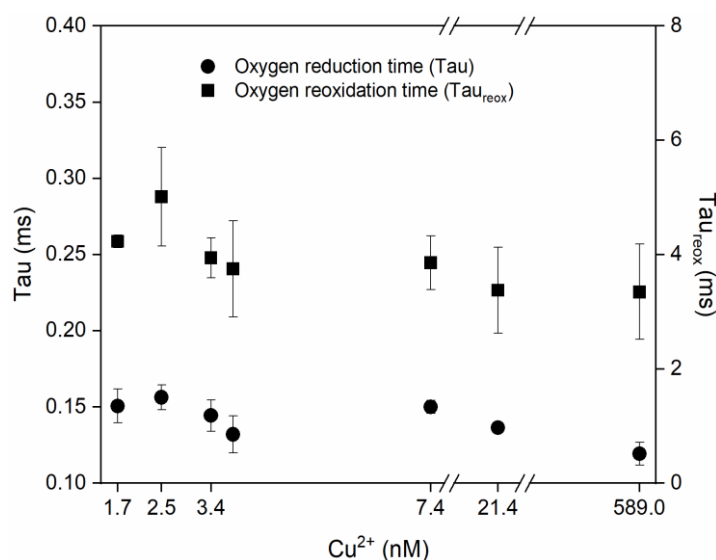
Fig. 27 Quinone A reduction and quinone A re-oxidation times as functions of Cu concentrations of *Monoraphidium* sp.

The uniform Tau values seen under all Cu exposure, except the highest, shows that the time taken for the reduction of the Q_A was reduced only high Cu. Yruela et al. (1993) showed that Cu^{2+} blocks the reduction of Q_A and that its binding site in the PSII is located after the pheophytin but before the Q_A. This blocking of the reduction of Q_A by Cu^{2+} could explain the reason for getting a low value of Tau under high Cu in this study. Contrary to

Tau, the Cu concentrations did not affect the time taken for the re-oxidation of Q_A (Tau (reox)), which means that once reduced upon giving up their electrons to Q_B, Q_A is readily oxidized irrespective of Cu concentration. The Q_B accepts 2 electrons from Q_A to be fully reduced and form quinol (MUH et al., 2012), so 1 Q_B is required to oxidize 2 Q_A. Therefore, half the number of Q_B are required to oxidize Q_A, and this could explain the reason for not recording a difference in the Tau (reox) in comparison to Tau.

3.3.6 Primary productivity of *Monoraphidium* sp. under different cu concentrations

The oxygen evolution rate (rO_2) and biomass-specific productivity (P^B) are both measures of primary productivity and are presented in Fig. 28. The primary productivities were not different ($p > 0.05$) for all the Cu concentrations, except in 589.0 nM Cu^{2+}, which resulted in the lowest primary productivity (rO_2, 79.7 ± 2.7 µmol O_2 mg Chl a^{-1} h^{-1}; P^B, 0.68 ± 0.02 mg C mg Chl a^{-1} h^{-1}).
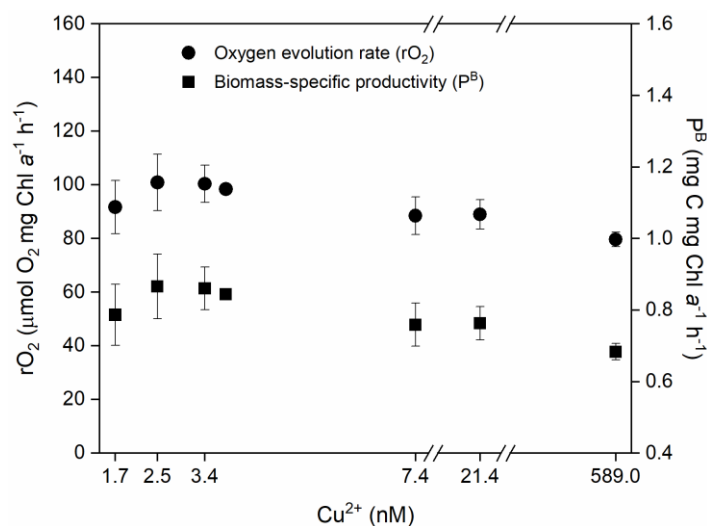
Fig. 28 Oxygen evolution rate and the biomass-specific productivity as functions of Cu concentrations of *Monoraphidium* sp.

