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

LARISSA LUIZA DOS REIS

Toxicidade dos metais cádmio, cobalto e níquel, isolados e em combinações binárias, para a clorofícea *Raphidocelis subcapitata*

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Ecologia e Recursos Naturais do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências, área de concentração em Ecologia e Recursos Naturais

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Folha de Aprovação

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Resumo

Nos ecossistemas aquáticos, os organismos estão expostos a diversos compostos capazes de interagir e gerar efeitos tóxicos. Os metais estão naturalmente presentes nesses ambientes e alguns são essenciais ao metabolismo dos organismos. Entretanto, atividades antrópicas têm aumentado a concentração desses elementos, os quais são capazes de causar danos desde o primeiro nível trófico. No presente estudo foram avaliados os efeitos dos metais cádmio (Cd), cobalto (Co) e níquel (Ni), isolados e em combinações binárias, sobre a densidade, rendimento quântico máximo (Φ_M), eficiência do complexo de evolução do oxigênio (F_0/F_v), geração de espécies reativas de oxigênio (ERO), síntese de carboidratos, fluorescência da clorofila *a*, tamanho e complexidade celular da clorofícea de água doce *Raphidocelis subcapitata*. O Cd diminuiu a densidade celular, o Φ_M, aumentou a geração de ERO, F_0/F_v, a fluorescência da clorofila *a*, o tamanho e a complexidade celular. O Co diminuiu a densidade, a fluorescência da clorofila *a*, a síntese de carboidratos, e o Φ_M, por outro lado, aumentou ERO, F_0/F_v, tamanho, e a complexidade celular. O Ni causou alterações (aumento e diminuição) na densidade celular e na geração de ROS, diminuiu a síntese de carboidratos, e a fluorescência da clorofila *a*. Em relação aos parâmetros fotossintéticos, o Ni não alterou Φ_M e causou o aumento de F_0/F_v apenas em 0.50 mg L^{-1}. A mistura de Cd-Co causou efeitos de sinergismo em altas doses de Co e baixas doses de Cd; e antagonismo em baixas concentrações de Co e altas de Cd. O modelo que melhor se ajustou aos dados foi CA (adição de concentração) com desvio DR (dependente da proporção da dose). Além disso, a mistura causou efeitos antagônicos ao Φ_M e F_0/F_v. Já a mistura de Cd-Ni resultou em efeitos de antagonismo em baixas doses e sinergismo em altas, com mudança do nível de dose superior a IC_{50}. O modelo IA (ação independente) com desvio DL (dependente do nível da dose) foi o que melhor explicou os dados. Os parâmetros fotossintéticos não foram alterados pela mistura dos metais, porém, houve aumento do tamanho e da complexidade celular, bem como alterações na geração de ERO e fluorescência da clorofila *a*. E a mistura de Co-Ni resultou em efeitos de sinergismo em baixas doses de Co e altas de Ni e antagonismo em altas doses de Co e baixas de Ni, os dados melhor se ajustaram ao modelo CA com desvio DR, com alterações em todos os parâmetros analisados. Considerando que as microalgas são a base das teias alimentares aquáticas e os metais podem ser bioacumulados e biomagnificados, os danos causados podem atingir níveis tróficos superiores e impactar o ecossistema como um todo. Portanto, conclui-se que as misturas dos metais causaram toxicidade às microalgas, evidenciando a importância da avaliação dos estudos de misturas, seus efeitos e mecanismos de toxicidade sobre os organismos, a fim de proteger a biodiversidade do ecossistema aquático.

Palavras-chave: Ecotoxicologia, Phyto-PAM, citômetro, metais, misturas, sinergismo, antagonismo.

Abstract

In aquatic ecosystems, organisms are exposed to several compounds capable of interacting and generating toxic effects. Metals are naturally present in these environments and some are essential to the metabolism of organisms. However, anthropic activities have increased the concentration of these elements, which are capable of causing damage from the first trophic level. In the present study, the effects of the metals cadmium (Cd), cobalt (Co) and nickel (Ni), isolated and in binary combinations, on density, maximum quantum yield (Φ_M), efficiency of the oxygen evolution complex (F_0 /F_v), generation of reactive oxygen species (ROS), carbohydrate synthesis, chlorophyll *a* fluorescence, cell size and complexity of the freshwater chlorophyte *Raphidocelis subcapitata*. Cd decreased cell density, Φ_M, increased ROS generation, F_0/F_v, chlorophyll *a* fluorescence, size and cell complexity. Co decreased density, chlorophyll *a* fluorescence, carbohydrate synthesis, and Φ_M and, on the other hand, increased ROS, F_0/F_v, size and the cellular complexity. Ni caused changes (increase and decrease) in cell density and in ROS generation, decreased carbohydrate synthesis, and chlorophyll *a* fluorescence. Regarding the photosynthetic parameters, Ni did not alter Φ_M and caused an increase in F_0/F_v only at 0.50 mg L^{-1}. The Cd-Co mixture caused synergistic effects at high doses of Co and low doses of Cd; and antagonism at low Co and high Cd concentrations. The model that best fitted the data was CA (concentration addition) with DR deviation (dependent on the dose ratio). Furthermore, the mixture caused antagonistic effects to Φ_M and F_0/F_v. The Cd-Ni mixture, on the other hand, resulted in antagonism effects at low doses and synergism at high doses, with a dose level change greater than IC_{50}. The IA model (independent action) with DL deviation (dose level dependent) best explained the data. Photosynthetic parameters were not altered by the mixture of metals, however, there was an increase in cell size and complexity, as well as changes in ROS generation and chlorophyll *a* fluorescence. And the Co-Ni mixture resulted in synergistic effects at low Co and high Ni doses and antagonism at high Co and low Ni doses, the data better fit the CA model with DR deviation, with changes in all analyzed parameters. Considering that microalgae are the basis of aquatic food webs and metals can be bioaccumulated and biomagnified, the damage caused can reach higher trophic levels and impact the ecosystem. Therefore, it is concluded that the metal mixtures caused toxicity to the microalgae, highlighting the importance of evaluating the studies of mixtures, their effects and mechanisms of toxicity on the organisms, in order to protect the biodiversity of the aquatic ecosystem.

Keywords: Ecotoxicology, Phyto-PAM, cytometer, metals, mixtures, synergism, antagonism.

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Effects of cadmium and cobalt mixtures on growth and photosynthesis of *Raphidocelis subcapitata* (Chlorophyceae)

dependent) deviation. Linear, concave and convex isoboles represent no interaction, synergy and antagonism, respectively.

Capítulo 2.

Effects of cadmium and nickel mixtures on multiple endpoints to the microalga *Raphidocelis subcapitata*

Figure 1. Cell density of *R. subcapitata* ((ln(cells mL^{-1})) exposed to Cd (A) and Ni (B) during 96h. The asterisks indicate significant difference to the control group ($p < 0.05$). Metal concentrations are expressed in mg L^{-1}; C = control group.

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Capítulo 3.

Isolated and combined effects of cobalt and nickel on the algae *Raphidocelis subcapitata*

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Figure. 4. Cellular reactive oxygen species (ROS) produced by *R. subcapitata* exposed to Co (Co1-Co4) and Ni (Ni1-Ni4), isolated and combined (M1-M23), at 96h. Asterisks represent significant differences ($p < 0.05$) from the control group (C). Metal concentrations are described in Table 1.

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Lista de abreviaturas e siglas

Φ_M – Rendimento quântico máximo de algas adaptadas ao escuro
Al – Alumínio
ANOVA – Análise de variância
As – Arsênio
CA – Adição de concentração
Cd – Cádmio
CEO – Complexo de evolução do oxigênio
Cel – Célula
$CdCl_2$ – Cloreto de cádmio
$CoCl_2$ – Cloreto de cobalto
Co – Cobalto
Co-Mb – Cobalto-Molibdênio
Cr – Cromo
Cu – Cobre
DL – Desvio dependendo do nível da dose
DR – Desvio dependente da proporção da dose
ERO – Espécies reativas de oxigênio
EUA – Estados Unidos da América
FAO – Organização Alimentar e Agrícola da Nações Unidas
Fe – Ferro
F_0 – Fluorescência inicial de algas adaptadas ao escuro
F_v – Fluorescência variável
F_m – Fluorescência máxima de algas adaptadas ao escuro
F_0/F_v – Eficiência do complexo de evolução do oxigênio
FSC-H – Tamanho celular (dispersão frontal)
FL3-H – Fluorescência da clorofila *a* (fluorescência vermelha)
Hg – Mercúrio
IA – Ação independente
IC_{50} – Concentração de inibição média
Ni – Níquel

OMS – Organização Mundial da Saúde

PAM – Pulso de amplitude modulada

Pb – Chumbo

PSI – Fotossistema I

PSII – Fotossistema II

SSC-H – Complexidade celular (dispersão lateral)

USEPA – Agência de Proteção Ambiental dos Estados Unidos

Zn – Zinco

Sumário

Estruturação da tese

Esta tese foi redigida e organizada em capítulos, os quais foram divididos em resumo, introdução, material e métodos, resultados e discussão, conclusões e referências bibliográficas, de acordo com as normas de formatação dos periódicos das editoras Elsevier (capítulo 1), Springer (capítulo 2) e Wiley (capítulo 3) às quais os capítulos foram submetidos para publicação. Os capítulos tratam dos efeitos da mistura de diferentes metais (Cd-Co, Cd-Ni e Co-Ni) sobre múltiplos *endpoints* de uma clorofícea de água doce, *Raphidocelis subcapitata*, e foram ordenados da seguinte forma:

Capítulo 1 – “Effects of cadmium and cobalt mixtures on growth and photosynthesis of *Raphidocelis subcapitata* (Chlorophyceae)”. Neste capítulo, avaliou-se o crescimento, parâmetros fisiológicos (fluorescência da clorofila *a*) e morfológicos (tamanho e complexidade celular), além da atividade fotossintética (rendimento máximo (Φ_M) e eficiência do complexo de evolução do oxigênio (F_0/F_v)) de *Raphidocelis subcapitata* exposta aos metais cádmio (Cd) e cobalto (Co), em mistura. A espécie *R. subcapitata* é sensível a esses metais, e por estarem na base das cadeias alimentares aquáticas, os danos causados a essas microalgas possivelmente podem atingir níveis tróficos superiores. Neste capítulo, foram discutidos os efeitos da mistura dos metais Cd-Co, que podem divergir dos efeitos isolados, reforçando a importância dos estudos com misturas.

Capítulo 2 – “Effects of cadmium and nickel mixtures on multiple endpoints to the microalga *Raphidocelis subcapitata”*. O objetivo deste estudo foi analisar os efeitos dos metais cádmio (Cd) e níquel (Ni), isolados e combinados, sobre diversos parâmetros da clorofícea *Raphidocelis subcapitata*. Os testes ecotoxicológicos avaliaram o crescimento, a complexidade e o tamanho celular, a fluorescência da clorofila *a*, a produção de espécies reativas de oxigênio (ERO) e o desempenho fotossintético através do Φ_M e F_0/F_v. A partir dos resultados obtidos, os efeitos isolados e combinados de Cd e Ni foram discutidos, destacando-se a importância das análises de misturas de metais, já que os efeitos podem ser diferentes (reduzidos ou potencializados).

Capítulo 3 – "Isolated and combined effects of cobalt and nickel on the algae *Raphidocelis subcapitata*". Neste estudo, foram avaliados os efeitos dos metais cobalto (Co) e níquel (Ni), isolados e combinados, sobre a microalga *Raphidocelis subcapitata*. Foram avaliados os parâmetros: crescimento, complexidade e tamanho celular, fluorescência da clorofila *a*, produção de ERO, síntese de carboidratos e a atividade fotossintética (Φ_M e F_0/F_v).

Ao final, baseando-se nestes três capítulos, foram elaboradas as conclusões gerais e as considerações finais da tese.

1. Introdução e justificativas

1.1. Metais e sua toxicidade

Os metais são definidos quimicamente como: “elementos que conduzem eletricidade, têm brilho metálico, são maleáveis e dúcteis, formam cátions e possuem óxidos básicos”. São compostos que ocorrem naturalmente no ambiente tendo como fontes naturais o intemperismo de rochas e erupções vulcânicas; por outro lado, as principais fontes antropogênicas são mineração, fundição, emissão industrial, atividade agrícola (pesticidas e fertilizantes fosfatados), e a queima de combustíveis fósseis que libera metais (ex. cádmio) no ambiente. A urbanização e industrialização tem contaminado o meio ambiente com metais, sendo que os mais comuns são: cromo (Cr), manganês (Mn), níquel (Ni), cobre (Cu), zinco (Zn), cádmio (Cd) e chumbo (Pb), sendo assunto de grande preocupação (Ali et al., 2019).

A poluição do meio ambiente por metais é um problema global. O ecossistema aquático é atingido por uma combinação de compostos tóxicos: antibióticos, cosméticos, hidrocarbonetos, pesticidas, inclusive metais (Verma et al., 2021). Kumar et al. (2019) realizaram uma avaliação global sobre o conteúdo de metais em águas superficiais e de acordo com as diretrizes da OMS (2017) e da USEPA (2009) apontaram os países e os metais que excederam os limites estabelecidos pelas agências reguladoras. Na China, os metais Fe, Mn, Co e Ni superaram os limites; na Índia os metais Fe, As, Cr, Mn, Ni e Cd extrapolaram os valores recomendados. Na Grécia as concentrações de Hg superaram os limites estabelecidos; na África do Sul, os metais que ultrapassaram os limites foram Cr e Ni. No Irã foram Ni e Cr; na Turquia o Ni extrapolou as diretrizes; nos EUA Ni, Cd e Cr superaram os valores de referência, no Paquistão os valores de Mn, Co, Cu, Ni, Cd, Cr e As excederam o limite; na Alemanha Mn e Ni excederam o valor recomendado. Os metais Co, Ni, Cr e As em Bangladesh e Nigéria ultrapassaram os valores mais altos recomendados pelas agências reguladoras.

No Brasil, em 2015, a ruptura de uma barragem na cidade de Mariana-MG lançou rejeitos de mineração por uma extensão de 663,2 km do Rio Doce (de Carvalho et al., 2017; Fernandes et al., 2016). Em 2019, na cidade de Brumadinho-MG, o rompimento de outra barragem descarregou rejeitos por aproximadamente 12 milhões de m^3 no Rio Paraopeba e imediações (Thompson et al., 2020). Consequentemente, cresce a preocupação da comunidade científica em ampliar o conhecimento da toxicidade de misturas metálicas, uma vez que, atualmente, a avaliação da toxicidade de metais sobre os organismos expostos aos mesmos e os procedimentos de análise de risco são, em grande parte, baseados na toxicidade de metais isolados (Nys et al., 2017).

Metais podem ser uma ameaça para ecossistemas aquáticos e terrestres. Eles são persistentes no ambiente, pois não são biodegradáveis, se acumulam nos organismos, podendo contaminar cadeias alimentares, o que conhecemos por biomagnificação, além de lixiviar para águas subterrâneas. Por outro lado, alguns metais são importantes ao metabolismo dos organismos com funções biológicas e bioquímicas muito importantes. Eles podem ser classificados como essenciais e não essenciais, sendo que deficiências ou excessos desses metais, podem ocasionar distúrbios no metabolismo, e consequentemente, doenças. Além disso, é importante ressaltar que os metais podem ser essenciais ou inócuos ao desenvolvimento dos seres vivos, dependendo do grupo de organismos, animais, plantas ou microrganismos (Ali et al., 2019; Lu et al., 2021).

A toxicidade dos metais varia de acordo com sua biodisponibilidade, a qual depende da especiação química, que por sua vez, no ambiente aquático, é influenciada pela condutividade, pH e concentração de outros compostos dissolvidos (Expósito et al., 2017). Metais no ecossistema aquático podem ter consequências danosas aos organismos contribuindo para o declínio populacional, além de que quase todos os metais podem ser tóxicos à biota, tudo depende da sua concentração e do tempo de exposição. Em algumas espécies, como os peixes, os metais são potenciais neurotoxinas e a exposição desses organismos aos metais é também capaz de causar deformidades, afetando sua sobrevivência e crescimento (Ali et al., 2019).

Metais como Cd, Co, Ni, entre outros (As, Cr, Mn) tiveram concentrações acima daquelas permitidas pela USEPA (2009) e pela OMS (Organização mundial da saúde) para água potável (Kumar et al., 2019), portanto, os metais escolhidos para serem avaliados neste estudo foram o cádmio (Cd), o cobalto (Co) e o níquel (Ni) devido ao seu uso, características e ocorrência no ambiente.

1.1.1. Cádmio

O Cd é um metal não essencial ao metabolismo dos organismos e é altamente tóxico a plantas, animais e seres humanos. É considerado um dos principais poluentes metálicos por sua elevada toxicidade e ampla distribuição nos ecossistemas aquáticos

(Wang et al., 2013). Este metal está presente na natureza geralmente como compostos de sulfeto de zinco, sendo utilizado em ligas, revestimentos, estabilizadores para plástico, mas a grande maioria é utilizada na produção de baterias. Devido à sua ampla comercialização e uso atividades antrópicas (produção industrial e agrícola), os níveis de Cd aumentaram no ambiente e no ecossistema aquático, consequentemente é importante entender suas rotas de exposição e toxicidade, uma vez que pode ser bioacumulado facilmente na cadeia alimentar (Zhang e Reynolds, 2019). Como em ambientes aquáticos o Cd é absorvido pelos organismos na forma iônica, sua toxicidade à biota está ligada diretamente à concentração de íons livres. O Cd^{2+} solúvel, por exemplo, induz mais danos que o Cd metálico e o pó de óxido de Cd (Sharma and Sachdeva, 2015).

Os organismos fotossintéticos são conhecidos por serem extremamente sensíveis aos íons Cd, o qual pode alterar a atividade do fotossistema II (PSII), do complexo de evolução do oxigênio e a respiração (Wang et al., 2013). Nas plantas, especificamente, vários processos fisiológicos são inibidos pelo Cd, tais como o metabolismo do nitrogênio, a síntese de clorofila e o transporte de nutrientes minerais (Sharma and Sachdeva, 2015). Alho et al. (2019) relataram alterações nos parâmetros fotossintéticos ao exporem a microalga *Raphidocelis subcapitata* ao Cd, que diminuiu o rendimento quântico máximo (Φ_M), influenciou de maneira negativa a eficiência do complexo de evolução do oxigênio (CEO), reforçando a capacidade do Cd em substituir o manganês (Mn) nas reações químicas do CEO, que pode interferir na transferência de elétrons, diminuindo o Φ_M.

1.1.2. Cobalto

O Co é um metal essencial aos seres vivos, pois é necessário para a síntese de vitamina B12. Distribuído por todo o planeta, é encontrado em solos, rochas, vegetação e água, além de ser comumente encontrado associado ao Ni. Além disso, pode ser encontrado em altas concentrações em águas residuais industriais, próximo a instalações de mineração de Co e escoamento de fertilizantes utilizados na agricultura (Pourkhabbaz et al., 2011; Stubblefield et al., 2020). O Co ocorre naturalmente nos ecossistemas aquáticos em baixas concentrações, em ambientes não contaminados a concentração deste metal (total e dissolvido) é menor que 0.005 mg L^{-1} (Nagpal, 2004), entretanto atividades antrópicas têm aumentado o nível deste metal nos ecossistemas aquáticos, provocando efeitos deletérios (Sridhar et al., 2020).

Os usos do Co são bastante diversos, sendo utilizado como pigmento catalisador na indústria de petróleo, nutriente em fertilizantes, aplicações industriais e tecnológicas como componente que melhora o desempenho de baterias recarregáveis e ligas magnéticas, além de ser componente em ligas resistentes a temperaturas elevadas para motores a jato (Stubblefield et al., 2020). O metal também é aplicado como pigmento na produção de vidro azul, tintas, na indústria de aço, cimento, na produção de ligas com estabilidade a altas temperaturas e resistentes ao desgaste e corrosão. Compostos com Co, como óxido de lítio cobalto, são usados em eletrodos de baterias e em baterias de Ni-Cd, que contém cobalto; composto de organocobalto tem função importante como catalisadores de oxidação e o Co-Mb é empregado como catalisador na remoção de impurezas sulfurosas do petróleo que dificultam o refino de combustíveis (Blust, 2011; Sridhar et al., 2020).

O Co está ligado a processos fisiológicos das células e tem função importante na regulação da síntese do DNA, ácidos graxos e metabolismo energético (Blust 2011). Por outro lado, concentrações elevados do metal podem ser tóxicas aos humanos (acima de 0.1 µg de Co por dia – recomendação da OMS e FAO (Organização Alimentar e Agrícola da Nações Unidas), animais (aves e mamíferos – 7.61 mg kg^{-1}, peso corporal) e plantas (ex. Hordeum vulgare EC_{20}=40 mg kg^{-1}), podendo levar à inibição da respiração celular e enzimas do ciclo do ácido cítrico (Gál et al., 2008; Pourkhabbaz et al., 2011). Efeitos tóxicos também já foram registrados em microalgas, como danos à atividade fotossintética, diminuição da síntese de clorofila (*a* e *b*), oxidação de proteínas, produção de ERO e danos ao DNA (El-Sheekh et al., 2003; Novak et al., 2013).

1.1.3. Níquel

O Ni é um metal de transição que ocorre na crosta terrestre geralmente associado ao oxigênio e enxofre como óxidos e sulfetos. É bem distribuído no ambiente por fontes naturais e atividades antrópicas. No solo, pode ser encontrado combinado com outros elementos, em meteoritos e emissão de vulcões; no ar através da combustão de combustíveis fósseis; na água pela lixiviação direta de rochas e sedimentos. Em fontes antropogênicas, é emitido como compostos solúveis, óxidos, sulfetos, e níquel metálico (menor teor) (Genchi et al., 2020).

O Ni é amplamente utilizado em atividades antrópicas devido a suas propriedades físico-químicas, baixo preço e brilho. O metal é resistente a corrosão, altas temperaturas, oxidação, além de ser reciclável, dúctil, e que se liga facilmente. O Ni é utilizado em metalurgia, como produção de ligas, galvanoplastia, produção de baterias de níquel-cádmio e como catalisador na indústria química alimentícia. É empregado na fabricação de chaves, clipes de papel, joias baratas, fechos de roupa (zíper, botão de pressão, fivela de cinto), equipamentos elétricos, moedas, utensílios domésticos de aço inoxidável, armamentos, ligas para indústrias metalúrgicas e de processamento de alimentos, pigmentos e catalisadores. Consequentemente, o amplo emprego do metal e a má gestão dos resíduos faz com que o metal seja levado ao ambiente causando poluição (García-García et al., 2018; Genchi et al., 2020).

Altos níveis de Ni no ambiente podem causar efeitos danosos aos organismos, afetando diversos *endpoints* em diferentes níveis tróficos nos ecossistemas aquáticos. Entre os táxons de água doce mais sensíveis ao Ni estão algas unicelulares, gastrópodes, cladóceros, insetos, anfíbios e peixes (menos sensíveis) (Wang et al., 2020). Este metal pode interferir em disfunções mitocondriais e no estresse oxidativo. As disfunções podem ocorrer pelo comprometimento do potencial de membrana da mitocôndria, redução da concentração de ATP e destruição do DNA mitocondrial. Então, esse processo pode interferir na cadeia respiratória de elétrons, aumentando ERO (Genchi et al., 2020).

1.2. Misturas tóxicas no ambiente - referências bibliográficas sobre as misturas

Em ambientes poluídos, os seres vivos estão expostos a uma mistura de diversos compostos químicos, os quais são capazes de causar toxicidade aos organismos mesmo que estejam presentes em concentrações mínimas, abaixo daquelas que causam efeito, o que é bastante preocupante, uma vez que mesmo essas baixas concentrações podem contribuir para toxicidade dos compostos em mistura (Beyer et al., 2014; Tang et al., 2013). Essas misturas complexas, proveniente de atividades industriais, agrícolas e urbanas, podem atingir a água, o solo e os sedimentos (Ferreira et al., 2008). As interações entre produtos químicos nos ecossistemas podem afetar processos biológicos, tais como excreção, metabolismo, transporte interno, absorção, ligações e biodisponibilidade (comum em combinações metálicas), que são importantes para analisar a toxicidade final dos compostos (Cedergreen, 2014).

A adição de concentração (CA) (Loewe, 1926) e a ação independente (IA) (Bliss, 1939) são modelos teóricos propostos com a finalidade de analisar a toxicidade das misturas para os seres vivos. O primeiro modelo supõe que os produtos químicos individuais tenham o mesmo modo de ação sobre um alvo biológico, contribuindo para uma resposta proporcional às suas respectivas toxicidades; enquanto o segundo supõe que os produtos químicos tenham modos de ação diferentes e, com isso, seus efeitos são estatisticamente independentes. Caso o modo de ação do químico não seja conhecido, ambos os modelos (CA e IA) são aplicados e aquele que melhor se ajustar aos dados é utilizado (Beyer et al., 2014; Mansano et al., 2017; Pavlaki et al., 2011).

Ainda que os modos de ação dos produtos químicos isolados sejam conhecidos, eles podem interagir dentro dos organismos. Com isso, tem-se diferentes desvios para ambos os modelos de referência dependendo da interação dos compostos. Por exemplo, o efeito pode ser maior que o aditivo, denominado sinergismo, ou menor que o aditivo, chamado antagonismo. Além disso, o efeito pode ser dependente da proporção da dose (DR – *dose ratio*), ou seja, a toxicidade é causada principalmente por um dos compostos químicos e depende da composição da mistura; ou dependente do nível da dose (DL – *dose level*), que varia de acordo com a concentração dos produtos químicos, ou seja, a toxicidade pode ser diferente em doses altas ou baixas dos compostos (Loureiro et al., 2010).

Os desvios podem ser identificados utilizando a ferramenta MIXTOX (Jonker et al., 2005). Essa ferramenta analisa qual modelo melhor se ajusta aos dados, CA ou IA, além de testar significativamente qual desvio (sinergismo, antagonismo, DR ou DL) melhor descreve os dados obtidos por meio de um conjunto de funções em uma estrutura aninhada (Jonker et al., 2005; Loureiro et al., 2010). Os desvios podem ser de natureza química, física e/ou biológica. Além disso, a interação entre os compostos pode ocorrer na fase toxicocinética (absorção, metabolismo, distribuição e excreção) ou na fase toxicodinâmica (efeitos do químico nos órgãos, sítios celulares ou receptores) (Ferreira et al., 2008).

A representação gráfica utilizada para ilustrar as interações dos compostos, de doses isoladas e combinadas, causando X% de efeito (e.g. X = 50%, CE_{50}), é chamada de isobolograma, onde os isoboles indicam aditividade, sinergismo e antagonismo (Figura 1). No eixo X, estão representadas as doses do composto A, enquanto que no eixo Y, as doses do composto B, sendo que cada ponto indica um par de doses que atinge a CE_{50} quando combinados. Como apresentado na figura 1, as CE_{50} obtidas através da aplicação isolada de cada produto são unidas, originando a isobole de aditividade. Com isso, os demais valores obtidos da combinação de diferentes concentrações dos produtos podem ser analisados em relação à posição do isobole de aditividade. Logo, tem-se uma ação de aditividade se os pontos estiverem em torno do isobole de aditividade; sinergismo se os pontos estiverem abaixo da linha e antagonismo se os pontos estiverem acima. Portanto, isoboles lineares representam não interação, isoboles côncavos representam sinergismo e convexos, antagonismo (Kruse et al., 2006; Ryall and Tan, 2015).

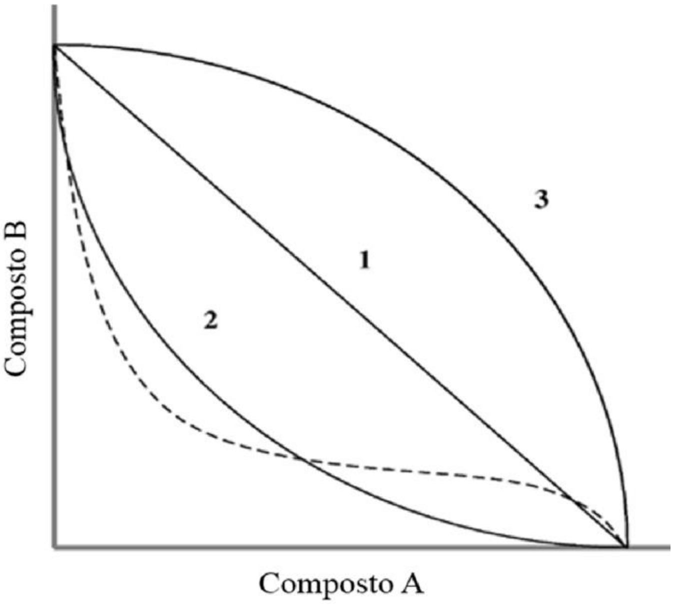

Figura 1. Isobolograma representando as interações entre os compostos A e B. A linha 1 representa ausência de interação, a curva 2 representa sinergismo e a curva 3 representa antagonismo. Uma isobole enviesada está representada pela linha pontilhada.

Diversos estudos investigaram as interações entre metais e diferentes espécies de microalgas, observando interações sinérgicas e antagônicas. Expósito et al. (2017) observaram que a combinação dos metais As (3.5 µg L^{-1}), Cu (5 µg L^{-1}), Ni (2.7 µg L^{-1}), Pb (2 µg L^{-1}) e Zn (21 µg L^{-1}) interagiu de modo antagônico sobre a clorofícea *Raphidocelis subcapitata*. Koppel et al. (2018) observaram interações antagônicas dos metais Cd, Cu, Ni, Pb, Zn sobre *Phaeocystis antarctica*, e interações sinérgicas (em altas concentrações) e antagônicas (em baixas concentrações) sobre *Cryothecomonas armigera*, sendo que a interação era dependente da concentração dos metais. Gebara et al. (2020) investigaram os efeitos dos metais Zn e alumínio sobre *R. subcapitata*, observando que o modelo CA e o desvio DL melhor se ajustaram aos dados obtidos, mostrando interações sinérgicas em baixas concentrações e antagônicas em altas concentrações. Alho et al. (2022) observou que a mistura dos metais Mn e Cr foi não interativa, ou seja, o efeito dos metais sobre *R. subcapitata* foi aditivo. Gebara et al. (2023) avaliou o desempenho do fotossistema II de *R. subcapitata* exposta a combinação de Zn e Al, observando o predomínio de interações antagônicas em altas concentrações dos metais e em alguns casos, interações sinérgicas (em baixas concentrações).

1.3. Ecotoxicologia e organismo-teste

O crescimento da produção e o uso de produtos químicos na medicina, agricultura e indústria acompanha o aumento dos estudos ecotoxicológicos nas últimas décadas, devido aos altos níveis de contaminantes que são liberados nos ecossistemas aquáticos. Com isso, análises sobre monitoramento da qualidade da água, bem como o estado de saúde ambiental são de grande relevância e os estudos ecotoxicológicos auxiliam na avaliação dos efeitos desses contaminantes ao meio ambiente e a saúde humana (Ceschin et al., 2021), adotando-se alguns organismos como modelos em testes de toxicidade. Na Ecotoxicologia aquática, geralmente são utilizados organismos de três níveis tróficos como, por exemplo as microalgas (produtores primários - base das cadeias tróficas), zooplâncton (consumidores – elo intermediário) e os peixes (consumidores – nível trófico superior) sendo que os testes ecotoxicológicos avaliam a toxicidade crônica e aguda dos compostos, dependendo do tempo de exposição e da concentração do químico (Expósito et al., 2017).

As microalgas são produtores primários capazes de transformar, através da fotossíntese, energia luminosa em energia química, produzindo biomassa e liberando oxigênio, compondo parte importante da biota aquática (Farrelly et al., 2013; Martínez-Ruiz and Martínez-Jerónimo, 2015). Com isso, compondo a base das cadeias alimentares de herbivoria, esses organismos são fonte de alimento ao zooplâncton que, por sua vez, servem de alimento para os peixes. Consequentemente, qualquer dano sofrido pelas microalgas pode afetar organismos de níveis tróficos superiores (Martínez-Ruiz and Martínez-Jerónimo, 2015). Além disso, microalgas são organismos de crescimento rápido e fácil cultivo, altamente sensíveis aos metais, sendo comumente usadas para avaliação dos efeitos desses compostos no ambiente aquático (Rodgher et al., 2012).

A espécie utilizada neste estudo, a clorofícea *Raphidocelis subcapitata*, conhecida anteriormente por *Pseudokirchneriella subcapitata* ou *Selenastrum capricornutum*, pertence à família Selenastraceae, ordem Sphaeropleales. Essa espécie é muito utilizada como modelo em estudos de toxicidade, uma vez que apresenta algumas vantagens, tais como sensibilidade a tóxicos, facilidade de cultivo, alta disponibilidade e relevância ecológica (Machado et al., 2015; Suzuki et al., 2018), características que favorecem o uso dessa clorofícea como indicador confiável de toxicidade (Machado and Soares, 2014).

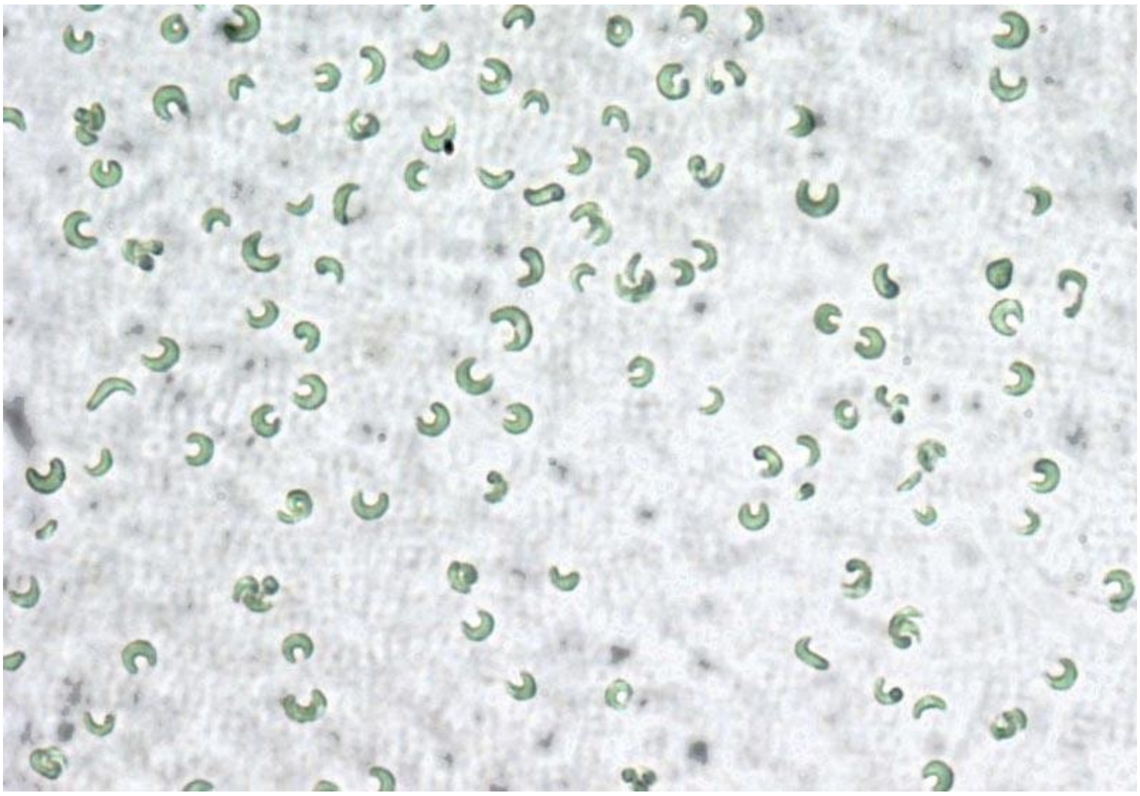

Figura 2. Clorofícea *Raphidocelis subcapitata* (Biotox Ambiental – Serviços - https://www.biotoxambiental.com.br/servicos/)

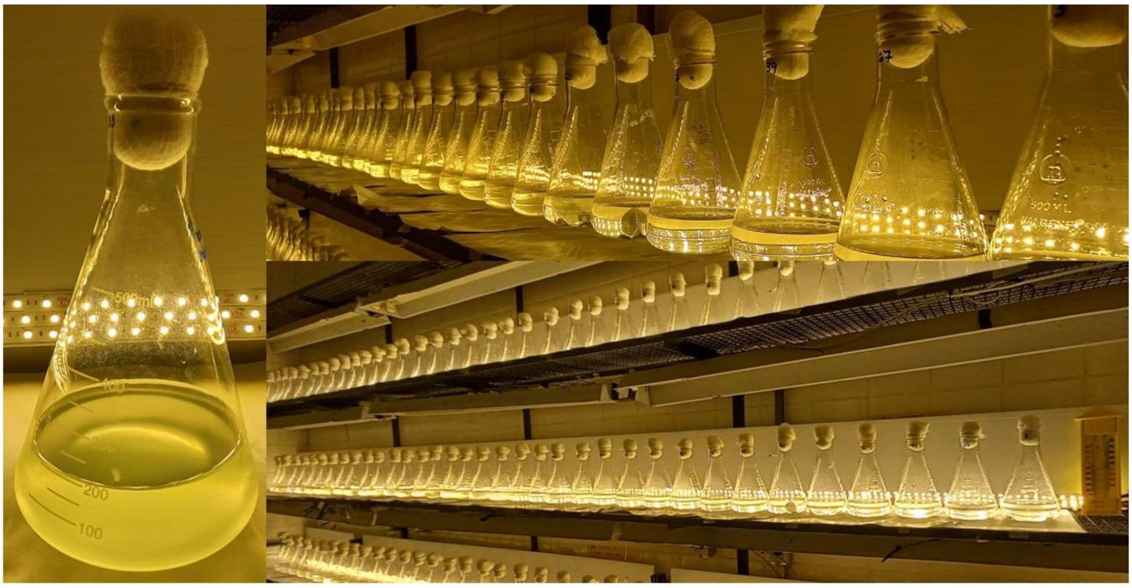

Figura 3. Cultivo da microalga *Raphidocelis subcapitata* e teste ecotoxicológico. Fonte: do autor.

1.4. Múltiplos *endpoints*

1.4.1. Parâmetros morfológicos e fisiológico

A presença de compostos tóxicos no ambiente faz com que cresça cada vez mais a necessidade de uma melhor compreensão sobre os mecanismos celulares e subcelulares de toxicidade. Com isso, a citometria de fluxo é uma ferramenta vantajosa, pois inclui a possibilidade de análise de multiparâmetros e análise rápida, superando limitações de técnicas tradicionais (Wlodkowic et al., 2022). Em relação às microalgas, a citometria de fluxo é capaz de analisar a fluorescência da clorofila *a*, o tamanho e a complexidade celular (Moreira et al., 2020). A análise desses parâmetros pode ser relacionada com a eficiência do processo fotossintético (no caso da fluorescência da clorofila *a*), com problemas no processo de divisão celular (no caso do tamanho celular) e com a internalização dos compostos por parte das microalgas (em relação a complexidade celular) (Gebara et al., 2020).

1.4.2. Espécies reativas de oxigênio (ERO)

As espécies reativas de oxigênio (ERO) são pró-oxidantes capazes de causar estresse oxidativo, sendo que as espécies mais comuns em organismos aeróbios são singleto de oxigênio ($1O_2$), radical hidroxila (OH), peróxido de hidrogênio (H_2O_2), ânion superóxido (O_2^-), e radicais livres (Gauthier et al., 2020). O estresse oxidativo é bastante utilizado para monitoramento da qualidade ambiental. O metabolismo celular (processos de respiração, fotossíntese - fluxo de elétrons) formam naturalmente radicais livres, como espécies reativas de oxigênio (ERO) e nitrogênio (ERN), que são utilizados em processos biológicos. Contudo, o constante fluxo de elétrons provenientes desses processos pode tornar as células dos organismos fotossintéticos suscetíveis a danos oxidativos. Com isso, ERO podem ser altamente prejudiciais aos organismos em elevadas concentrações, ocasionando danos ao DNA, lipídeos e proteínas (Pikula et al., 2019).

Nas microalgas, níveis elevados de ERO podem causar danos em ácidos nucleicos, lipídeos, proteínas e na sinalização da transdução, podendo levar a disfunção metabólica e morte celular. O sistema de defesa antioxidante das algas compreende a proteção enzimática e não enzimática, a fim de reduzir os danos causados por ERO, sabendo que os antioxidantes são diferentes entre as espécies de algas (Gauthier et al., 2020). Estudos anteriores registraram aumento de ERO ao expor diferentes espécies de microalgas a metais (isolados e combinados), evidenciando os danos causados por esses compostos (Alho et al., 2019; Gebara et al., 2020).

1.4.3. Carboidratos

Carboidratos são moléculas importantes para as microalgas, pois participam das reações fotossintéticas e respiratórias, além de funcionarem como armazenamento de energia e componente estrutural da parede celular (Martínez-Ruiz and Martínez-Jerónimo, 2015). Situações de estresse ambiental e alterações no cultivo podem ser fatores responsáveis por alterações na biomassa e na composição bioquímica, alterando a quantidade de carboidratos (Rocha et al., 2018; Rossi et al., 2018). A síntese desta molécula pode diminuir, indicando que ocorreu uma despolarização da membrana, causada pelo excesso de íons, que pode levar a outros danos como a deformação da parede celular ou alterações nas reações da fotossíntese (Martínez-Ruiz and Martínez-Jerónimo, 2015). Além disso, estudos anteriores já relataram alteração na síntese de carboidratos com diferentes espécies de microalgas expostas a diversos metais (Alho et al., 2019; Chia et al., 2015; Silva et al., 2018).

1.4.4. Parâmetros fotossintéticos

O sistema fotossintético é formado por dois fotossistemas, PSII e PSI. O PSII é composto por um complexo proteico que acelera a transferência de elétrons induzida pela luz, da água para a plastoquinona. Contam com mais de 25 polipeptídeos, um centro de reação (P680), além de um sistema "watersplitting", também chamado de complexo de evolução do oxigênio – CEO (Lombardi e Maldonado, 2011). A atividade fotossintética das microalgas pode ser inibida por metais. A análise dessa atividade pode ser realizada por um fluorímetro de pulso de amplitude modulada (PAM), que é eficiente na identificação dos efeitos da exposição de metais sobre microalgas, além de analisar múltiplos parâmetros rapidamente, fornecendo respostas de maneira simples, rápida e aprimorada (Expósito et al., 2021; Herlory et al., 2013). Tal equipamento fornece informações a respeito do rendimento quântico máximo (Φ_M), ou seja, a capacidade de células adaptadas ao escuro em converter energia luminosa em energia química, além de medir a fluorescência inicial (F_0) e a fluorescência variável (F_v), cuja razão (F_0/F_v) fornece informações sobre a eficiência do complexo de evolução de oxigênio (OEC) que indica o estado da reação de fotólise de água (Echeveste et al., 2017; Juneau et al., 2002). Estudos anteriores demonstraram que alguns metais têm capacidade de substituir o magnésio (átomo central da clorofila), causando queda da atividade fotossintética e, também, causar danos ao complexo de evolução do oxigênio (CEO), pela substituição do manganês no processo de foto-oxidação da água (Herlory et al., 2013; Juneau et al., 2002).