We recorded no alteration in primary productivity under low Cu, despite seeing an increase in $rETR_m$ for the first three copper concentrations. This could be related to the recorded unaltered Chl a/b ratio under these Cu exposures, because primary productivity is related to the Chl content of the cells. Additionally, not all the electrons that pass through the ETC are used for primary production. Some are utilized for photo-protection and assimilation of N into amino acids (PODGÓRSKA et al., 2020), especially where nitrate is

used as a N source, like in this study. Similarly, the oxygen evolution rate of *Fucus serratus* was unchanged by Cu exposure (NIELSEN; NIELSEN, 2005). In contrast, other studies reported a stimulation in oxygen evolution of tobacco (BURDA et al., 2002) and *Scenedesmus incrassatulus* (PERALES-VELA et al., 2007) by low Cu^{2+}. A stimulation of oxygen evolution of up to 68.5% was obtained in *Porphyra haitanesis* under 1μM Cu^{2+} (LI et al., 2010). Relating to tobacco oxygen evolution stimulation, Burda et al. (2002) excluded the possibility of Cu^{2+} substituting other ions in the PSII, but they rather suggested that there exists a specific high-affinity Cu-binding site within PSII, hence the reason for the stimulatory effect at low Cu.

The decrease in primary productivity we obtained under high Cu can be due to the fact that oxygen consumption by respiration may have outweighed its production through photosynthesis (LI et al., 2010), hence resulting in reduced oxygen liberation. The primary target sites in the PSII for Cu are $tyrosine_z$, both cytochrome b_{559} forms and $chlorophyll_z$, and these sites are the sources of the Cu-induced inhibition of oxygen evolution (BURDA et al., 2003). High Cu affects oxygen evolution by causing the disintegration of the extrinsic proteins of the oxygen-evolving complex of the PSII (YRUELA, 2005).

4.0 CONCLUSION

This study focused on examining the possible stimulatory effect of environmentally relevant Cu levels, on microalgae biomass, growth, biomolecules production, antioxidant response, photosynthesis, and primary productivity. In general, the results from this study demonstrates that Cu within environmentally relevant concentrations, causes different physiological responses in different microalgae species. While some species are susceptible to increase in Cu, others are better adapted to slight increases in the metal. Such nuances could potentially change microalgae diversity, favouring the dominance of better adapted species, a scenario that is not desired for ecosystem balance and stability. The major highlights of the present study are presented below.

- Cu concentrations up to 9.1 nM Cu^{2+} did not alter biomass (cell density and dry weight), pigments (Chl a and total carotenoids), and growth rates in the microalgae. But *Monoraphidium* sp. had the highest growth rates among the microalgae species.

- β-carotene and lutein contents were similar in *C. chlorastera* between 0.1–0.4 nM Cu^{2+}, and in *Monoraphidium* sp. between 1.7–3.8 nM Cu^{2+}. *C. chlorastera* was the best β-carotene (1.2 mg g^{-1}; 0.3–0.4 nM Cu^{2+}) and lutein (6.14 mg g^{-1}; 0.4 nM Cu^{2+}) producer.

- Total carbohydrates content per cell increased in *C. pantanale* (28.3 ± 2.1 pg $cell^{-1}$) in 589.0 nM Cu^{2+}. However, *C. chlorastera* had the highest carbohydrates content among all the species, higher than 50 pg $cell^{-1}$ across the Cu concentrations tested.