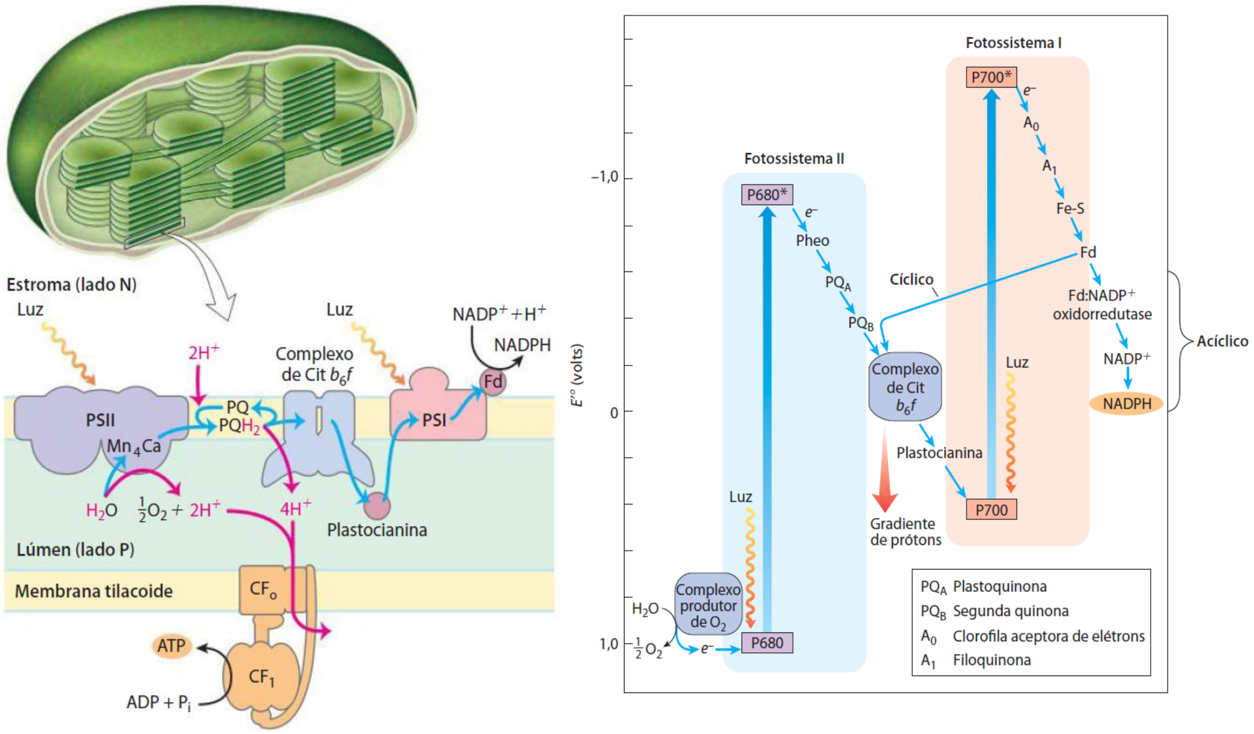

Figura 4. Diagrama geral da fotossíntese com esquema do circuito de prótons e elétrons (Nelson e Cox, 2014. Princípios de Bioquímica de Lehninger, 6 ed, pg 779).

1.5. Justificativas

Alguns metais são utilizados pelos organismos em processos biológicos essenciais. Por outro lado, diversos metais não têm função biológica conhecida e causam apenas efeitos tóxicos aos organismos. Além disso, metais classificados como essenciais ao desenvolvimento dos organismos também podem causar danos aos mesmos quando presentes em concentrações acima daquelas consideradas necessárias (Gauthier et al., 2014). No ambiente, os organismos estão expostos a uma grande variedade de metais, ainda que grande parte das avaliações de risco seja pautada nos efeitos desses compostos isolados (Nys et al., 2017).

A ocorrência de metais nos ecossistemas aquáticos é um problema de âmbito global. Países como África do Sul, Alemanha, Bangladesh, China, Estados Unidos, Grécia, Índia, Irã, Nigéria, Paquistão e Turquia, excederam os valores máximos permitidos a diversos metais em água potável, segundo órgãos de regulamentação como OMS (2017) e USEPA (2009) (Kumar et al., 2019). No Brasil, foram registrados dois grandes desastres ambientais que espalharam metais em diversos ecossistemas aquáticos e terrestres. Em 2015, ocorreu no estado de Minas Gerais, na cidade de Mariana, o rompimento da barragem do Fundão, pertencente a uma indústria de mineração. Os rejeitos de mineração foram lançados numa extensão de 663,2 km do Rio Doce (Fernandes et al., 2016; Quadra et al., 2019). O outro desastre aconteceu em 2019, com o rompimento de uma barragem na cidade de Brumadinho, que espalhou rejeitos de mineração em pelo menos 12 milhões de m^3 dentro e ao redor do Rio Paraopeba (Thompson et al., 2020).

No Brasil, os metais Cd, Co e Ni já foram quantificados concomitantemente em ecossistemas aquáticos em diferentes concentrações. No córrego de Água Boa (Dourados, Mato Grosso do Sul, Brasil) foram encontrados 0,033 mg L^{-1} de Cd; 0,253 mg L^{-1} de Co e 0,296 mg L^{-1} de Ni (Dourado et al., 2017); no Rio Paraopeba, após o desastre de Brumadinho, foram registrados 0,007 mg L^{-1} de Cd; 0,011 mg L^{-1} de Co e 0,014 mg L^{-1} de Ni (Thompson et al., 2020). O CONAMA (Conselho Nacional do Meio Ambiente) resolução n° 357 prevê que os limites desses metais para garantir a qualidade da água (classe 1, tabela 1, padrão 1) são 0,001 mg L^{-1} de Cd; 0,05 mg L^{-1} de Co, e 0,025 mg L^{-1} de Ni. Assim, sabendo que os metais coocorrem nos ecossistemas aquáticos, não são biodegradáveis e têm comportamento cumulativo (Zhao et al., 2017), é importante e necessário que os efeitos desses metais combinados sejam investigados, uma vez que esses compostos podem interagir, potencializando sua toxicidade, ainda que estejam presentes no ambiente em concentrações abaixo das que causam efeito visível (Beyer et al., 2014).

Este estudo tem como objetivo avaliar a toxicidade dos metais Cd, Co e Ni, isolados e combinados, sobre a densidade, parâmetros fotossintéticos (rendimento quântico máximo (Φ_M) e eficiência do complexo de evolução do oxigênio (F_0/F_v)), produção de espécies reativas de oxigênio (ERO) e síntese de carboidratos de uma clorofícea de água doce (*Raphidocelis subcapitata*). Os efeitos danosos causados a essas microalgas podem atingir toda a cadeia trófica, por biomagnificação, pois elas são produtoras primárias e compõem a base das cadeias alimentares aquáticas de herbivoria (Wan et al., 2021). Essa espécie é fácil de ser cultivada, apresenta rápido tempo de duplicação e sensibilidade a tóxicos (Machado et al., 2015), sendo um bom modelo para estudos ecotoxicológicos. Além disso, até onde sabemos, nosso trabalho é o primeiro a analisar os efeitos dos metais combinados sobre parâmetros além da densidade, como ROS, carboidratos totais e desempenho fotossintético.

2. Objetivos e hipóteses

2.1. Objetivo geral

Este estudo objetivou avaliar os efeitos dos metais cádmio, cobalto e níquel, isolados e em combinações binárias, sobre a densidade celular, parâmetros morfológicos (tamanho e complexidade celular), fisiológicos (fluorescência da clorofila *a*), síntese de ERO intracelular, carboidratos totais e atividade fotossintética (Φ_M e F_0/F_v) da clorofícea de água doce *Raphidocelis subcapitata*.

2.2. Objetivos específicos

- Avaliar os efeitos da mistura dos metais cádmio e cobalto sobre a densidade celular e parâmetros fotossintéticos (Φ_M e F_0/F_v) da microalga.

- Investigar os efeitos dos metais cádmio e níquel (isolados e combinados) sobre a densidade celular, fluorescência da clorofila *a*, tamanho e complexidade celular, produção de ERO e atividade fotossintética (Φ_M e F_0/F_v) de *R. subcapitata.*

- Analisar os efeitos dos metais cobalto e níquel (isolados e combinados) sobre a densidade celular, fluorescência da clorofila *a*, tamanho e complexidade celular, produção de ERO, síntese de carboidratos e atividade fotossintética (Φ_M e F_0/F_v) de *R. subcapitata.*

2.3. Hipóteses

- Os metais Cd, Ni e Co isolados causam efeitos deletérios sobre a densidade da alga *R. subcapitata*. O Cd em concentrações mais baixas, já que é um metal não essencial, o Co em concentrações mais elevadas (pois é um metal essencial) e o Ni provavelmente em concentrações intermediárias, porque é considerado metal essencial, mas não para a espécie de alga estudada.

- A exposição aos metais isolados (Cd, Co, Ni) causa estresse na microalga alterando os parâmetros analisados: aumentando produção de ERO, tamanho e complexidade celular (efeito observado na exposição a metais) e diminuindo rendimento máximo, F_0/F_v e fluorescência da clorofila *a*.

- A mistura dos metais Cd e Co resulta na potencialização dos efeitos tóxicos destes metais caracterizando efeitos sinérgicos sobre o crescimento e desempenho fotossintético de *R. subcapitata*; pois ambos causam efeitos deletérios quando expostos a isoladamente a diferentes espécies de microalgas.

- A mistura dos metais Cd/Ni e Co/Ni tem seus efeitos adversos potencializados sobre os parâmetros analisados: densidade celular, produção de ERO, fluorescência da clorofila a, tamanho e complexidade celular, síntese de carboidratos, Φ_M e F_0/F_v. Caracterizando a existência de sinergismo entre os metais.

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Capítulo 1. Effects of cadmium and cobalt mixtures on growth and photosynthesis of *Raphidocelis subcapitata* (Chlorophyceae)

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Abstract

Metals occur simultaneously in the environment, and therefore it is important to know their toxicity and mechanism of action when associated with another metal. Furthermore, anthropogenic actions increase their concentrations in the environment where they can interact and undergo transformations that can even increase their toxicity. This study aimed to evaluate the effects of cadmium (Cd) and cobalt (Co), isolated and combined, on the microalgae *Raphidocelis subcapitata*. Regarding the toxicity of isolated metals, the IC_{50}96 h was 0.08 mg L^{-1} of Cd and 0.16 mg L^{-1} of Co. Cell density decreased at all concentrations of the Cd tested. The parameters related to cell size, cell complexity and mean cell chlorophyll *a* (Chl *a*) fluorescence were significantly affected by both metals. According to species sensitivity curves (SSD), the microalgae *R. subcapitata* was the second most sensitive organism to Co exposure and the tenth concerning Cd. Metal mixture data were best fitted to the concentration addition (CA) model and dose-ratio dependence (DR) deviation, showing synergism at high concentrations of Co and low concentrations of Cd. Besides that, antagonism was observed at low concentrations of Co and high concentrations of Cd. Photosynthetic performance, assessed by maximum quantum yield (Φ_M) and oxygen evolving complex (OEC), presented antagonism effects for both analyzed parameters. Thus, the mixture of Cd and Co showed synergistic and antagonistic interactions for the parameters analyzed in *R. Subcapitata*, indicating the importance of understanding the mechanisms of toxicity of metal mixtures in phytoplankton.

Keywords: Metal toxicity, Combined effects, Synergism, Species sensitivity distribution, Ecotoxicology.

1. Introduction

Metals occur naturally in aquatic environments. However, the development of agriculture and industry has increased the levels of metals found in ecosystems. Metal pollution has gained attention due to the possible interactions and transformations that it can undergo in the environment. Some metals can transform into highly persistent compounds with high toxicity, which can be bioaccumulated in organisms, persisting in the food chain and, finally, threaten human health (Zhou et al., 2008). Recently, in Brazil, two environmental disasters have been recorded that excessively raised the concentrations of metals in aquatic ecosystems. In 2015, the rupture of a dam in the city of Mariana launched tailings for 663.2 km of the Doce river (Fernandes et al., 2016; Quadra et al., 2019) and, in 2019, the rupture of a dam in the city of Brumadinho spread at least 12 m^3 of tailings in and around the Paraopeba river and its surroundings (Thompson et al., 2020). Ecotoxicological studies and risk assessments for the regulation of chemicals are generally based on the toxicity of isolated compounds. On the other hand, freshwater organisms are exposed to mixtures of metals in the environment (Barata et al., 2006; Nys et al., 2017). Studies carried out in Brazilian water bodies have found a series of essential and non-essential metals in alarming concentrations (da Silva et al., 2017; Dourado et al., 2017; Meche et al., 2010; Weber et al., 2013). Two of these metals were chosen to be used in this study: cobalt (Co) and cadmium (Cd). Previous studies have shown environmental concentrations of metals between 0.0023 (Weber et al., 2013) to 0.25 mg L^{-1} (Dourado et al., 2017) of Co and 0.0009 (Weber et al., 2013) to 0.047 mg L^{-1} (Dourado et al., 2017) of Cd. In addition, Meche et al. (2010) evaluated the presence of metals in muscle samples from fish collected in the Rio Piracicaba (SP, Brazil). In the species *Hypostomus punctatus*, 0.92 mg of Cd and 1.40 mg of Co were quantified, and other metals; in *Serrasalmus spilopleura* were quantified 0.72 mg of Cd and 0.74 mg of Co, in addition to other metals.

Cobalt is an essential micronutrient constituent of vitamin B12, and it is vital for the growth of phytoplankton, plants and animals, although in high concentrations it can be toxic to terrestrial and aquatic organisms (Nagpal, 2004). Some sectors have an extensive demand for Co, such as chemical products (mining and processing; sewage effluents; agricultural products); in addition to the paint, cement, steel, magnet and fertilizer industry (Sridhar et al., 2020). Stubblefield et al. (2020) analyzed the toxicity of Co for different organisms, *Oncorhynchus mykiss* (survival) - EC50–96 h: 1.51 mg L^{-1};

Ceriodaphnia dubia (survival) - EC50–48 h: 2.15 mg L^{-1}; *Pimephales promelas* (survival) - EC50–96 h: 3.09 and 54.1 mg L^{-1} (larval and juvenile, respectively); *Hyalella azteca* (survival) - EC50–96 h: 3.29 mg L^{-1}; *Daphnia magna* (survival) - EC50–48 h: 5.89 mg L^{-1}; *Danio rerio* (survival) - EC50–96 h: 15.98 and 85.3 mg L^{-1} (larval and juvenile, respectively); *Chironomus tentans* (survival) - EC50–96 h: 429 mg L^{-1}.

Cadmium is a non-essential metal for the development of organisms and it affects the environment due to various human activities (use of phosphate fertilizers, burning of fossil fuels, mining, metal smelting and refining, waste incineration and urban traffic) (Samadani et al., 2018). Previous studies have investigated the toxicity of Cd exposed to different organisms: *Daphnia magna* (immobilization) - EC50–48 h: 0.02 mg L^{-1} (Traudt et al., 2017); *Ankistrodesmus fusiformis* (cell density) - EC50–96 h: 0.14 mg L^{-1}, *Chlorella ellipsoidea* (cell density) - EC50–96 h: 0.43 mg L^{-1}, *Monoraphidium contortum* (cell density) - EC50–96 h: 0.19 mg L^{-1} and *Scenedesmus acuminatus* (cell density) - EC50–96 h: 0.40 mg L^{-1} (Magdaleno et al., 2014); *Ceriodaphnia dubia* (survival) - EC50–48 h: 0.04 mg L^{-1} (Brooks et al., 2004); *Oncorhynchus mykiss* (mortality) - LC50–96 h: 0.003 mg L^{-1} (Calfee et al., 2014).

Microalgae have a fundamental role in the balance of the aquatic environments, since they are at the base of the food chain, supplying oxygen and organic substances to other forms of life. As a result, microalgae are usually the first organisms to be affected by metal pollution (Carfagna et al., 2013; Mansano et al., 2017). Previous studies that evaluated the effects of Cd on microalgae *R. subcapitata* indicated a decrease in cell density and photosynthetic activity; while it increased the synthesis of chlorophyll *a*, total lipids and lipid classes, carbohydrates, proteins, sugars, amino acids and the production of ROS; in addition, the metal stimulated photoprotection mechanisms and oxidative stress (Alho et al., 2019; Bradshaw et al., 2019; Čypaitė et al., 2014; Dos Reis et al., 2021; Koukal et al., 2003; Maršálek and Rojíčková, 1996). While *R. subcapitata* under exposed to Co presented decreased cell density, inhibited growth and impaired photosynthetic activity, in addition to increasing the synthesis of total lipids and chlorophyll a (Al-Hasawi et al., 2020; Dos Reis et al., 2021; Hussein, 2009).

Since metals are not found isolated in the environment, the assessment of metal mixture toxicity has gained attention due to its ecological relevance (Nys et al., 2017). Rachlin and Grosso (1993) analyzed the effects of Cd, Co and copper (Cu) on the microalgae

Chlorella vulgaris. The authors showed that Cd and Co mixture resulted in antagonistic interactions after 96 h of exposure and reduced 63.4% of cell growth. Furthermore, mixtures between Cd and Cu; Co and Cu showed synergistic interactions and reduced 93.9% and 92.7% cell growth, respectively. Fulladosa et al. (2005) evaluated the effect of binary mixtures of the metals Cd, Co, Cu, Pb (lead), Zn (zinc) on the bacterium *Vibrio fischeri*, and the mixture between Cd and Co also showed antagonistic interactions. There are few studies regarding the toxicity of Co and Cd mixtures, especially for microalgae. Most studies have analyzed the effects of the mixture based only on growth (Fulladosa et al., 2005; Rachlin and Grosso, 1993), although studies that address the impacts at the intracellular level are of great importance as the alterations caused by the metals in the microalgal cell can reach other trophic levels in the food chain (Rodgher et al., 2009). In this context, this study aimed to evaluate for the first time the effects of Cd and Co mixtures on photosynthetic parameters of *Raphidocelis subcapitata* and, besides that, we also evaluated the mixtures' effects on the microalgae growth, in order to obtain a better understanding of the toxicity of metal mixtures for this Chlorophyceae.

2. Material and methods

2.1. Algae culture and toxicity tests

The microalgae *Raphidocelis subcapitata* (also named *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) was cultured in L.C. Oligo medium (Table S1. Supplementary material) (AFNOR – Association Française de Normalisation, 1980), at 25 ± 0.5 °C, 4000 lux, photoperiod of 12/12 h light/dark, and periodic manual shaking. The culture medium was previously autoclaved (20 min at 121 °C, 1 atm above standard pressure) and the pH was adjusted to 7 ± 0.05.

In toxicity tests, exponential growth cultures were inoculated at a concentration of 1×10^5 cells mL^{-1} in polycarbonate flasks. *R. subcapitata* was exposed to Cd and Co in their isolated and combined forms. During the toxicity tests, the nominal concentrations used were 0.13 (control group with no metal addition – amount of Co from the L. C. Oligo medium), 0.05, 0.10, 0.20, and 0.25 mg L^{-1} of Co; and 0 (control group – no metal addition); 0.025, 0.050, 0.075, and 0.100 mg L^{-1} of Cd. Three toxicity tests were performed, in triplicate for each metal concentration and control as recommended by

OECD (2011) guidelines, for 96 h of exposure. Mixture toxicity tests were done using a full factorial design, with 16 treatments (Table S2. Supplementary material).

2.2. Cell density and cytometer measurements

Samples of control and treatments were fixed with formaldehyde buffered with borax (final concentration of 1%) and left in the dark for 10 min before frozen in liquid nitrogen. They were stored at -20 °C until analyses. Cell count was performed in FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) with a 15 mW Argon- ion laser (emission 488 nm), using 10 μL of 6 μm fluorescent beads (Fluoresbrite carboxylate microspheres; Polysciences, Inc., Warrington, PA, USA.) as the internal standard. To determine cell density, we used SSC-H (lateral dispersion) versus FL3-H (red fluorescence), according to Sarmento et al. (2008) procedures. Data were analyzed in FlowJo software, version V10.0 (Treestar.com, USA). Mean values of SSC-H; FL3-H and FSC-H, correspond to cellular complexity, cellular chlorophyll *a* (Chl *a*) fluorescence and cell size, respectively. They were used for the calculation of the relative SSC-H (SSC-H_{algae}/SSC-H_{beads}); relative FSC-H (SSC-H_{algae}/$SSCH_{beads}$), and relative FL3-H (FL3-H_{algae}/FL3-H_{beads}) (Mansano et al., 2017).

2.3. Metal determination

Metal solutions of Cd ($CdCl_2$, Titrisol – Merck, Germany) and Co ($CoCl_2$, Titrisol– Merck, Germany) were obtained commercially and diluted to the final concentration of 1 g of metal L^{-1}. These metal solutions were used to make the stock solutions of Cd (10 mg L^{-1}) and Co (1000 mg L^{-1}), which were acidified with 1% (v/v) ultrapure HNO_3, and then quantified using 228.80 and 240.73 nm absorption lines; 0.7 and 0.2 nm slid width, respectively. Measurements were done by a flame atomic absorption spectrometry (PerkinElmer PinAAcle 900 T, USA) and a burner 50 mm. Nominal concentrations of Cd and Co were used in our study as they did not differ more than 10% from the measured concentrations (Table S3. Supplementary material).

2.4. Photosynthetic activity

Measurements of the maximum quantum yield (Φ_M – capacity of the dark-adapted cells to convert light energy into chemical energy (Juneau et al., 2002)) and efficiency of the oxygen evolving complex (OEC - protein complex that indicates the state of the photolysis reaction of water (Echeveste et al., 2017)) were obtained by pulse amplitude modulated fluorometer (PHYTO-PAM® Fluorometer Analyzer, Heinz Walz, Germany). After 96 h of exposure, 3 mL of sample was taken from control and the treatments. The samples remained in the dark for 15 min to allow oxidation of all PSII reaction centers (state adapted to the dark) (Herlory et al., 2013). Phyto-PAM provides values of Φ_M, F_m (maximum fluorescence) and F_0 (minimum fluorescence) (Genty et al., 1989). The efficiency of OEC was calculated with dark-acclimated cells through the equation F_0/F_v (where $F_v = F_m - F_0$) (Kriedemann et al., 1985).

2.5. Data analysis

The inhibitory concentrations (IC_{10}, IC_{20} and IC_{50}-96 h) were calculated by non-linear regression logistic curves using Statistica 7.0 software (Statsoft Inc, 2004). Statistical analyses were performed in the SigmaPlot 12.0 software. For normal data, Dunnett's post-hoc tests were carried out to verify a statistically significant difference ($p < 0.05$) between treatments and control. Non-normal data were compared using the Kruskal-Wallis test and Dunn's post hoc test.

Species sensitivity distribution (SSD) curves were plotted using lognormal values of IC/EC_{50} toxicity data for freshwater aquatic species exposed to Cd and Co (Tables S4 and S5) with the ETX 2.0 program (Van Vlaardingen et al., 2005) and assuming a 5% log-normality according to the Anderson-Darling test.

The cell density from mixture toxicity tests was analyzed through the reference models CA (concentration addition – this model defines that individual compounds have the same mode of action and act on the same target, which contributes to a common response in proportion to their toxicities (Mansano et al., 2017)) and IA (independent action – this model defines that individual compounds have different modes of action, therefore, their effects are statistically independent of each other (Mansano et al., 2017), using the MIXTOX tool (Jonker et al., 2005). In the following step, three models were extended to analyze if there were deviations: S/A (synergism/antagonism); DR (dose ratio-dependent) and DL (dose level-dependent), by the addition of two parameters "a" and "b". In synergism/antagonism deviation, parameter "a" becomes negative or positive, respectively. In DR, the values of the parameters "bDR" and "a" indicate that the deviation is controlled by the composition of the mixture. In DL, "a" indicates deviation at low and high doses, in other words, $a > 0$ = antagonism, and $a < 0$ = synergism; while "bDR" values indicate which dose level of the deviation changed (see Table S6 in supplementary material). More information about the interpretation of MIXTOX parameters is available in Jonker et al. (2005). After the analyses, the best fit was chosen by the maximum likelihood method and the best descriptive deviation was statistically identified.

In order to evaluate the Co and Cd combined effects in photosynthesis, data were analyzed according to the method used by Gottardi et al. (2017). In mixtures, regarding the photosynthetic efficiency, antagonism occurs when the observed values are higher than the predicted effects. Synergism is the opposite; it occurs when the observed effects are lower than the predicted values. To the efficiency of OEC (determined by F_0/F_v), antagonism occurs when the observed values are lower than the predicted effects and synergism occurs when the observed effects are higher than the predicted values. We only present statistically significant data from the mixing treatments compared to the control group and to single metal exposure (one-way ANOVA, Tukey's post-hoc test) (see Table S4 in the Supplementary Material for p values).

The predicted effect caused by Cd and Co was calculated in relation to the control:

$$\text{Predicted} = (Cd / C) * (Co / C)$$

where (Cd) refers to the results of the treatment with isolated Cadmium, (Co) to the treatment with isolated Cobalt and (C) to the control.

The observed effect was calculated with the mixture of metals, Cd and Co, in relation to the control:

$$\text{Observed} = M/C$$

where (M) refers to the mixture of the metals Cd and Co and (C) to the control.

3. Results and discussion

3.1. Single toxicity

Our data showed that Cd inhibited cell growth as the concentration increased ($p < 0.05$, one-way ANOVA and Dunnett's post-hoc test) (Fig. 1A). Co significantly inhibited the cell growth only at 0.10 mg L^{-1} ($p < 0.05$, Kruskal–Wallis and Dunn's post-hoc test) (Fig. 1B). The $IC_{50–96h}$ (Table 1) for Cd was 0.08 ± 0.003 mg L^{-1}, similarly to the reported values in the literature for *R. subcapitata*: $IC_{50–96h}$ of 0.06 mg L^{-1} (Thellen et al., 1989) and $IC_{50–96h}$ of 0.07 mg L^{-1}, quantified as free Cd^{2+} (Rodgher et al., 2012). For Co, the $IC_{50–96h}$ (Table 1) was 0.11 ± 0.03 mg L^{-1} similarly to $IC_{50–72h}$ of 0.14 mg L^{-1} reported by Stubblefield et al. (2020).

In our study, inhibition concentration 10, 20, and 50 (Table 1) for Cd and Co, indicated higher toxicity of Cd than Co to *R. subcapitata*. Determining inhibition concentrations is valid in ecotoxicological studies as they indicate the amount of compound that is toxic to organisms. In addition, IC10 is used to construct SSD curves for chronic toxicity values (Mansano et al., 2018), and can be used as a guideline in determining water quality (Golding et al., 2015).

The interference of Cd in the growth of microalgae has already been analyzed in previous studies (Källqvist, 2009; Rocha et al., 2020), pointing out that the causes for this effect may be interference of the metal on the enzymatic and photosynthetic activities and also alterations in the membrane potential (Pistocchi et al., 2000). In relation to Co, other studies have reported that lower concentrations of this metal may favor the growth of microalgae, while higher concentrations show toxicity to their physiological performance (Cheng et al., 2015; Ghafari et al., 2018; Liyanaarachchi et al., 2020).

Cadmium and Cobalt caused physiological (cellular Chl *a* fluorescence – FL3-H) and morphological (cell size – FSC-H, and cell complexity – SSC-H) changes (Fig 1CD). Cell size, cell complexity and Ch *a* fluorescence increased ($p < 0.05$, one-way ANOVA, followed by Dunnett's post-hoc test) in algal cells exposed to all concentrations of Co (Fig. 1D). The exposure to Cd (Fig. 1C) led to an increase ($p < 0.05$, one-way ANOVA, followed by the Dunnett's post-hoc test) in the cell complexity in all tested concentrations, except for 0.025 mg L^{-1}; increase in the Chl *a* fluorescence at 0.025, 0.075 and 0.100 mg L^{-1}; and an increase in the cell size at all Cd concentrations.

According to previous studies, metals and pesticides often lead to an increase in the size and cellular complexity of *R. subcapitata* (Franklin et al., 2001; Gebara et al., 2020; Machado and Soares, 2014; Mansano et al., 2017; Moreira et al., 2020). This result may be indicative of failures in the regulation of cell volume by membranes, a consequence of high levels of oxidative radicals formed (Franklin et al., 2001; Jamers et al., 2013), interruption in the cell cycle before the division step (Machado and Soares, 2014), change in membrane potential and permeability, interfering with the end of cell division, since they do not divide but, on the other hand, continue to fix carbon (through photosynthetic activity) get increased cells as photosynthetic products (Franklin et al., 2001).

Machado and Soares (2014) observed an increase in cell size in the exposure of Cd to *R. subcapitata*. The authors pointed that eukaryotic organisms have cell cycle checkpoints. In unicellular algae, one of these points is linked to obtaining an ideal cell volume to proceed with the process. Controlling cell volume is critical in regulating the cell cycle in order to maintain cell size. Furthermore, they observed that Cd interrupts cell growth after the second nuclear division and before cytokinesis. Thus, the results suggest that the metals, Cd and Co, probably interfered in the cell division process, being internalized in algal cells and resulting in an increase in cell size and complexity.

Exposure of microalgae to metals and pesticides are known to induce an increase in Chl *a* fluorescence, which has been attributed as a strategy to maximize light capture and, consequently, increasing the photosynthetic efficiency, even though the microalgae was under metal-stress condition (Alho et al., 2019; Mansano et al., 2017; Moreira et al., 2020). It could explain the increase of this parameter that we observed in response to the exposure by both Cd and Co (Fig. 1C and D).

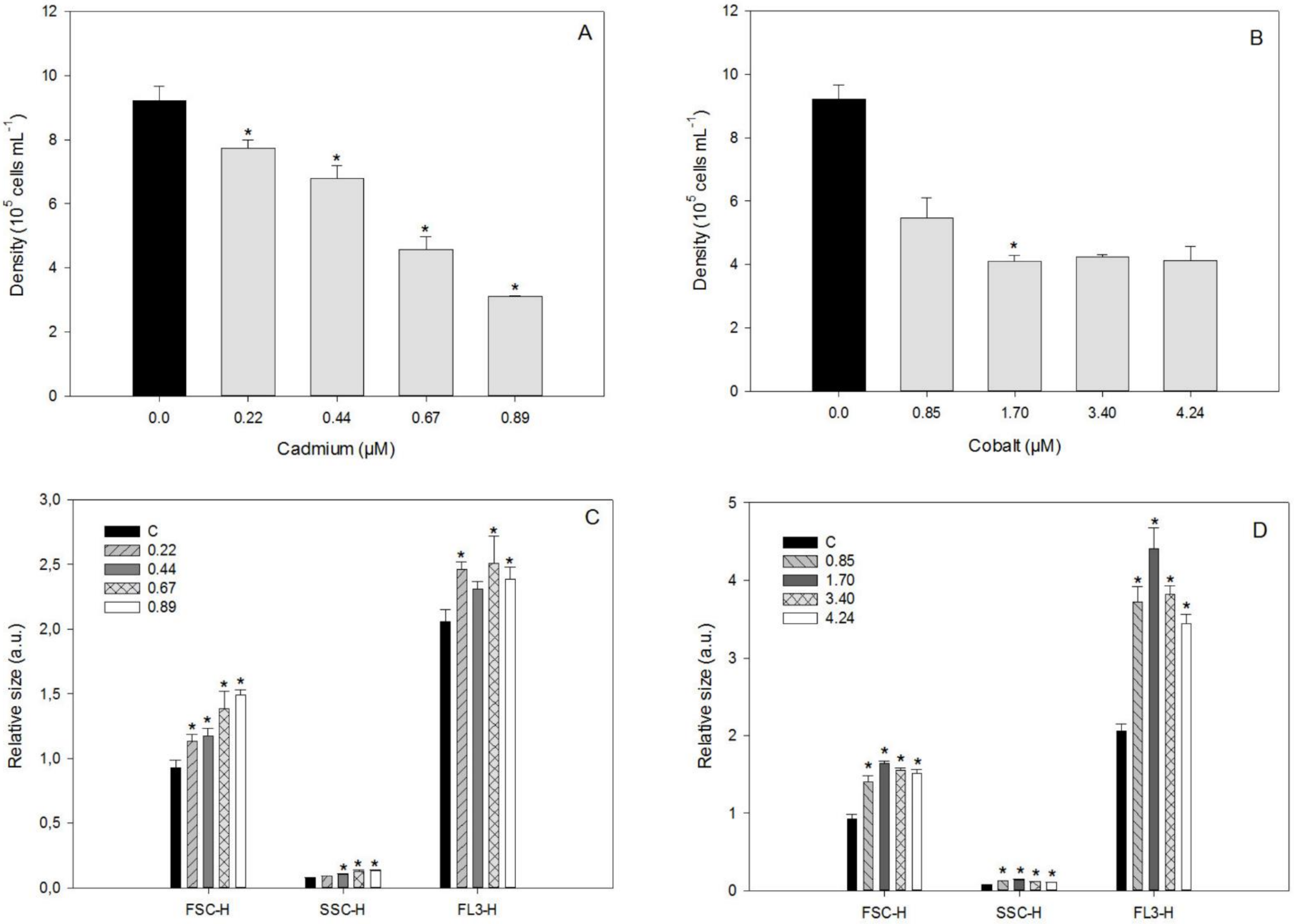


Fig. 1. Cellular parameters of *Raphidocelis subcapitata* exposed to Cd (A and C) and Co (B and D) during 96 h. Density (10^5 cells mL^{-1}) are expressed in panels A-B; while cell size (FSC-H), cellular complexity (SSC-H) and chlorophyll *a* fluorescence (FL3-H) are shown in panels C-D. The asterisks indicate significant difference ($p < 0.05$, one-way ANOVA and the Dunnett's post-hoc test – panels A, C and D); $p < 0.05$, Kruskal–Wallis and Dunn's post-hoc test – panel B) when compared to the control group (C). Metal concentrations are expressed in mg L^{-1}.

Table 1. Values of inhibition concentration IC_{10}, IC_{20} and IC_{50} and standard error of *Raphidocelis subcapitata* after 96h of exposure to cadmium and cobalt. NC means not calculated.

	Cadmium (mg L^{-1})	Cobalt (mg L^{-1})
IC_{10}	0.03 ± 0.004	NC
IC_{20}	0.04 ± 0.004	NC

IC$_{50}$	0.08 ± 0.003	0.11 ± 0.03

3.2. SSD analyses

Species sensitivity curves (SSD curves) (Fig. 2) showed that *R. subcapitata* is one of the most sensitive organisms to Co, being only less sensitive than *Hyalella azteca*. Moreover, regarding Cd, *R. subcapitata* was less sensitive than some species of microcrustaceans, e.g., *Ceriodaphnia dubia*, *Daphnia magna* and *Simocephalus serrulatus*; and more sensitive than the algae *Microcystis aeruginosa*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*.

The levels of Cd and Co allowed by CONAMA (National Environment Council) to guarantee the water quality are, respectively: 0.001 to 0.01 mg L^{-1} and 0.05 to 0.2 mg L^{-1} (Brazil, 2005). According to USEPA, the permitted level of Cd in water is 0.002 mg L^{-1} (CMC – criterion maximum concentration) (USEPA, 1995). For WHO (World Health Organization), the concentration of Co in surface and groundwater is low, being less than 0.001 mg L^{-1} in pristine areas and between 0.001 to 0.01 mg L^{-1} in populated areas. On the other hand, concentrations of 4.5 (Mineral Creek, Arizona, USA) and 6.5 (Little St. Francis river, Missouri, USA) mg L^{-1} of Co have already been quantified in some rivers (Kim et al., 2006). Analyzing the permitted limits and the data presented, the concentration of Cd allowed by CONAMA and USEPA would be harmful to *Oncorhynchus mykiss* (eggs); *Paramecium trichium* and *Salmo gairdneri* (juvenile) (Fig. 2A, Table S4). In relation to Co, the species affected by the permitted levels of Co would be *Hyalella azteca*; *Raphidocelis subcapitata* (present study) and *Spirodela polyrhiza* (Fig. 2B, Table S5).

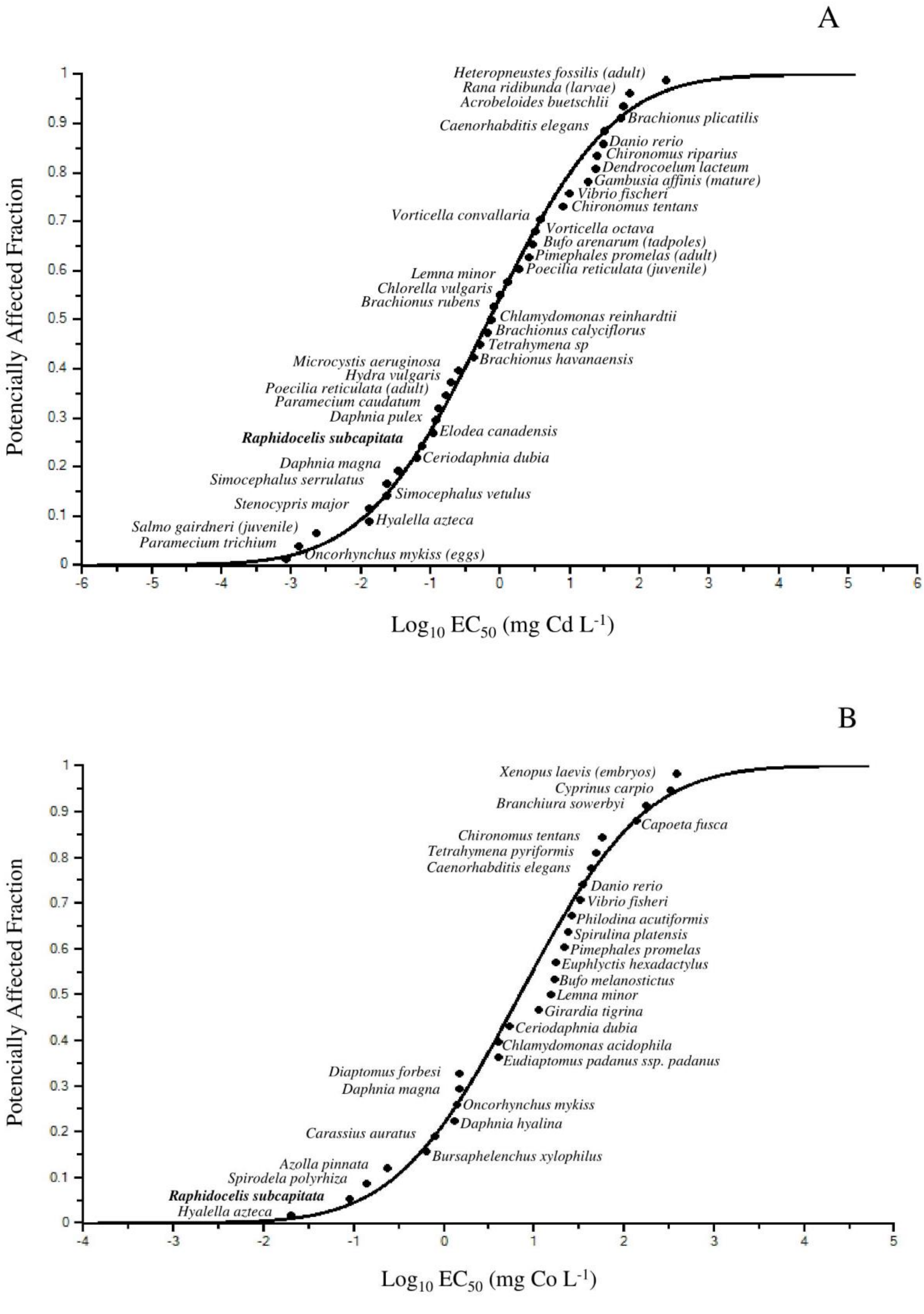


Fig. 2. SSD (Species sensitivity distribution) based on EC$_{50}$ values (mg metal L^{-1}) of aquatic organisms, obtained from literature data (Tables 4 and 5 Supplementary material), exposed to Cd (A) and Co (B). Data from *Raphidocelis subcapitata* (present study) are in bold.

3.3. Mixture toxicity tests

Cell density results of the mixture tests were analyzed by the MIXTOX tool, all parameters and significance values are shown in Table 2. For CA model (Table 2), the SS (sum of squared residuals) was 5.02 ($p < 0.05$; $r^2 = 0.86$). The "*a*" was added to describe S/A deviation, but it was not statistically significant ($p = 0.114$). The "*a*" and "b_{DR}" were added to describe the deviation DR (dose ratio-dependent) and the SS value decreased to 3.94, which was statistically significant ($p < 0.05$; $r^2 = 0.89$). DL (dose level-dependent) deviation was not significant ($p = 0.195$). Thus, for CA model, DR deviation was the best fit to our data, which indicated that at high concentrations of Co and low concentrations of Cd occurred synergism; and at low concentrations of Co and high concentrations of Cd occurred antagonism (Fig. 3).

Table 2. Summary of the modelled data of cell density of *Raphidocelis subcapitata* exposed to mixtures of Cd and Co during 96 h.

	Concentration addition				Independent action			
	CA	S/A	DR	DL	IA	S/A	DR	DL
Max	8.70	8.64	8.51	8.57	8.16	9.06	8.64	8.67
β Cd	1.67	2.14	2.10	2.17	1.12	1.31	1.35	1.40
β Co	0.37	0.36	0.35	0.45	0.45	0.10	1.96×10^{-8}	1.10×10^{-8}
IC_{50} for Cd	0.78	0.73	0.73	0.74	1.21	0.72	0.88	0.89
IC_{50} for Co	1.82	1.47	2.16	1.80	4.15	0.46	0.24	0.22
A	-	1.04	-1.82	-0.04	-	2.49	0.24	-0.09
$b_{DR/DL}$	-	-	4.55	10.94	-	-	0.05	2.01
SS	5.02	4.55	3.94	4.41	9.63	5.97	16.74	17.41
r^2	0.86	0.87	0.89	0.87	0.72	0.83	0.52	0.50
X^2 or F test	29.59	2.49	6.11	3.27	13.05	11.95	NC	NC
Df	-	1.00	2.00	2.00	-	1.00	1.00	1.00
p (X^2/F)	3.8×10^{-8}	0.114	0.047	0.195	2.19×10^{-5}	0.0005	NC	NC

This data were obtained from methodology used in Jonker et al. (2005). Max = maximum response value, β = slope response of isolated compounds, IC_{50} = median growth inhibition concentration; *a* and $b_{DR/DL}$ = function parameters; SS = sum of the squared residuals; r^2 = regression coefficient; χ^2 or *F*test = statistical test; d*f* = degrees of freedom; $p(\chi^2/F)$ = level of significance of statistical test; CA = concentration addition model; IA = independent action model; S/A = synergism/antagonism deviation; DR = dose ratio-dependent deviation; DL = dose level-dependent deviation.

For IA model (Table 2), the SS was 9.63 ($p < 0.05$; $r^2 = 0.72$). The "*a*" parameter was added in S/A deviation, and the SS value was 5.97 ($p < 0.05$; $r^2 = 0.83$). DR and DL deviations were not statistically significant (*p* value was not calculated). Then, for this model, S/A deviation was the best fit to our data, which indicated antagonism in the mixture of Cd and Co.