- Total proteins content per cell increased in *C. pantanale* (90.9 ± 37.0 pg $cell^{-1}$) in 589.0 nM Cu^{2+}. However, *C. chlorastera* had the highest proteins content among all species, producing 270.2 ± 44.5 pg $cell^{-1}$ in 0.3 nM Cu^{2+}.

- An increase in Cu concentrations caused a corresponding increase in lipids content in *C. chlorastera* up to 1.2 nM Cu^{2+}, with the highest lipids (61.9 ± 14.3 % dw) being produced in the said Cu exposure.

- The microalgae antioxidant activity as measured by DPPH radical activity, was generally not altered by Cu. Similarly, their lipids quality was not compromised through oxidation in low Cu, but high Cu caused the peroxidation of the cellular lipids. Similarly, the microalgae cellular antioxidant enzymes activities (GST, POD, and SOD) were not affected by environmentally significant Cu exposure, they were increased only in high Cu.

- The microalgae $\Delta F/F_m'$ showed a more sensitive response to Cu exposure than their F_v/F_m.

- In *Monoraphdium* sp., an increase in Cu (3.4–7.4 nM Cu^{2+}) stimulated an increase in photosynthesis, as recorded in the $\Delta F/F_m'$, $rETR_m$, E_k, qP, and qL.

- The NPQ progressively increased with Cu increase, while the antenna size increased only in high Cu.

- *Monoraphidium* sp. was the least inhibited species by high light exposure, as evidenced by its low β across all Cu exposures compared to the other species.

- The primary productivity of *Monoraphidium* sp. was unaltered by low Cu exposure (1.7–21.4 nM Cu^{2+}), but reduced in high Cu (589.0 nM Cu^{2+}).

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6.0 APPENDICES

Appendix 1 Total Cu concentration; free Cu^{2+} ions concentration and its dissolved %; % of Cu complexed by ligands in BG-11 medium (contains citrate and EDTA as chelating agents)

Total Cu (nM)	Cu^{2+} (nM)	Cu^{2+} % Conc.	Cu-citrate % Conc.	CuEDTA-2 % Conc.
80	1.7	2.106	94.985	1.109
120	2.5	2.108	94.982	1.109
160	3.4	2.109	94.978	1.109
180	3.8	2.110	94.976	1.110
350	7.4	2.116	94.962	1.112
1000	21.4	2.138	94.905	1.119
19600	589.0	3.007	92.823	1.375

Appendix 2 Total Cu concentration; free Cu^{2+} ions concentration and its dissolved %; % of Cu complexed by ligands in WC medium (contains only EDTA as chelating agent)

Total Cu (nM)	Cu^{2+} (nM)	Cu^{2+} % Conc.	CuEDTA-2 % Conc.
80	0.1	0.145	99.718
120	0.2	0.174	99.66
160	0.3	0.203	99.60
180	0.4	0.219	99.59
350	1.2	0.352	99.34
1000	9.1	0.913	98.38
19600	5478.0	27.95	50.72

Appendix 3 Chromatogram showing the β-carotene peak in the extract of *Chlamydomonas chlorastera*

Empower™ 3 SOFTWARE

Untitled

S A M P L E   I N F O R M A T I O N

Sample Name:	CCA1 re dil	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	vanessa_re_diluida
Vial:	31	Acq. Method Set:	carotenoides_MS
Injection #:	1	Processing Method	B_PM
Injection Volume:	20,00 ul	Channel Name:	2998 Ch1 450nm@1.2nm
Run Time:	60,0 Minutes	Proc. Chnl. Descr.:	2998 Ch1 450nm@1.2nm
Date Acquired:	21/10/2021 16:02:14 BRST		
Date Processed:	28/10/2021 17:45:56 BRST		