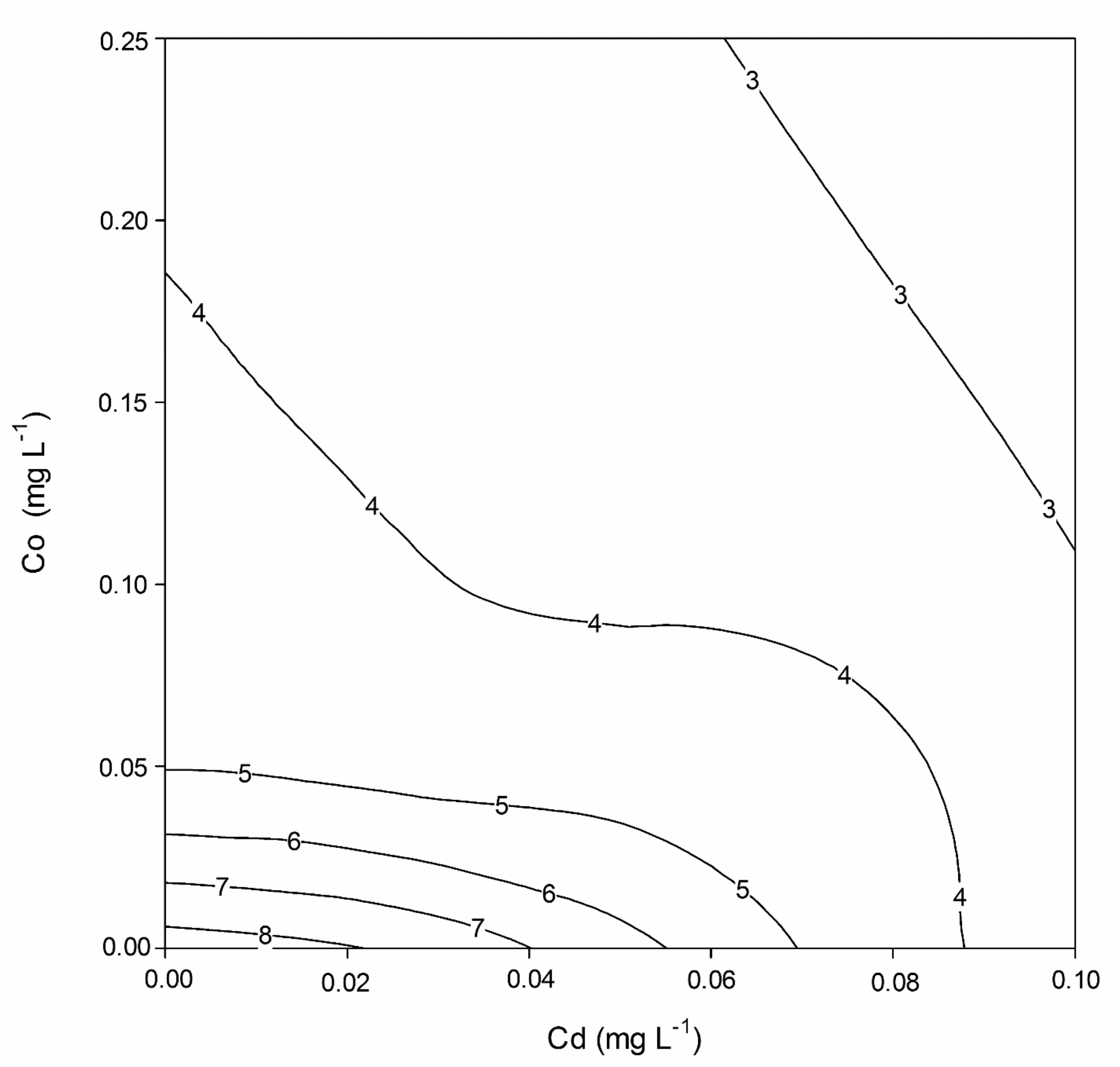
Both CA and IA models fitted well to the mixture data, but the CA model was the one that better explains the results, as the dose-ratio dependent (DR) deviation presented the best fit (significant *p* value, the smallest SS and largest r^2 value). Based on this, synergism occurred in high Co concentrations and low Cd concentrations and antagonism occurred in high Cd concentrations and low Co concentrations (Fig. 3).

Taking into account Cd and Co levels quantified in the environment, we draw attention to the imminent risk of synergism of these metals in aquatic environments. The Cd environmental levels ranged from 0.00005 mg L^{-1} in Pardo river (Alves et al., 2014), 0.04 mg L^{-1} in Água Boa stream (Dourado et al., 2017) and 4.51 mg L^{-1} in the Kor river (Ebrahimi and Taherianfard, 2010). Meanwhile, Co levels in freshwater varied from 0.0023 mg L^{-1} in Sinos river (Weber et al., 2013), 0.25 mg L^{-1} in Água Boa stream (Dourado et al., 2017) and 2.39 mg L^{-1} in Tanjero river (Aziz et al., 2012).

As far as we know, only a few studies evaluated the toxicity of Cd and Co mixtures to algae (e.g. Rachlin and Grosso, 1993) and the existing studies do not address recent mixture modeling techniques. Mo et al. (2019) exposed the microalgae *Chlorella pyrenoidosa* to five binary combinations of metals: Ni–Fe, Ni–Zn, Ni–Pb, Ni–Cd, and Ni–Cr. The data were analyzed by the CA model, and the Ni–Cd mixture showed synergism in high concentrations. Another study with mixtures involving the metal Cd with the microalgae *Chlorella vulgaris* showed synergism in the combination Cd–Cu and antagonism Cd–Zn (Franklin et al., 2002). The authors argue that synergism between metals may occur if one metal facilitates the absorption of the other, while antagonism can occur if both compete for the same transport sites on the cell membrane (Franklin et al., 2002). Therefore, we may indicate that antagonism occurs with Cd and Co, because they probably compete for the same transport sites on the membrane, since they are divalent metals (Starodub et al., 1987). Synergism could happen because the exposure to different metals may result in an increase of permeability of the plasma membrane followed by an increased cellular uptake and consequently toxicity (Starodub et al., 1987).

Binary (Cd–Co; Cd–Cu; Cu–Co) and tertiary (Cu–Co–Cd) metal mixture tests with the microalgae *Chlorella vulgaris* indicated the occurrence of synergism with the mixture between Cd–Cu; Cu–Co and antagonism with the tertiary mixture (Cd + Co + Cu) and with Cd–Co (Rachlin and Grosso, 1993). Thus, we see the importance of studying mixtures, as they are present in the environment in this way, and their absorption can have several interactive effects modifying the toxicity of individual compounds (Batool and Javed, 2015).

Fig. 3. Isobologram of *Raphidocelis subcapitata* (cell density) exposed to Cd and Co mixtures, following



the CA (concentration addition) model and DR (dose ratio-dependent) deviation. Linear, concave and convex isoboles represent no interaction, synergy and antagonism, respectively.

According to the literature data, this is the first study that evaluates the maximum quantum yield (Φ_M) and F_0/F_V of the microalgae *Raphidocelis subcapitata* exposed to the Cd and Co mixture. The results obtained are shown in Table 3 and we only present the data that obtained a statistically significant difference ($p < 0.05$, one-way ANOVA and Tukey's post-hoc test).

Regarding the photosynthetic parameters during single exposure, Cd did not alter the parameters Φ_M e F_0/F_v in a statistically significant way, while Co changed Φ_M and F_0/F_v at the highest concentrations tested (0.20 and 0.25 mg L^{-1}). The effect of the combined metals was antagonistic for both analyzed parameters (Φ_M and F_0/F_v), since, in relation to the Φ_M, the observed values were higher than the predicted ones; and in the F_0/F_v data showed that the observed values were lower than the predicted ones for all combinations tested.

Maleva et al. (2012) investigated the effects of the metals Ni, Cd, Cu, Zn, and Mn, single and combined, on different parameters of *Elodea densa*. They observed that binary metal mixtures did not change significantly the maximum quantum efficiency of PSII (Φ_M), while the isolated Cd and Cu metals decreased this endpoint. Starodub et al. (1987), when evaluating the toxicity of copper (Cu), zinc (Zn), and lead (Pb) in binary mixtures on *Scenedesmus quadricauda*, observed that synergistic interactions predominated over the growth and antagonistic relationships for photosynthesis, similar to the results observed here.

Table 3. Predicted and observed Φ_M and F_0/F_v values of *Raphidocelis subcapitata* exposed to single and combined Cd and Co after 96 h. We observed antagonistic interactions in all treatments.

Cd	Co	Φ_M						F_0/F_v					
(mg L^{-1})	(mg L^{-1})	Control	Cd	Co	Mixture	Predicted	Observed	Control	Cd	Co	Mixture	Predicted	Observed
0.025	0.05	0.60	0.60	0.59	0.64	0.97	1.07	0.66	0.66	0.70	0.56	1.06	0.84
0.025	0.10	0.60	0.60	0.58	0.64	0.96	1.07	0.66	0.66	0.73	0.56	1.10	0.84
0.025	0.20	0.60	0.60	0.57	0.63	0.95	1.04	0.66	0.66	0.75	0.59	1.14	0.89
0.025	0.25	0.60	0.60	0.56	0.63	0.93	1.04	0.66	0.66	0.79	0.60	1.20	0.90
0.050	0.05	0.60	0.61	0.59	0.64	0.98	1.06	0.66	0.65	0.70	0.57	1.04	0.86
0.050	0.10	0.60	0.61	0.58	0.64	0.97	1.06	0.66	0.65	0.73	0.57	1.07	0.87
0.050	0.20	0.60	0.61	0.57	0.62	0.96	1.03	0.66	0.65	0.75	0.61	1.11	0.92
0.050	0.25	0.60	0.61	0.56	0.62	0.94	1.04	0.66	0.65	0.79	0.60	1.17	0.91
0.075	0.05	0.60	0.59	0.59	0.63	0.95	1.05	0.66	0.71	0.70	0.58	1.14	0.88
0.075	0.10	0.60	0.59	0.58	0.63	0.94	1.05	0.66	0.71	0.73	0.58	1.17	0.88
0.075	0.20	0.60	0.59	0.57	0.62	0.92	1.03	0.66	0.71	0.75	0.61	1.22	0.92
0.100	0.05	0.60	0.55	0.59	0.61	0.89	1.01	0.66	0.81	0.70	0.64	1.31	0.97
0.100	0.10	0.60	0.55	0.58	0.61	0.88	1.01	0.66	0.81	0.73	0.64	1.35	0.97
0.100	0.20	0.60	0.55	0.57	0.60	0.87	0.99	0.66	0.81	0.75	0.67	1.40	1.01
0.100	0.25	0.60	0.55	0.56	0.59	0.85	0.99	0.66	0.81	0.79	0.68	1.47	1.03

These data were obtained from the methodology used in Gottardi et al. (2017). Φ_M = maximum quantum yield and F_0/F_v = Efficiency of the oxygen-evolving complex.

Therefore, in our study, both metals, when combined, did not cause negative effects, but antagonistic interactions on the *R. subcapitata* photosynthetic performance. Therefore, we can assume that the reduced growth in response to the mixture of Co and Cd was not due to the impairment of photosynthetic process.

The Φ_M has been used as an indicator of stress, giving information about the physiology of the alga and being related to photosynthetic performance (Herlory et al., 2013). The drop in Φ_M in response to high concentrations of Co in the isolated form may be related to a reduction or partial blocking of electron transport from photosystem II (PSII) to photosystem I (PSI) due to the reoxidation of quinone A (Herlory et al., 2013; Mallick and Mohn, 2003). The efficiency OEC indicates how the water photo-oxidation process is going. Increased values of parameter F_0/F_v indicates that this system was affected (Alho et al., 2019). This can happen due to a substitution of manganese (Mn) at the division site, interfering with the reaction and impairing the production of oxygen (Herlory et al., 2013; Juneau et al., 2002). Although the changes in these endpoints were subtle for Cd and, especially, Co highest concentrations when in the isolated test, it indicates damage to the water splitting process in *R. subcapitata*. When in the mixture, Co and Cd lead to antagonistic responses in these parameters.

Thus, we can see how the effects of mixtures can be different from the effects of isolated metals, and may not be predicted based on the toxicity of single metals (Franklin et al., 2002). Regarding algal density, synergism was mainly caused by Co, which significantly affected only one concentration tested when analyzed alone, while isolated Cd inhibited the density in all treatments. Regarding photosynthetic parameters, isolated Co negatively affected Φ_M and F_0/F_v, while Cd did not show significant changes. On the other hand, when the metals were combined, the effect was antagonistic for both analyzed parameters. Therefore, we reinforce the importance of analyzing the effects of mixed metals in further studies.

4. Conclusion

Our results showed that Cd was more toxic than Co regarding the effects on cell density during single exposure. On the other hand, both metals increased the cellular Chl *a* fluorescence, and caused changes in cell morphology (cell size and complexity). Concerning the mixture treatments, the effects on algal growth better fitted the concentration addition model (CA) and dose ratio-dependent (DR) deviation. Therefore, when the metals were combined, the mixture presented synergism at high concentrations of Co and low concentrations of Cd, and antagonism at high concentrations of Cd and low concentrations of Co. In relation to the mixture effects of Co and Cd on photosynthetic parameters, we observed antagonistic interactions on the maximum quantum yield (Φ_M) and the efficiency of the oxygen evolving complex (OEC). The synergistic interactions of Cd and Co metals and their occurrence in the environment reinforce the concern about the effects of mixed compounds on organisms. In addition, as microalgae, the test organism used in this work, are primary producers, the impacts on these organisms may extend throughout the trophic chain.

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Supplementary material

Table S1. LC Oligo culture medium composition (AFNOR, 1980).

Solutions	Reagent	Concentration (M)
1	$Ca(NO_3)_2.4H_2O$	1.7×10^{-4}
2	KNO_3	$1.0 \text{ x } 10^{-3}$
3	$MgSO_4.7H_2O$	1.2×10^{-4}
4	K_2HPO_4	2.3×10^{-4}
	$CuCl_2.H_2O$	6.0×10^{-8}
	$(NH_4)6Mo_7O_{24}.4H_2O$	2.4×10^{-8}
	$ZnSO_4.7H_2O$	1.0×10^{-7}
5	$CoCl_2.6H_2O$	$1.3 \text{ x } 10^{-7}$
	$Mn(NO_3)_2.H_2O$	1.5×10^{-7}
	$C_6H_8O_7.H_2O$	1.4×10^{-7}
	H_3BO_3	4.9×10^{-7}
	$C_6H_5FeO_7.H_2O$	3.1×10^{-6}
6	$FeCl_3.6H_2O$	1.9×10^{-6}
	$FeSO_4.7H_2O$	1.1×10^{-6}
7	$NaHCO_3$	1.8×10^{-4}

Table S2. Full factorial design of the toxicity test of *Raphidocelis subcapitata* exposed to Cd and Co, isolated and in mixture. Treatments T1 to T8 refer to the isolated metals, and T9 to T24 refer to the mixture of metals. The control group is represented as "C".

Full factorial		Cadmium (mg L^{-1})				
		0.0	0.025	0.050	0.075	0.100
Cobalt (mg L^{-1})	0.01	C	I5	I6	I7	I8
	0.05	I1	M1	M2	M3	M4
	0.10	I2	M5	M6	M7	M8
	0.20	I3	M9	M10	M11	M12
	0.25	I4	M13	M14	M15	M16

Table S3. Comparison between nominal and real concentrations of cadmium (Cd) and cobalt (Co) in the stock solutions, quantified by flame atomic absorption spectrometry.

Metal	**Nominal concentration ($mg\ L^{-1}$)**	**Real concentration ($mg\ L^{-1}$)**
Cd	10	10.25 ± 0.07
Co	10	10.42 ± 0.14

Table S4. *P* values referring to the statistical tests of the parameters Φ_M and F_0/F_v *of R. subcapitata* exposed to the mixture of metals Cd and Co.

Combined metals		*p* values	
Cd ($mg\ L^{-1}$)	Co ($mg\ L^{-1}$)	Φ_M	F_0/F_v
0.025	0.05	<0.05	<0.05
0.025	0.10	<0.05	<0.05
0.025	0.20	<0.05	<0.05
0.025	0.25	<0.05	<0.05
0.050	0.05	<0.05	<0.05
0.050	0.10	<0.05	<0.05
0.050	0.20	<0.05	<0.05
0.050	0.25	<0.05	<0.05
0.075	0.05	<0.05	<0.05
0.075	0.10	<0.05	<0.05
0.075	0.20	<0.05	<0.05
0.075	0.25	0.99	0.99
0.100	0.05	<0.05	<0.05
0.100	0.10	<0.05	<0.05
0.100	0.20	<0.05	<0.05
0.100	0.25	<0.05	<0.05

Table S4. Toxicity data of aquatic organisms exposed to cadmium (datum from the present study is in bold) obtained from scientific studies.

Group	Scientific Name	Duration	Value (mg/L)	Endpoint	Reference
Fish	*Oncorhynchus mykiss* (eggs)	4 days	0.00086	LC_{50}	Mebane et al. (2007)
Protozoan	*Paramecium trichium*	2 days	0.0013	IC_{50}	Miyoshi et al. (2003)
Fish	*Salmo gairdneri* (juvenile)	4 days	0.0023	LC_{50}	Spehar and Carlson (1984)
Crustacean	*Hyalella azteca*	4 days	0.013	LC_{50}	Mackie (1989); Suedel et al. (1997); Sims and Steevens (2008)
Ostracod	*Stenocypris major*	4 days	0.013	LC_{50}	Shuhaimi-Othman et al. (2013)
Crustacean	*Simocephalus vetulus*	2 days	0.024	LC_{50}	Mount and Norberg (1984)
Crustacean	*Simocephalus serrulatus*	2 days	0.024	LC_{50}	Spehar and Carlson (1984)
Crustacean	*Daphnia magna*	2 days	0.035	EC_{50} / LC_{50}	Mount and Norberg (1984); Hall et al. (1986); Hatakeyama and Sugaya (1989); Stuhlbacher et al. (1993); Suedel et al. (1997); Meyer et al. (2015).
Crustacean	*Ceriodaphnia dubia*	2 days	0.063	LC_{50}	Suedel et al. (1997)
Algae	***Raphidocelis subcapitata***	**4 days**	**0.075**	$\mathbf{IC_{50}}$	**Present study**
Macrophyte	*Elodea canadensis*	7 days	0.11	EC_{50}	Basile et al. (2012)
Crustacean	*Daphnia pulex*	2 days	0.12	LC_{50}	Mount and Norberg (1984); Hall et al. (1986); Slabbert and Venter (1999)
Protozoan	*Paramecium caudatum*	2 days	0.13	IC50 / LC50	Madoni et al. (1992); Miyoshi et al. (2003)
Fish	*Poecilia reticulata (adult)*	4 days	0.17	LC50	Shuhaimi-Othman et al. (2013)
Cnidarian	*Hydra vulgaris*	4 days	0.20	LC50	Beach and Pascoe (1998); Karntanut and David Pascoe (2000)

Algae	*Microcystis aeruginosa*	4 days	0.25	EC50	Qian et al. (2012)
Rotifer	*Brachionus havanaensis*	1 day	0.419	LC50	Juárez-Franco et al. (2007)
Ciliate	*Tetrahymena sp*	1 day	0.52	LC50	Rico et al. (2009)
Rotifer	*Brachionus calyciflorus*	1 day	0.65	LC50	Couillard et al. (1989)
Algae	*Chlamydomonas reinhardtii*	3 days	0.742	IC50	Tanaka et al. (2011)
Rotifer	*Brachionus rubens*	1 day	0.81	LC50	Snell and Persoone (1989)
Algae	*Chlorella vulgaris*	4 days	1.02	EC50	Lam et al. (1998)
Macrophyte	*Lemna minor*	4 days	1.27	EC50	Hu et al. (2017)
Fish	*Poecilia reticulata (juvenile)*	4 days	1.85	LC50	Slabbert and Venter (1999)
Fish	*Pimephales promelas (adult)*	4 days	2.60	LC50	Spehar and Carlson (1984); Benson and Birge (1985)
Amphibians	*Bufo arenarum (tadpoles)*	4 days	2.97	LC50	Muino et al. (1990); Ferrari et al. (1993)
Ciliate	*Vorticella octava*	1 day	3.24	LC50	Madoni et al. (1996)
Ciliate	*Vorticella convallaria*	1 day	3.81	LC50	Madoni et al. (1996)
Insect	*Chironomus tentans*	4 days	8.00	LC50	Suedel et al. (1997)
Bacteria	*Vibrio fischeri*	30 min	9.96	EC50	Wang et al. (2009)
Fish	*Gambusia affinis* (mature)	4 days	18.51	LC_{50}	Sharma et al. (2009)
Platyhelminthes	*Dendrocoelum lacteum*	4 days	23.22	LC_{50}	Ham et al. (1995)
Insect	*Chironomus riparius*	4 days	24.90	LC_{50}	Williams et al. (1986)
Fish	*Danio rerio*	4 days	30.33	LC_{50}	Zhang et al. (2012)
Nematode	*Caenorhabditis elegans*	3 days	31.36	LC_{50} / EC_{50}	Kammenga et al. (1994); Chu and Chow (2002)
Rotifer	*Brachionus plicatilis*	1 day	54.90	LC_{50}	Snell and Persoone (1989)
Nematode	*Acrobeloides buetschlii*	3 days	59.28	LC_{50}	Kammenga et al. (1994)
Amphibians	*Rana ridibunda (larvae)*	4 days	71.80	LC_{50}	Loumbourdis et al. (1999)
Fish	*Heteropneustes fossilis* (adult)	4 days	240.94	LC_{50}	Kasherwani et al. (2009)

Table S5. Toxicity data of aquatic organisms exposed to cobalt (datum from the present study is in bold) obtained from scientific studies. *For species with more than one available endpoint data on literature, the geometric means were calculated.

Group	Scientific Name	Duration	Value (mg/L)	Endpoint	Reference
Crustacean	*Hyalella azteca*	7 days	0.016	LC50	Borgmann et al. (2005)
Algae	***Raphidocelis subcapitata***	**4 days**	**0.09**	**IC50**	**Present study**
Macrophyte	*Spirodela polyrhiza*	4 days	0.135	EC50	Gaur et al. (1994)
Macrophyte	*Azolla pinnata*	4 days	0.242	EC50	Gaur et al. (1994)
Nematode	*Bursaphelenchus xylophilus*	1 day	0.648	LD50	Kobamoto and Izumi (1984)
Fish	*Carassius auratus*	7 days	0.81	EC50	Birge (1980); Marr (1998)
Crustacean	*Daphnia hyalina*	2 days	1.32	LC50	Baudouin and Scoppa (1974)
Fish	*Oncorhynchus mykiss*	4 days	1.406	LC50	Marr et al. (1998)
Crustacean	*Daphnia magna*	2 days	1.50	LC50	Khangarot et al. (1987)
Crustaceans	*Diaptomus forbesi*	4 days	1.50	LC50	Mukherjee and Kaviraj (2011)
Crustacean	*Eudiaptomus padanus ssp. Padanus*	2 days	4.00	LC50	Baudouin and Scoppa (1974)
Algae	*Chlamydomonas acidophila*	4 days	4.096	EC50	Nishikawa and Tominaga (2001)
Crustacean	*Ceriodaphnia dubia*	2 days	5.30	LC50	Diamond et al. (1992)
Platelminto	*Girardia tigrine*	4 days	11.35	LC50	Ewell et al. (1986)
Macrophyte	*Lemna minor*	7 days	15.70	EC50	Ince et al. (1999)
Amphibians	*Bufo melanostictus*	4 days	17.20	LC50	Mukherjee and Kaviraj (2011)
Amphibians	*Euphlyctis hexadactylus*	4 days	17.59	LC50	Khangarot et al. (1985)
Fish	*Pimephales promelas*	4 days	21.79	LC50	Ewell et al. (1986)
Algae	*Spirulina platensis*	4 days	23.80	EC50	Sharma et al. (1987)
Rotifer	*Philodina acutiformis*	4 days	26.78	EC50	Buikema et al. (1974)

Bacteria	*Vibrio fisheri*	15 min	32.71	EC50	Ince et al. (1999)
Fish	*Danio rerio*	4 days	35.30	LC50	Reinardy et al. (2013)
Nematode	*Caenorhabditis elegans*	2 days	43.37	EC50	Chu and Chow (2002)
Ciliate	*Tetrahymena pyriformis*	15 h	50.00	IC50	Sauvant et al. (1995)
Insect	*Chironomus tentans*	2 days	56.87	EC50	Khangarot and Ray (1989)
Fish	*Capoeta fusca*	4 days	137.04	LC50	Pourkhabbaz et al. (2011)*
Annelida	*Branchiura sowerbyi*	4 days	179.00	LC50	Mukherjee and Kaviraj (2011)
Fish	*Cyprinus carpio*	4 days	327.50	LC50	Saravi et al. (2009)
Amphibians	*Xenopus laevis* (embryos)	4 days	384.24	LC50	Saka (2004)

Table S6. Interpretation of parameters referring to the addition of concentration (CA) and independent action (IA) models by Jonker et al. (2005).

Parameter	Value		Meaning
	CA	IA	
		Antagonism/Synergism	
A	>0	<0	Antagonism
	<0	>0	Synergism
		Dose ratio dependence	
A	>0	>0	Antagonism, except for those mixture ratios where significant negative b_is indicate synergism
	<0	<0	Synergism, except for those mixture ratios where significant positive b_is indicate antagonism
b_i	>0	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
	<0	<0	Synergism where the toxicity of the mixture is caused mainly by toxicant *i*
		Dose level dependence	
A	>0	>0	Antagonism low dose level and synergism high dose level
	<0	<0	Synergism low dose level and antagonism high dose level
b_{DL}	>1	>2	Change at lower dose level than the EC50
	=1	=2	Change at the EC50 level
	$0 < b_{DL} < 1$	$1 < b_{DL} < 2$	Change at higher dose level than the EC50
	<0	<1	No change, but the magnitude of synergism/antagonism is dose level (CA) or effect level (IA) dependent

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Capítulo 2. Effects of cadmium and nickel mixtures on multiple endpoints to the microalga *Raphidocelis subcapitata*

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Abstract

Investigating the effects of mixtures of contaminants on aquatic organisms is highly important, as it reflects what actually occurs in the environment. Cadmium (Cd) and nickel (Ni) are metals that co-occur in aquatic ecosystems, and there is rare information of their joint toxicity on Chlorophyceae using multiple endpoints. We evaluate the effects of isolated and combined Cd and Ni metals on multiple endpoints (cell density, chlorophyll *a* fluorescence, cell size and complexity, reactive oxygen species (ROS), maximum quantum yield (Φ_M) and efficiency oxygen evolving complex OEC - F_0/F_v) of the chlorophycean *Raphidocelis subcapitata*. Results showed that single Cd decreased cell density, increased ROS production (up to 308% at 0.075 mg L^{-1} of Cd), chlorophyll *a* (Chl *a*) fluorescence (0.050 to 0.100 mg L^{-1} of Cd), cell size (0.025 to 0.100 mg L^{-1} of Cd) and cell complexity in all concentrations tested. Ni exposure decreased ROS production up to 25% at 0.25 mg L^{-1} of Ni and Chl *a* fluorescence in all concentrations tested. Cell density and oxygen evolving complex - OEC (F_0/F_v) - were only affected at 0.5 mg L^{-1} of Ni. Mixture toxicity showed antagonistic effects at low doses and synergism at high doses, with a dose level change greater than IC_{50}. The independent action (IA) model and dose-level dependent (DL) deviation were best fitted to our data. Cd-Ni mixtures resulted in a significant increase in cell size and cell complexity, changes in ROS production and in Chl *a* fluorescence, and did not affect the photosynthetic parameters. Therefore, we reinforce the importance of a better understanding of the toxicity mechanisms of metals mixtures in order to guide resolutions on concentrations of metals in freshwater ecosystems, as microalgae are the base of the food chain and metals are bioaccumulative, damage can reach higher trophic levels.

Keywords: Metal mixture, synergism, antagonism, ROS, photosynthetic activity, toxicity, combined effects.

1. Introduction

In the environment, organisms are exposed to a wide range of contaminants although the vast majority of risk assessments are based on the effects of the isolated compounds (Nys et al., 2017). The mode of action of contaminants, whether the same or different, can influence their toxicity, which can result in additive, synergistic or antagonistic effects (Beyer et al., 2014), being able to reach the biological processes of organisms such as metabolism, connections, internal transport, absorption, excretion and bioavailability (common to be affected in metallic mixtures) (Cedergreen 2014). The analysis of the toxicity of the mixtures can be done by theoretical models of concentration addition (CA) (Loewe 1926) and independent action (IA) (Bliss 1939). The CA model assumes that individual compounds have the same mode of action on a biological target, while the AI proposes that chemicals have a different mode of action (Pavlaki *et al.* 2011).

Metals are classified as essentials, i.e., when they are necessary for developing organisms, or non-essentials, i.e., with no known biological function. Several metals reach surface waters from different routes such as surface runoff, atmospheric deposition or effluent discharge (Sun et al., 2021). The metal contamination of the aquatic environments has increased since the beginning of the industrialization process (Gauthier et al., 2014). In Brazil, areas of recent environmental disasters, such as the cities of Mariana and Brumadinho, where the collapse of mining dams spread tailings along 663.2 km of the Doce river, in addition to 12 m^3 of tailings through the Paraopeba river and its surroundings, also contributed to the increase of metal concentrations in the environment (Fernandes et al., 2016; Quadra et al., 2019; Thompson et al., 2020). The concern around this contamination is the effects of these metals, high toxicity, and persistence in the environment, in addition to bioaccumulation at trophic levels, which can affect human health (Chen et al., 2000). In addition, several metals have already been quantified in contaminated effluents in Brazil (Cruz et al., 2019; Silva et al., 2019; Souza et al., 2016).

Cadmium (Cd) is a non-essential metal, commonly found in freshwater environments (Arambawatta-Lekamge et al., 2021) and it is present in various human activities such as metal smelting and refining, urban traffic, waste incineration, mining, burning fossil fuels and in phosphate fertilizers (Samadani et al., 2018). It has been detected in several Brazilian freshwater environments at concentrations above the limit allowed by the National Environment Council (CONAMA), i.e., 0.001 mg L^{-1} of Cd – class 1 (Brazil, 2005): 0.00005 mg Cd L^{-1} in Vacacaí River (Kochhann et al., 2013); 0.007 mg L^{-1} of Cd in Paraopeba River after Brumadinho disaster (Thompson et al., 2020) and 0.033 mg L^{-1} of Cd in the Água Boa stream (Dourado et al., 2017).

Arambawatta-Lekamge et al. (2021) evaluated the toxicity of the metal in organisms of different trophic levels: *Chlorella vulgaris* (growth inhibition) – $EC_{50\text{-}72h}$: 4.23 mg L^{-1}; *Lemna perpusilla* (growth inhibition) – $EC_{50\text{-}7d}$: 0.405 mg L^{-1}; *Daphnia magna* (immobilization) – $EC_{50\text{-}48h}$: 0.045 mg L^{-1}; *Moina macropa* (immobilization) – $EC_{50\text{-}48h}$: 0.012 mg L^{-1}; *Poecilia reticulata* (mortality) – $LC_{50\text{-}96h}$: 0.076 mg L^{-1}, and *Duttaphrynus melanostictus* (mortality) – $LC_{50\text{-}96h}$: 0.431 mg L^{-1}. For the microalga *R. subcapitata* (growth inhibition) some values are – $IC_{50\text{-}96h}$: 0.075 mg L^{-1} (Dos Reis *et al.* 2022) and $IC_{50\text{-}72h}$: 0.036 mg L^{-1} (Alves *et al.* 2017). Previous studies that analyzed the effects of Cd on *R. subcapitata* observed an increase in the synthesis of carbohydrates (0.019 mg L^{-1}), total lipids (0.009 – 0.019 mg L^{-1}) and intracellular ROS (0.002 – 0.019 mg L^{-1}) (Alho *et al.* 2019); decrease in maximum performance and impairment of the oxygen evolution complex (F_0/F_v) – 0.100 and 0.125 mg L^{-1} (Dos Reis *et al.* 2021); loss of membrane integrity and decrease in chl *a* content – 0.21 mg L^{-1} (Machado *et al.* 2015); in addition to the increase in cell size and the interruption of the cell cycle after the second division of the nucleus and before cytokinesis – 0.21 mg L^{-1} of Cd (Machado & Soares 2014).

Nickel (Ni) is classified as an essential metal and its physical and chemical properties have led to its wide use in metallurgy (electroplating, nickel-cadmium batteries and alloy production); it is also used as a catalyst in the chemical and food industry. Consequently, the metal is found in the environment by the diffusion of its products in the manufacturing, recycling and disposal phases (Genchi et al., 2020). Ni were quantified at 0.004 mg L^{-1} in the Vacacai River (Kochhann et al., 2013); 0.013 mg L^{-1} of Ni in the Paraopeba River (Brumadinho disaster) (Thompson et al., 2020); and 0.296 mg L^{-1} of Ni in the Água Boa stream (Dourado et al., 2017). The last concentration exceeds the Ni limit allowed by CONAMA in freshwater, which is 0.25 mg L^{-1} of Ni (Brazil, 2005).

Several authors evaluated the toxicity of Ni at different trophic levels: the microalgae *Ankistrodesmus falcatus* (growth inhibition) – $IC_{50\text{-}96h}$: 0.017 mg L^{-1} (Martínez-Ruiz and Martínez-Jerónimo, 2015); the cladoceran *Daphnia magna* (immobilization) – $EC_{50\text{-}48h}$: 3.85 mg L^{-1} (Kim et al., 2017); the macrophyte *Spirodela polyrrhiza* (growth inhibition) – $EC_{50\text{-}14d}$: 0.9 mg L^{-1} (Montvydienė and Marčiulionienė, 2004); Chironomidae

Chironomus javanus (mortality) – $EC_{50\text{-}96h}$: 5.32 mg L^{-1} (Shuhaimi-Othman et al., 2011); the fish *Danio rerio* (mortality) – $LC_{50\text{-}96h}$: 24.3 mg L^{-1} (Alsop et al., 2014) and the frog *Duttaphrynus melanostictus* (mortality) – $EC_{50\text{-}96h}$: 8.8 mg L^{-1} (Shuhaimi-Othman et al., 2012). For the microalga *R. subcapitata* (growth inhibition) – $EC_{50\text{-}96h}$: 0.50 mg L^{-1} (Filová *et al.* 2021) and $IC_{50\text{-}96h}$: 0.675 mg L^{-1} (Mo *et al.* 2016). Filová *et al.* (2021) evaluated the toxicity of Ni to *R. subcapitata*, highlighting the decrease in the synthesis of chlorophyll *a* (0.10 – 1.50 mg L^{-1}), chlorophyll *b* (0.10 – 1.50 mg L^{-1}) and carotenoids (0.15 – 0.75 mg L^{-1}). In addition, the authors monitored the intensity of stress using the thiol group (-SH) and TBARS (thiobarbituric acid reactive substances) as factors, observing an increase in these indicators at 0.4 – 1.5 mg L^{-1} (-SH) and 0.25 – 1.5 mg L^{-1} (TBARS).

Since both metals are used in anthropic activities, they occur in the environment (Dourado et al., 2017; Kochhann et al., 2013; Thompson et al., 2020), therefore it is important to investigate what their combined effects and how they will affect organisms. To the best of our knowledge, a few studies have evaluated the effects of Cd-Ni on freshwater organisms, including: the diatom *Navicula pelliculosa* (Nagai and De Schamphelaere, 2016); the macrophyte *Lemna gibba* (Martinez et al., 2021); rainbow trout *Oncorhynchus mykiss* (Dew et al., 2016); zebrafish *Danio rerio* (Aldavood et al., 2020) and yellow perch *Perca flavescens* (Fadhlaoui and Couture, 2016). Therefore, there is a lack of knowledge about the combined effects of Cd and Ni on freshwater microalgae that analyze multiple endpoints.

Microalgae make up the base of the food chain as primary producers. Thus, any adverse effect caused by environmental pollutants on these organisms can reach higher trophic levels, including affecting human health (Wan et al., 2021). As a result, microalgae are widely used in ecotoxicological tests. The species used in this study was the Chlorophyceae *Raphidocelis subcapitata*, with characteristics that contribute to its wide use in this area, such as: easy cultivation in the laboratory, high sensitivity to contaminants and no formation of aggregates under cultivation and test conditions (Expósito et al., 2017). Thus, this study aimed to fill the knowledge gap by investigating the effects of combined Cd and Ni metals on multiple endpoints, such as density, cell size and complexity, chlorophyll *a* fluorescence, production of reactive oxygen species

(ROS), Φ_M (maximum quantum yield) and oxygen evolution complex from a freshwater green alga.

2. Material and methods

2.1. Test organisms and cultivation condition

The inoculum of the microalga *R. subcapitata* was obtained from the Department of Ecology and Evolutionary Biology (DEBE, Federal University of São Carlos - UFSCar, São Carlos - SP, Brazil) and cultured in CHU 12 medium (Chu, 1942) (Table S1), at 25 ± 1°C; with light intensity of ≅ 130 μmol photon m^{-2} s^{-1} (LED light) and in a 12h/12h of light/dark photoperiod. Culture medium was previously autoclaved (121°C, 1 atm above standard pressure, 20 min) and the pH was adjusted (7 ± 0.05) before inoculating the microalgae.

2.2. Toxicity tests

For toxicity tests, *R. subcapitata* was inoculated at 5 x 10^4 cells mL^{-1}, in the exponential growth phase, on 500 mL polycarbonate flasks containing 200 mL of test solutions. The microalgae were exposed to different concentrations of Cd (0.010; 0.025; 0.050; 0.075; 0.100 mg L^{-1}) and Ni (0.10; 0.15; 0.25; 0.35; 0.50 mg L^{-1}), in isolated and combined forms, all in triplicate, during 96 h. There was no metal addition in the control group, only culture medium. It was performed three toxicity tests in triplicate to analyze cell density, then we performed one test in triplicate for each metal concentration, control and mixture analyzing all the endpoints (cell density, ROS, F_0/F_v and Φ_M). Mixture tests were carried out using a partial fixed-ratio design (Cassee et al., 1998) to avoid the combination of high metal concentrations that could lead to cell death. This methodology calculates the concentrations of the metal mixture based on the expected toxic strengths, that is, 0.375 (0.125+0.25; 0.25+0.125), 0.5 (0.125+0.375; 0.25+0.25; 0.375+0.125), 0.75 (0.125+0.625; 0.25+0.5; 0.375+0.375; 0.5+0.25; 0.625+0.125), 1 (0.125+0.875; 0.25+0.75; 0.375+0.625; 0.5+0.5; 0.625+0.375; 0.75+0.25; 0.875+0.125), 1.5 (0.5+1; 0.75+0.75; 1+0.5), 1.75 (0.75+1; 1+0.75) and 2 (1+1) toxic units (TU) (Pérez *et al.* 2011;

Mansano *et al.* 2017). One TU was equal the IC50-96h calculated from single toxicity tests to Cd and Ni. The 23 combinations of metals used in the test are described in Table 1. We used Stock solutions of Cd (100 mg L^{-1}) and Ni (1000 mg L^{-1}), obtained from Titrisol standard solutions of $CdCl_2$ (1000 mg L^{-1} of Cd) and $NiCl_2$ (1000 mg $L^{-1}$of Ni) (Merck, Germany).

Table 1. Metal concentrations (mg L^{-1}) used in isolated and mixture toxicity tests. Concentrations of Cd1 to Cd5 and Ni1 to Ni5 refer to isolated tests, while M1 to M23 refer to mixture tests and the C = control group.

	Metal concentration (mg L^{-1})	
	Cd	Ni
C	0	0
Cd1	0.010	0
Cd2	0.025	0
Cd3	0.050	0
Cd4	0.075	0
Cd5	0.100	0
Ni1	0	0.10
Ni2	0	0.15
Ni3	0	0.25
Ni4	0	0.35
Ni5	0	0.50
M1	0.006	0.063
M2	0.006	0.094
M3	0.006	0.156
M4	0.006	0.219
M5	0.013	0.031
M6	0.013	0.063
M7	0.013	0.125
M8	0.013	0.188
M9	0.019	0.031
M10	0.019	0.094
M11	0.019	0.156
M12	0.025	0.063
M13	0.025	0.125
M14	0.025	0.250
M15	0.031	0.031
M16	0.031	0.094
M17	0.038	0.063

M18	0.038	0.188
M19	0.038	0.250
M20	0.044	0.031
M21	0.050	0.125
M22	0.050	0.188
M23	0.050	0.250

2.3. Metal determination

Metal determination was carried out in the stock solutions using inductively coupled plasma optical emission spectrometry (ICP OES, iCAP 7000 - Thermo Fischer Scientific, Madison, WI, EUA). The results are available in Table S2. The nominal concentrations were maintained as they did not vary more than 20% from the measured concentrations (OECD, 2002). The detection limit was 0.0002 mg L^{-1} of Cd and 0.005 mg L^{-1} of Ni; while the limit of quantification was 0.01 mg L^{-1} of Cd and 0.1 mg L^{-1} of Ni.

2.4. Flow cytometry analysis

For cell density, control and treatment samples were fixed with formaldehyde buffered with borax (1% final concentration), left in the dark for 10 minutes and frozen in liquid nitrogen, daily.

The relative ROS determination was done according to Hong et al. (2009). Samples (495 µL) were collected after 96h of exposure, and 5 µL of DCFH-DA (2′,7′-Dichlorofluorescein diacetate, CAS number 2044-85-1, Sigma Aldrich) diluted in dimethylsulfoxide (10^4 µM) were added (final concentration of 10 µM). The samples were kept in the dark for 1 h and then analyzed by flow cytometry.

Cell counting and relative ROS were performed in a FACSCalibur flow cytometer (Becton & Dickinson Franklin Lakes, NJ, U.S.A.) equipped with a 15 mW Argon-ion laser (emission 488 nm), adding 10 µL of 6 µm fluorescent beads (Fluoresbrite carboxylate microspheres; Polysciences, Inc., Warrington, PA, USA.) as the internal standard.

Algal cells were identified using SSC-H (lateral dispersion) versus FL3-H (red fluorescence), according to Sarmento et al. (2008) procedures. Mean values of FSC-H; FL3-H and SSC-H correspond to the cell size; cellular chlorophyll *a* (Chl *a*) fluorescence and cellular complexity, and were calculated as described in Mansano et al. (2017). Relative ROS were calculated by the equations (1 and 2) described below (Hong et al., 2009):

$\text{FL1-H}_{\text{relative}} = \log(\text{FL1-H}_{\text{samples}}) / \log(\text{FL1-H}_{\text{beads}})$ (1)

$\text{ROS}_{\text{relative}}(\%) = (\text{FL1-H}_{\text{relative[treatments]}} / \text{FL1-H}_{\text{relative[control group]}}) \text{ X } 100$ (2)

The data were analyzed by FlowJo software, version V10.0 (Treestar.com, USA).

2.5. Analysis of the photosynthetic activity

After 96 h of exposure, samples (3 mL) were collected from all treatments and remained 15 min in the dark. To analyze the photosynthetic activity, measurements of the maximum quantum yield (Φ_M) and efficiency of the oxygen evolving complex (OEC) were obtained by pulse amplitude modulated fluorometer (PHYTO-PAM® Fluorometer Analyzer, Heinz Walz, Germany). The equipment provides the values of F_0 (minimum fluorescence), F_m (maximum fluorescence) and Φ_M (Genty et al., 1989); while the efficiency of OEC was calculated by the equation F_0/F_v (where $F_v = F_m - F_0$) (Kriedemann et al., 1985).

2.6. Data analysis

Statistical analyses were performed using SigmaPlot 12.0 software. Normal data were compared using ANOVA (one-way analysis of variance) and Dunnett's post-hoc test; and non-normal data were tested by the Kruskal-Wallis test and Dunn's post-hoc test. Statistical significances were considered when $p < 0.05$. Uniquely for the ROS mixture data, we normalized these data using log transformation. The values of IC_{50}-96h (cell density data) were performed using nonlinear regression, with a sigmoidal three-parameter logistic curve in the SigmaPlot 12.0 software.

Mixture test data (cell density) were analyzed by the MIXTOX tool (Jonker et al., 2005), through the CA (concentration addition) and IA (independent action) reference models. Then, the deviations were analyzed: synergism/antagonism (S/A), dose ratio-dependent (DR), dose level dependent (DL), by adding two parameters (‘‘a’’ and ‘‘b’’). In the S/A deviation, parameter “a” becomes positive to antagonism and negative to synergism. In the DR deviation, the addition of the parameter b_{DR} indicates that the deviation from the reference model is controlled by the composition of the mixture. In the DL deviation, the value of “a” determines the deviation ($a > 0$ = antagonism; while $a < 0$ = synergism), and the b_{DL} parameter indicates at which dose level the deviation changes. Details and additional information on these deviation functions are available in Jonker et al. (2005). After the evaluation, the best fit was chosen by the maximum likelihood method and the best deviation was defined statistically.

3. Results and discussion

3.1. Cell density

Our data showed that Cd interfered with cell density after 48h of exposure to the metal (Fig. 1A). The statistically significant decrease occurs at 72h at the two highest concentrations tested, 0.075 and 0.100 mg L^{-1}. After 96h of exposure, the metal decreased cell density at 0.025; 0.050; 0.075 and 0.100 mg L^{-1} of Cd. Effects of Ni on cell density (Fig. 1B) also appear within 48h of exposure, however, in this case, the concentration of 0.35 mg L^{-1} of Ni contributes to the increase in cell density. In 72h and 96h of exposure, the concentration of 0.50 mg L^{-1} significantly reduces the cell density of *R. subcapitata*.