Auto-Scaled Chromatogram
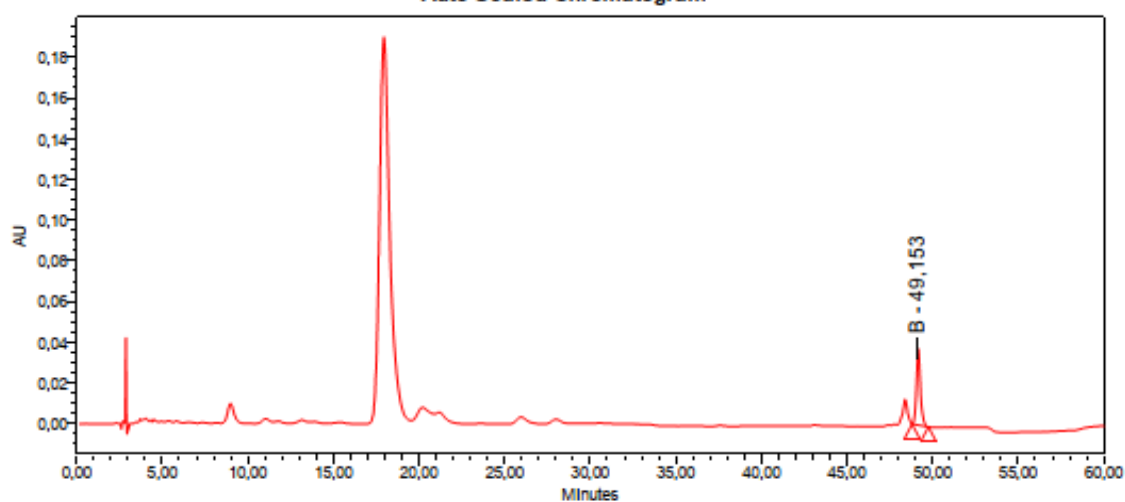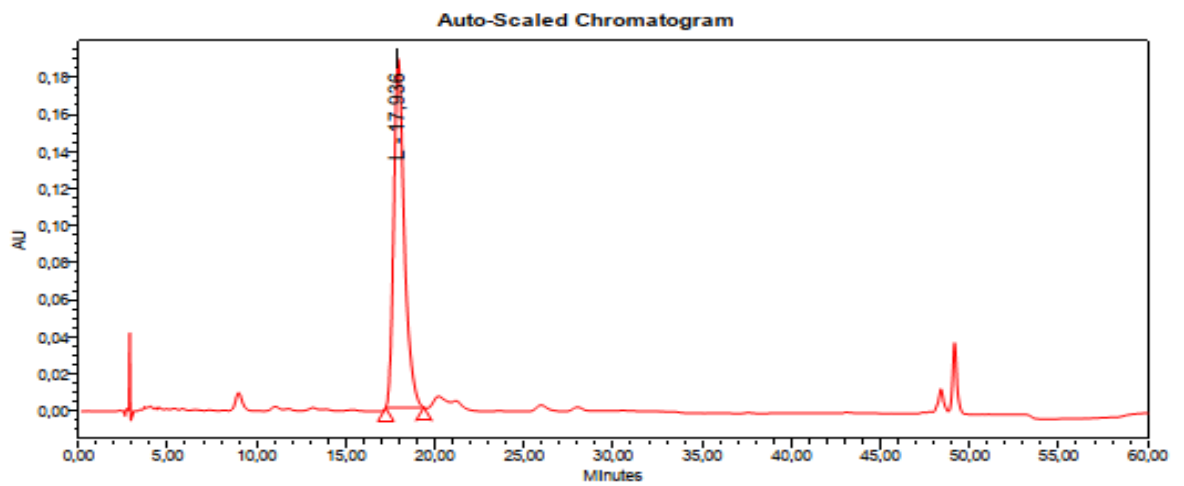
Peak Results

	Name	RT	Area	Height	Amount	Units
1	B	49,153	612923	37638	7,081	ug/mL

Reported by User: System
Report Method: Untitled
Report Method II 125
Page: 1 of 1

Project Name: NST C30\Vanessa_18102021
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Appendix 4 Chromatogram showing the lutein peak in the extract of *Chlamydomonas chlorastera*