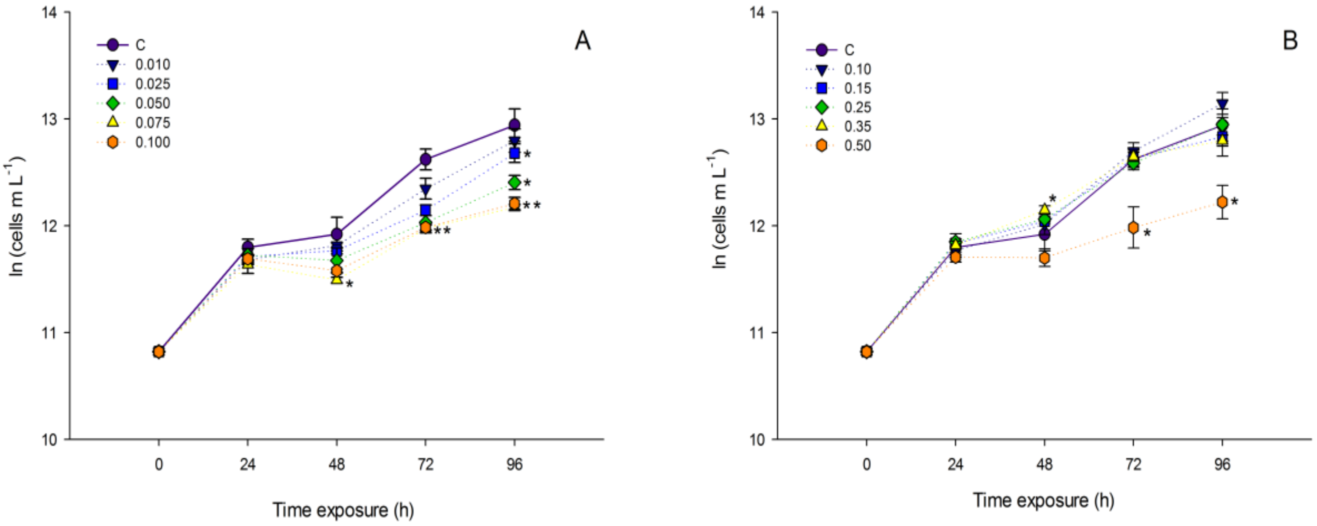

Fig.1. Cell density of *R. subcapitata* ((ln (cells mL^{-1})) exposed to Cd (A) and Ni (B) during 96h. The asterisks indicate significant difference to the control group ($p < 0.05$). Metal concentrations are expressed in mg L^{-1}; C = control group.

The decrease in cell density caused by Cd can occur by the action of the metal on the membrane potential, alteration of the photosynthetic and enzymatic activity of the cell (Pistocchi et al., 2000). Previous studies have also reported the negative effect of the metal on the growth of different species of microalgae such as *Selenastrum gracile* (Rocha et al., 2020); *Scenedesmus acutus* and *Chlorella pyrenoidosa* (Chandrashekharaiah et al., 2021). Čypaitė *et al.* (2014) observed growth inhibition of *R. subcapitata* at 0.001 mg L^{-1} of Cd, and the two highest concentrations tested, 0.1 and 1 mg L^{-1}, caused cell death; Alho *et al.* (2019) observed that 0.019 mg L^{-1} of Cd was sufficient to completely inhibit the growth of *R. subcapitata*.

Nickel (Ni) is an essential nutrient for phytoplankton metabolism, although it has not been found to be essential to the *R. subcapitata* species (Filová et al., 2021). The entry of Ni into the cell can occur through ion exchange, specific transporters or transient receptor potential channels, and concentrations above those necessary for organisms can cause toxic effects (Martínez-Ruiz and Martínez-Jerónimo, 2015). In addition, the effects of Ni on the growth have already been reported to *Ankistrodesmus falcatus* (Martínez-Ruiz and Martínez-Jerónimo, 2015); *Scenedesmus vacuolatus*, *Dunaliella bioculata*, *Chlorella vulgaris* and *Chlorella sorokiniana* (Haiduc et al., 2009). Filová *et al.* (2021) observed that after 48h of Ni exposure at 0.494, 0.741, and 1.482 mg L^{-1}, decreased *R. subcapitata* growth.

3.2. Toxicity Tests

Data from mixture toxicity tests (96h cell density) were analyzed by the MIXTOX tool and all parameters and significance results were presented in Table 2. Regarding our data, both models, CA (concentration addition) and IA (independent action), were statistically validated, although the model that best fitted to Cd-Ni data was IA. For the CA model (Table 2), the sum of squared residuals (SS) was 7.43 ($p < 0.05$; $r^2 = 0.77$). After adding the “a” parameter in synergism/antagonism (S/A) deviation, the SS value decreased to 7.22 ($p = 0.32$; $r^2 = 0.78$), with no significance. In dose-ratio-dependent (DR) deviation, parameters “a” and “bDR” were added and there was a decrease in SS to 6.05 ($p = 0.03$; $r^2 = 0.81$) and it was the best deviation for the CA model. In the dose-level dependent (DL) deviation, the SS value was 5.79 ($p = 0.05$; $r^2 = 0.81$) with no significance.

For the IA model (Table 2), the SS value was 7.38 ($p < 0.05$; $r^2 = 0.77$). Parameter “a” was added in S/A deviation and the SS decreased to 7.36 ($p = 0.79$; $r^2 = 0.77$), with no significance. In DR deviation, parameters “a” and “bDR” were added and the SS value decreased to 6.42 ($p = 0.09$; $r^2 = 0.80$), although with no significance. In the DL deviation, the SS value was 4.74 ($p < 0.05$; $r^2 = 0.85$) and it was the best deviation for the IA model. Thus, IA was the model with DL deviation that best explains our mixture data (significant p value, lower SS and higher r^2 value), indicating that there was antagonism at low doses and synergism at high doses ($a > 0$) and change from the 'antagonism to synergism dose level higher than IC_{50} ($1 < b_{DL} < 2$). Based on that, synergism occurs at dose levels above IC_{50} values for Cd (0.06 mg L^{-1}) and Ni (0.46 mg L^{-1}) (Fig. 2).

Table 2. Data from reference models of concentration and independent action applied to cell density of *Raphidocelis subcapitata* exposed to mixtures of Cd and Ni during 96 h.

	Concentration addition				Independent action			
	CA	S/A	DR	DL	IA	S/A	DR	DL
Max	4.52	4.56	4.6	4.67	4.83	4.88	4.85	4.76
β Cd	1.69	1.64	1.66	0.81	1.36	1.28	1.42	0.4
β Ni	3.77	3.5	4.64	2.68	2.16	2.09	2.49	2.2
IC$_{50}$ for Cd	0.07	0.07	0.06	0.08	0.06	0.06	0.05	0.08
IC$_{50}$ for Ni	0.47	0.46	0.46	0.46	0.43	0.43	0.45	0.46
A	-	0.46	-2.38	6.98	-	0.24	-3.3	12.48
$b_{DR/DL}$	-	-	5.44	1.02	-	-	5.75	1.94
SS	7.43	7.22	6.05	6.27	7.38	7.36	6.42	4.74
r^2	0.77	0.78	0.81	0.81	0.77	0.77	0.8	0.85
χ^2 or F test	24.28	0.99	7.01	5.79	24.51	0.07	4.73	15.01
Df	-	1	2	2	-	1	2	2
p (χ^2/F)	6.74×10^{-9}	0.32	0.03	0.05	6.08×10^{-9}	0.79	0.09	5×10^{-4}

These data were obtained from methodology used in Jonker et al. (2005). Max = maximum response value, β = slope response of isolated compounds, IC$_{50}$ = median growth inhibition concentration; a and $b_{DR/DL}$ = function parameters; SS = sum of the squared residuals; r^2 = regression coefficient; χ^2 or F test = statistical test; df = degrees of freedom; *p*(χ^2/F) = level of significance of statistical test; CA = concentration addition model; IA = independent action model; S/A = synergism/antagonism deviation; DR = dose ratio-dependent deviation; DL = dose level-dependent deviation.

Cd and Ni concentrations may vary in aquatic environments, e.g.: 0.1 mg L^{-1} of Ni (Souza et al., 2016) to 1.08 mg L^{-1} of Ni (de Carvalho et al., 2017); and 0.017 mg L^{-1} of Cd (Manfrin et al., 2021) to 0.047 mg L^{-1} of Cd (Dourado et al., 2017). In addition, these are the main metals responsible for freshwater contamination by mining, smelting, and atmospheric deposition in Canada (Bougas et al., 2013). The combination of metals can change their toxicity, differing from the isolated effect, due to the interaction that can occur in their absorption (Batool and Javed, 2015). In our study, the results of the mixture of concentrations close to those indicate synergism (Fig. 2), highlighting the importance of studying mixtures of these metals as they co-occur in the environment at relevant concentrations.

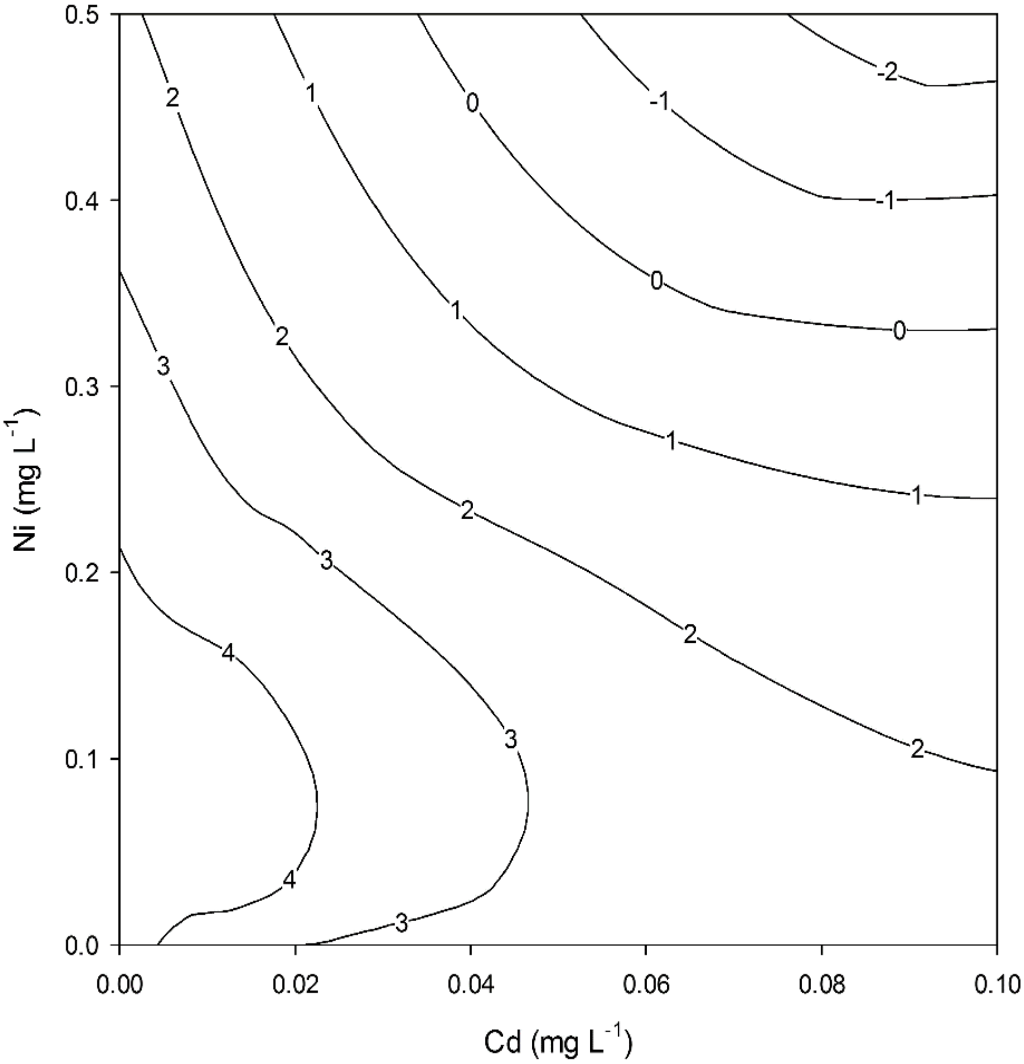

Fig. 2. Isobologram of *R. subcapitata* (cell density) exposed to Cd and Ni mixtures, following the IA (independent action) model and DL (dose-level dependent). Concave and convex isoboles represent synergism and antagonism, respectively.

CA model indicates that the mode of action of the compounds is the same, while the IA model assumes that the mode of action is different (De Zwart and Posthuma, 2005). Nagai and De Schamphelaere (2016) evaluated the effects of binary mixtures of four metals (Zn, Cu, Cd, Ni) on the diatom *Navicula pelliculosa*. For the Cd-Ni mixture, the model that best represented their results was also IA, however they observed only an antagonistic effect, suggesting that the metals have different modes of action and the effects of the mixtures are species-specific. Mo et al., 2016 analyzed Cd-Ni effects on *Chlorella pyrenoidosa* and *Selenastrum capricornutum* (the previous name of *Raphidocelis* *subcapitata*). For *C. pyrenoidosa*, they observed antagonism and synergism effects; and for *S. capricornutum*, they observed the three possible interactions, i.e., antagonism and synergism (as observed in the present study), and additive effect.

Our works differ in some points, Mo et al. (2016) analyzed the Cd-Ni mixture only by the concentration addition (CA) model. The authors observed that in the area where the synergistic effect had an increasing tendency, the Ni concentration proportion decreases with the increase of the Cd concentration proportion. Furthermore, the work was carried out in microplates, the culture medium was BG11 and the metal used was $Ni(NO_3)_2$. In this work, the Cd-Ni mixture was evaluated by the CA and IA models, and our data best fitted to the IA model, noting that the synergism occurs at dose levels above IC_{50} values for Cd and Ni. The tests were performed with a volume of 200mL in Erlenmeyer flasks, CHU 12 culture medium and nickel chloride ($NiCl_2$). Therefore, even if these experiments were performed with *R. subcapitata* our results differ because methodology, experimental design, culture medium and Ni source were different.

Interactions between compounds can affect processes such as bioavailability, absorption, internal transport, metabolism, binding at the target site and excretion, which interfere with the resulting toxicity of the compounds (Cedergreen, 2014). Therefore, synergistic interactions are likely due to alterations caused by the metals in one or more of these processes. Bioavailability is commonly altered by metals, i.e., one can interfere with the availability and also the composition of the other. Furthermore, one can alter the absorption of the other by competing for biological ligands or competitive inhibition of transport proteins, which are normally observed for metallic interactions (Cedergreen, 2014).

3.3. Flow cytometry

Cd exposure caused morphological (cell size – FSC-H, and cell complexity – SSC-H), and physiological (cellular Chl *a* fluorescence – FL3-H) changes. It led to an increase in all parameters, cell size (Fig. 3A); cell complexity (Fig. 3B) and cellular Chl *a* fluorescence (Fig. 3C). On the other hand, Ni exposure only decreased cellular Chl *a* fluorescence (Fig.3C); cell size (Fig. 3A) and complexity (Fig. 3B) were not altered significantly. When Cd and Ni were combined at different concentrations, we have a statistically significant increase in cell size (Fig.3A) in the M5; M12; M13; M14; M15; M16; M17; M18; M19; M20; M22 and M23 combinations.

Concerning the cellular complexity (Fig. 3B), metal mixtures in low concentrations of Cd and varied concentrations of Ni did not show a significant alteration. On the other hand, a significant increase in M14; M19; M20; M22 and M23 treatments was observed, i.e., concentrations of 0.025 to 0.05 mg L^{-1} of Cd (intermediate to higher concentrations) combined with different concentrations of Ni (from 0.03 to 0.25 mg L^{-1}), indicating that probably the Cd at higher concentrations induces an increase in this parameter.

The response of the mixed metals in Chl *a* fluorescence (FL3-H; Fig. 3C) varied according to the metal concentration. Treatments M5 and M7 (statistically different from the control group) have the same Cd concentration (0.0125 mg L^{-1}) combined with increasing Ni concentrations and had opposite results. M5 (lower Ni concentration) significantly increased Chl *a* fluorescence, while M7 (higher Ni concentration) decreased it. We have similar behavior in the M11 treatment. With the increase in Cd concentration, from the M14 treatment, the results showed an increase in the Chl *a* fluorescence, as seen in M19, M20, M22, M23, indicating that at higher concentrations, the effect of a metal potentiates the other.

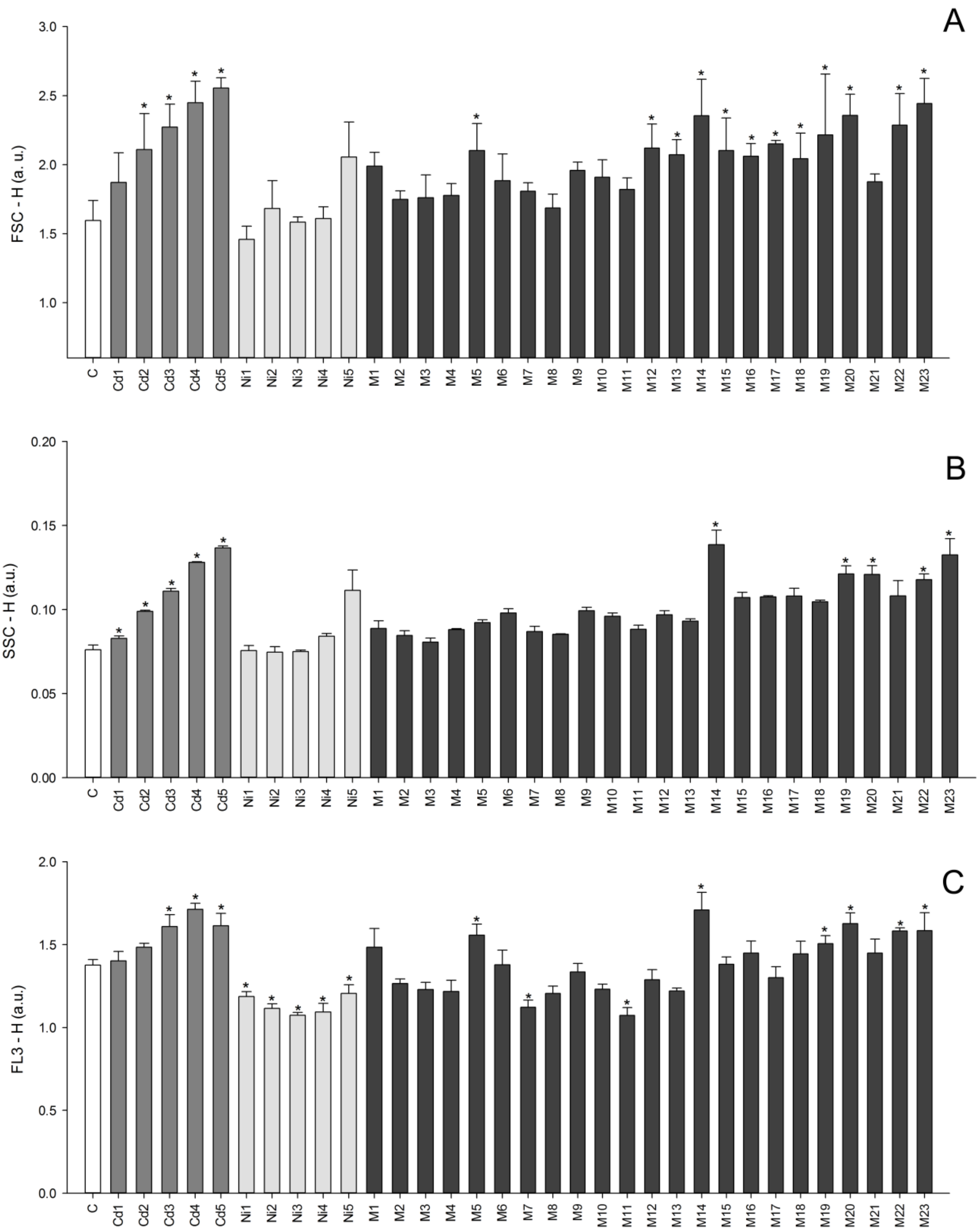

Fig. 3. Cell size (FSC-H) (A), cellular complexity (SSC-H) (B) and chlorophyll *a* fluorescence (FL3-H) (C) of *R. subcapitata* exposed to Cd and Ni, isolated (Cd1-Cd5 and Ni1-Ni5) and combined (M1-M23) after 96h of exposure. The asterisks indicate a significant difference (p <0.05) when compared to the control group.

The increase in cell size (FSC-H) indicates impairment in the cell division process (Gebara et al., 2020) and occurs due to different factors. Membrane potential can be altered by the metal, causing damage at the end of cell division, leading to an accumulation of products of photosynthetic activity, increasing cell size (Franklin et al., 2001). Furthermore, high levels of oxidative radicals can lead to failures in cell volume regulation (Jamers et al., 2013) and the cell cycle can be interrupted before the division step (Machado and Soares, 2014). Previous studies with the microalgae *R. subcapitata* also observed an increase in cell size when exposed to 0.005; 0.010; 0.015 mg L^{-1} of Zn; 0.67; 0.89; 1.11 mg L^{-1} of Al (Gebara *et al.* 2020); 0.4; 0.8 mg L^{-1} of Mn and 0.2 mg L^{-1} of Cr (Alho *et al.* 2022). Jamers *et al.* (2009) exposed *Chlamydomonas reinhardtii* to Cd and observed an increase in cell size at 0.56 mg L^{-1}.

Franklin *et al.* (2001) observed an increase in cell size in *Selenastrum capricornutum* (*R. subcapitata*) at 24h, 48h and 72h of exposure to copper (Cu). The greatest increase (up to 45% greater than the control) occurred at 0.03 mg L^{-1} of Cu. The authors argued that the increase in cell size may result in an increased cell permeability or an uncoupling of cell division and photosynthesis. Then, the cells continue the process of carbon fixation, however, as they cannot complete the division, they accumulate the products of photosynthesis. Our results support the hypothesis that Cd compromised the cell division process as the metal affected cell density, induced ROS formation, and compromised photosynthetic yield only at the highest concentration tested. In addition, this parameter was the most affected by the mixture of Cd-Ni, indicating that the combined metals can interfere with the cell division process.

The SSC-H parameter points to the complexity and internal granularity of the cell. Jamers *et al.* (2009) observed an increase in this parameter in the exposure of *Chlamydomonas reinhardtii* at 0.56 and 11.2 mg L^{-1} of Cd. The authors argued that this could be due to ultrastructural changes inside the cell. Previous studies exposed the microalgae *Dunaliella minuta* (Visviki & Rachlin 1992) and *Skeletonema costatum* (Nassiri *et al.* 1997) to Cu and Cd and observed the appearance of cytoplasmic vacuoles, arguing that this response possibly occurs due to an osmotic disorganization caused by the alterations suffered by the membrane in the exposure to metals. Nassiri *et al.* (1997) observed that Cd was captured in vacuoles enriched with sulfur and nitrogen, proposing that the metal was possibly bound to organic molecules to decrease its concentration in the cytoplasm.

Nishikawa *et al.* (2003) exposed the microalgae *Chlamydomonas acidophila* at 2.25 mg L^{-1} of Cd after 72h and observed an increase in starch granules and vacuoles. Aguilera and Amils (2005) also observed an increase in the volume and number of cytoplasmic vacuoles when *Chlamydomonas sp* was exposed to Cd. Therefore, the increase in cellular complexity (SSC-H) may indicate the accumulation of metal internalized by the microalgae cells. It is a defense mechanism to deal with the presence of metals in order to minimize (reduce or prevent) their toxic effects (Juarez *et al.* 2008).

Based on that, the results obtained under exposure to Cd suggest that *R. subcapitata* cells internalized the metal seeking to minimize its damage, at all concentrations tested. Ni did not change the cellular complexity, indicating that the cells did not present this defense mechanism, and probably activated another protective mechanism, such as extracellular adsorption (Juarez *et al.* 2008). Although Ni did not significantly change this parameter, the mixture of Cd-Ni, at M14, M19, M20, M22 and M23 treatments indicates that the metals were possibly internalized by the microalgae, activating the defense mechanism.

Decreased Chl *a* fluorescence (FL3-H) may indicate damage to photosynthetic processes (Alho et al., 2022). Studies indicate that Ni can interfere with photosynthesis, inhibits electron transport, decreases yield, as it can replace Mg in the chlorophyll molecule and also compete with Ca^{2+} at its binding site (Baumann et al., 2009; Boisvert et al., 2007). On the other hand, exposure to Cd led to increased fluorescence of Chl *a*. This result indicates that the cells used a strategy to increase the photosynthetic rate, enhancing light capture, even under stress conditions (Alho et al., 2019; Dos Reis et al., 2022; Mansano et al., 2017). Mixing the metals increased (M5, M14, M19, M20, M22 and M23) or decreased (M7 and M11) Chl *a* fluorescence. The increase in fluorescence and the strategy of increasing the photosynthetic rate corroborate the data obtained as the mixture of metals did not affect the Φ_M - maximum quantum yield (Fig. 5A). On the other hand, although the decrease in fluorescence indicates some damage to the photosynthetic process, our results did not show damage to the Φ_M and F_0/F_v, which suggest that the combined metals affected another element of the photosynthetic process.

3.4. ROS measurements

Cd significantly increased the percentage of ROS at all concentrations tested (Fig. 4); while Ni decreased the percentage of ROS in the first concentrations, 0.10; 0.15 and 0.25 mg L^{-1}; while at the highest concentration, 0.50 mg L^{-1} of Ni, the opposite behavior, was observed, with an increase in the percentage of ROS (Fig. 4). All results were compared to the control group. In the metal mixture, we have a decrease in the percentage of ROS at low concentrations of Cd and varied concentrations of Ni, in M2; M3; M10 and M15. On the other hand, when Cd concentrations increased combined with varying Ni concentrations, there was an increase in the percentage of ROS in M13; M16; M20; M21 and M23.

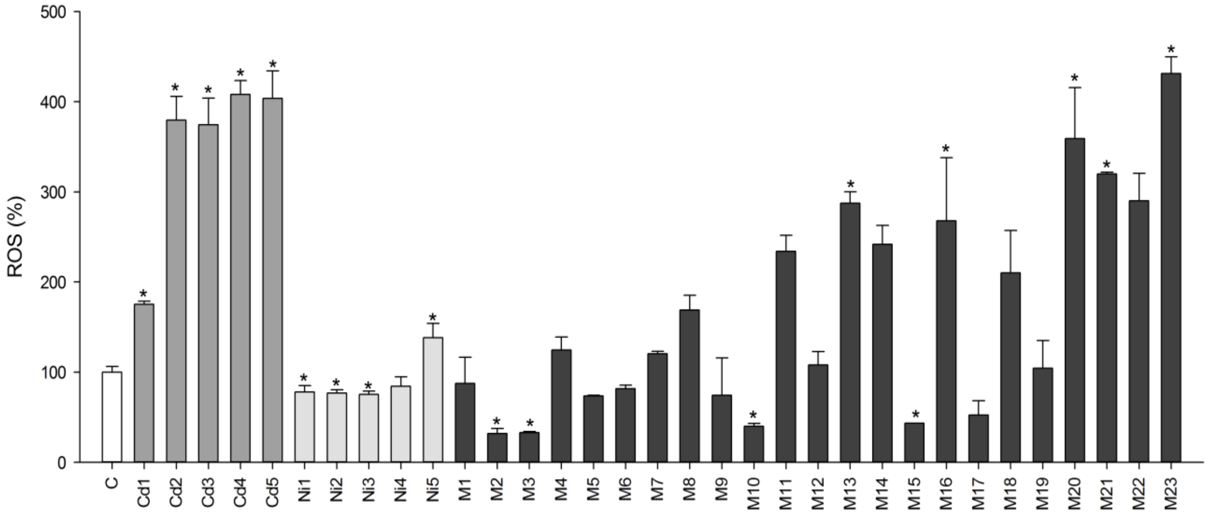

Fig. 4. Cellular reactive oxygen species (ROS) produced by *R. subcapitata* exposed to Cd and Ni, isolated (Cd1-Cd5; and Ni1-Ni5) and combined (M1-M23) after 96h of exposure. Metal concentrations are described in table 1. Asterisks represent significant differences from the control group ($p < 0.05$).

Metals can induce oxidative stress by directly increasing ROS formation and decreasing the antioxidant capacity of cells. ROS can be signaling molecules, altering gene expression and modulating the activity of proteins, however they can be very harmful to organisms in high concentrations as they are capable of oxidizing lipids, nucleic acids and proteins, which can lead to changes in structure, mutagenesis (Pinto et al., 2003), as well as damage to the photosynthetic apparatus and cell death (Sharma et al., 2012).

Machado and Soares (2016) observed an increase in ROS production in exposed *R. subcapitata* to 0.21 mg L^{-1} of Cd, suggesting that the increase in intracellular ROS stimulates the increase of antioxidant defenses seeking to repair the imbalance caused by oxidative stress. Supporting this claim, the authors observed that oxidative stress caused by H_2O_2, Cd, Cu and Zn increased glutathione (GSH) synthesis. Sziválk et al. (2009) also observed an increase in ROS production in Cd exposure to *Chlamydomonas reinhardtii*, suggesting that the effect may have occurred due to the accumulation of H_2O_2 and consequent oxidative stress, together with lipid peroxidation and protein carbonylation in algae cells. Ni increases ROS production and leads to oxidative stress in aquatic organisms (Brix et al., 2017) and different plant species (García-García et al., 2018), which are able to trigger lipid peroxidation and plasma membrane damage (Zheng et al., 2013).

Gebara *et al.* (2020) and Alho *et al.* (2022) observed a decrease in intracellular ROS to *R. subcapitata* at 0.03 mg L^{-1} of Zn (after 5h of exposure) and 0.4; 1.0 mg L^{-1} of Cr (after 48 of exposure) respectively. These results suggest that this may be due to the antioxidant mechanisms that algae have, such as low molecular weight compounds and enzymes (ascorbate, glutathione, ascorbate peroxidase, catalase, glutathione peroxide and superoxide dismutase) that allow algae to decrease their intracellular content of ROS (Pinto *et al.* 2003). Supporting this argument, Martínez-Ruiz and Martínez-Jerónimo (2015) observed an increase in antioxidant enzymes (e.g., catalase, glutathione peroxidase, superoxide dismutase) when exposing the microalgae *Ankistrodesmus falcatus* to different Ni concentrations (0.001 – 0.017 mg L^{-1}) for 96h. In our study, the combination of Ni and Cd resulted in both behaviors, i.e., under exposure to some mixtures, the microalgae were able to activate the antioxidant mechanisms, decreasing ROS; however, some mixtures caused an oxidative stress, increasing ROS, possibly damaging the cellular structure and cell death.

3.5. Photosynthetic parameters

Our data to single exposure showed that Cd at 0.010 and 0.025 mg L^{-1} contributed to an increase in Φ_M, while at 0.100 mg L^{-1} decreased this parameter, compared to the control (Fig. 5A). On the other hand, Ni exposure did not significantly alter the Φ_M (Fig. 5A).

The combination of Cd-Ni did not lead to changes in this parameter (Fig. 5A). Regarding F_0/F_v in the single exposure, the metals, increased F_0/F_v at the highest concentrations tested, 0.100 mg L^{-1} of Cd and 0.50 mg L^{-1} of Ni (Fig. 5B). On the other hand, mixture tests did not significantly alter this parameter (Fig. 5B).

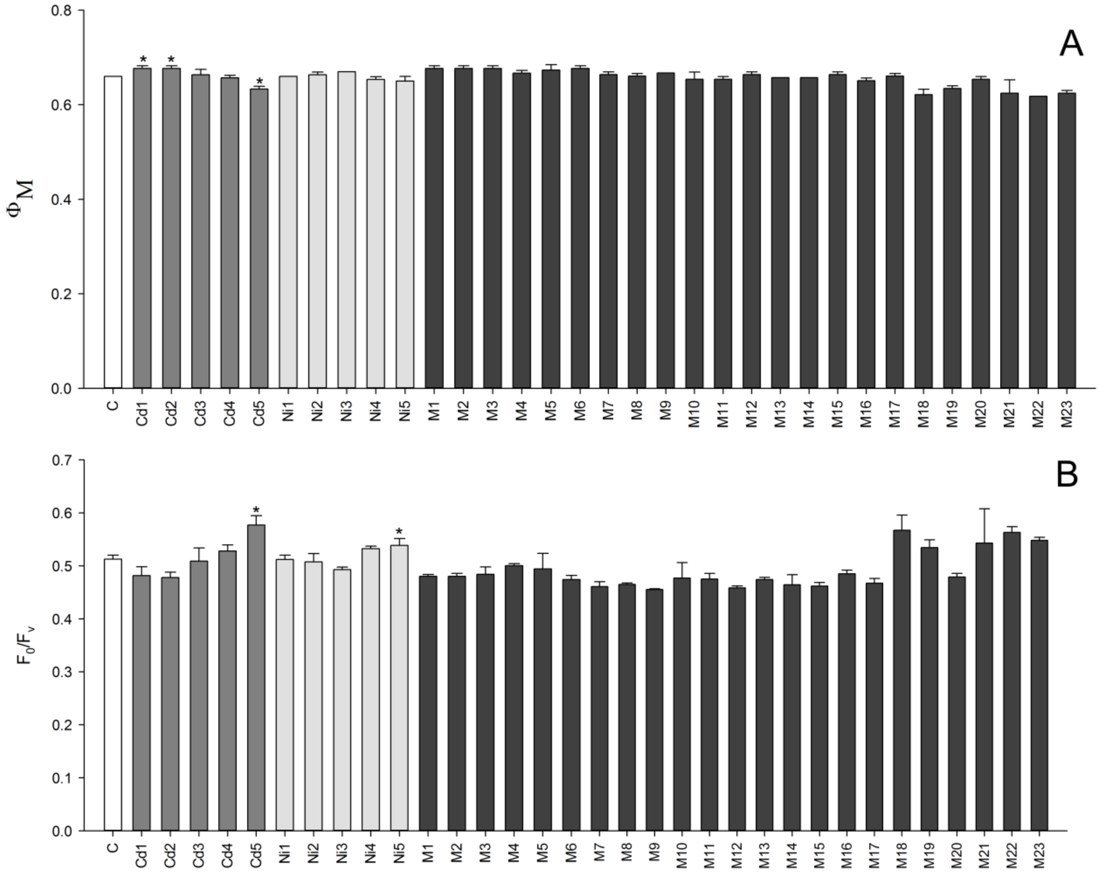

Fig. 5. Maximum quantum yield (Φ_M) (A) and measurement of the efficiency of Oxygen Evolving Complex (F_0/F_V) (B) of *R. subcapitata* exposed to Cd and Ni isolated (Cd1-Cd5 and Ni1-Ni5) and combined (M1-M23), after 96h of treatment. The symbol * means the significant difference ($p < 0.05$) when compared to the control group.

The maximum quantum yield (Φ_M) indicates the amount of light used in photosynthesis, providing information about the physiological state of algae (Herlory et al., 2013). The F_0/F_V ratio indicates the efficiency of the oxygen evolution complex (OEC), which in turn, is the place where the hydrolysis reaction, water molecule breakdown and oxygen production occur. The increase in the values of this ratio means that the system has been affected by the metals (Herlory et al., 2013). Photosynthetic parameters are sensitive to metal exposure and can lead to inhibition of chlorophyll synthesis, energy capture and transfer, and photochemistry quantum yield of photosystem II (PSII) (Wang et al., 2013). In addition, some metal ions (Hg - mercury, Cd, Zn, Ni, Cu) are capable of replacing magnesium – the central atom of the chlorophyll molecule – interfering with the photosynthetic process, forming an unstable molecule (Baumann et al., 2009).

Cd shows toxicity to PSII as it can interfere with the acceptor or donor side and also inhibit the activity of the oxygen evolution complex (F_0/F_v). In addition, the metal interferes with enzymatic activity, inhibiting or inactivating its activity, through bonds in functional groups, which can cause inhibition of growth, photosynthesis and respiration in algae and plant cells (Wang et al., 2013). Our data indicate that Cd were capable of doing this only at the 0.100 mg L^{-1}, where the Φ_M decreased and F_0/F_v increased in comparison to control group. In addition, previous studies also reported changes in the Φ_M and F_0/F_v to Cd exposure to some microalgae: *Chlorella pyrenoidosa* (Wang et al., 2013); *Raphidocelis subcapitata* (Alho et al., 2019; Machado et al., 2015), *Selenastrum gracile* (Rocha et al., 2020) and *Ankistrodesmus densus* (Rocha et al., 2021).

Ni is able to reach the PSII, inhibit electron transport and consequently decrease the maximum yield. The metal acts on the active site of the OEC, competing with the Ca^{2+} binding site. The inhibitory action of the metal may also be related to the removal of OEC associated polypeptides from PSII, which causes a conformational change causing membrane dissociation and inhibition of electron transport (Boisvert et al., 2007). Our results indicate that Ni concentrations tested did not affect the potential of microalgae to perform photosynthesis (Φ_M), and that F_0/F_v only changed at the maximum concentration tested.

To the best of our knowledge, this is the first study that investigates the effect of Ni-Cd metal mixture to photosynthetic parameters (Φ_M and F_0/F_v) in *R. subcapitata*. Our results indicate that the metal mixture did not change the photosynthetic parameters analyzed, showing an antagonistic interaction. Other studies have also observed this effect. The Cd-Co mixture also showed antagonistic effects in the evaluation of Φ_M and F_0/F_v in *R. subcapitata* (Dos Reis et al., 2022); however, isolated Co negatively affected the endpoints. The effects of the metals Cd, Cu, Mn, Ni and Zn, isolated and combined, showed an antagonistic effect for Φ_M from *Elodea densa*. On the other hand, the isolated metals Cd and Cu affected this parameter (Maleva et al., 2012).

4. Conclusion

In general, our study shows that Cd negatively affected cell density and photosynthetic parameters of *R. subcapitata*. Cd also increased ROS production, Chl *a* fluorescence, cell size and complexity. Ni exposure decreased cell density only at the highest concentration tested, altered ROS production and decreased Chl *a* fluorescence. Concerning the photosynthetic parameters, Ni only affected F_0/F_v. For the metal mixture, IA was the model that best fitted to our data with DL deviation, indicating antagonism at low doses and synergism at high doses, with dose level change greater than IC_{50}. In addition, Cd-Ni mixture caused morphological and structural changes, increasing cell size and cellular complexity; and affecting the fluorescence of Chl *a* and ROS production. On the other hand, the photosynthetic parameters analyzed were not altered by the mixture of metals. Thus, we reinforce the importance of investigating the effects of metals in mixtures, as they can be different or potentiated, in addition to highlighting the relevance of studies with microalgae since they are organisms sensitive to contaminants and the base of the food chain, i.e., the effects in these organisms can be transferred to higher trophic levels. In addition, it can help develop strategies and guidelines to reduce the impacts of metal mixtures on aquatic ecosystems.

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Supplementary Material

Table S1. CHU 12 culture medium composition (Chu, 1942).

Reagent	Concentration (g L^{-1})
$Ca(NO_3)_2.4H_2O$	4.3
K_2HPO_4	0.5
$MgSO_4.7H_2O$	7.5
KCl	0.5
Na_2CO_3	2
$FeCl_3.6H2O$	0.05

Table S2. Comparison between nominal and measured concentrations of cadmium (Cd) and nickel (Ni) in the stock solutions, quantified by inductively coupled plasma optical emission spectrometry (ICP OES, iCAP 7000 - Thermo Fischer Scientific, Madison, WI, USA).

Metal	**Nominal concentration (mg L^{-1})**	**Measured concentration (mg L^{-1})**
Cd	100.00	86.90 ± 0.039
Ni	1000.00	956.0 ± 0.109

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Capítulo 3. Isolated and combined effects of cobalt and nickel on the algae *Raphidocelis subcapitata*

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Abstract

Aquatic organisms are exposed to several compounds that occur in mixtures in the environment. Thus, it is important to investigate their effects to organisms because these combined effects can be potentiated. Cobalt (Co) and nickel (Ni) are metals that occur in the environment and are used in human activities. To the best of our knowledge, there are no studies that investigated the effects of mixtures of these metals on a freshwater Chlorophyceae. Therefore, this study analyzed the isolated and combined effects of Co and Ni in cell density, physiological and morphological parameters, reactive oxygen species (ROS), carbohydrates and photosynthetic parameters of the microalga *Raphidocelis subcapitata*. The data obtained showed that Co affected the cell density from 0.25 mg Co L^{-1}; the fluorescence of chlorophyll *a* (Chl *a*) at 0.10 mg Co L^{-1}; ROS production at 0.50 mg Co L^{-1}, total carbohydrates and efficiency of the oxygen evolving complex (OEC) at all tested concentrations; and the maximum quantum yield (Φ_M) from 0.50 mg Co L^{-1}. Ni exposure did not alter photosynthetic parameters, but it decreased ROS, inhibited the cell density at 0.35 mg Ni L^{-1}, and it altered Chl *a* fluorescence and carbohydrate at all tested concentrations. Regarding the Co-Ni mixtures, our data best fitted the concentration addition (CA) model and dose-ratio dependent (DR) deviation in which synergism was observed at low doses of Co and high doses of Ni and antagonism occurred at high doses of Co and low doses of Ni. The combined metals caused morphological and physiological changes in *R. subcapitata*, in addition to changes in ROS production, carbohydrates, Φ_M and OEC. Since we observed synergistic and antagonistic effects on algae exposed to Co-Ni combinations, it is important to investigate the toxicity mechanisms of these metal mixtures, in order to guide regulations regarding metal concentrations in the aquatic environments.

Keywords: mixture, photosynthesis, carbohydrates, ROS, metal

1. Introduction

Contamination of aquatic environments by metals is a persistent problem as these compounds are not degradable and they can bioaccumulate and cause toxicity to organisms, which are exposed to a wide variety of metallic mixtures (Filová et al., 2021; Liu et al., 2017). Contaminants can have a different or similar mode of action, which, in a mixture, can result in antagonistic, synergistic or additive effects (Beyer et al., 2014).

Metals such as cobalt (Co) and nickel (Ni) co-occur in the environment (Dourado et al., 2017; Thompson et al., 2020). The statistics of the concentrations of mean values of Co and Ni in surface water bodies were 3.995 mg L^{-1} and 0.946 mg L^{-1} respectively (Kumar et al., 2019). According to WHO (World Health Organization - 2017) and USEPA (United States Environmental Protection Agency - 2009) guidelines, countries such as China, Pakistan, Bangladesh and Nigeria exceed the average values allowed for metals Co and Ni for drinking water (Kumar et al., 2019). In Brazil in the Paraopeba River at concentrations of 0.011 mg Co L^{-1} and 0.014 mg Ni L^{-1} after the Brumadinho dam collapse (Thompson et al., 2020) and in the Água Boa stream in concentrations of 0.253 mg Co L^{-1} and 0.296 mg Ni L^{-1} (Dourado et al., 2017).

Co is an essential metal for the synthesis of vitamin B12 and it can be found in soil, water, vegetation, rocks, and is regularly found associated with nickel (Pourkhabbaz et al., 2011). The demands and applications of Co are diverse, such as the production of wear-resistant superalloys that are corrosion-resistant and high-temperature, battery electrodes and Ni-Cd batteries (which contain Co), chemicals (agricultural products, sewage effluents, mining and processing); paint, steel, cement, magnets and fertilizer industries, in addition to acting as a catalyst in the removal of sulfur impurities from petroleum (Blust, 2011; Sridhar et al., 2020). Therefore, Co has already been found in high concentrations near mining and agricultural fertilizer runoff regions, and can be toxic to humans, animals and plants (Pourkhabbaz et al., 2011). Co has been quantified in surface waters in USA in concentrations of less than 0.0001 to 1 mg L^{-1} with the highest values found in streams polluted by mining (Stubblefield et al., 2020). In view of this, some studies have evaluated the effects of the metal on organisms, reporting protein oxidation, DNA damage, ROS production, decrease of chlorophyll *a* and *b* synthesis and impairment of photosynthetic activity in microalgae (El-Sheekh et al., 2003; Novak et al., 2013); decrease in fertilization rate, embryo survival and reduction in reproductive success in fish (Reinardy et al., 2013); inhibition of active transport, leaf fall, inhibition of greening, stimulation of ROS production and formation of hydrogen peroxide (H_2O_2) radicals in plants (Mahey et al., 2020).

Ni is a metal that occurs naturally in the environment and the main sources of metal entry into the aquatic ecosystem are urban runoff, landfill leaching, municipal and industrial effluents (mining debris), natural sources, and soil or substrate disturbances (Wang et al., 2020). In addition, human activities and poor management of waste paints, electronic equipment, Ni-Cd batteries, cigarettes, stainless steel, jewelry, and coin-making alloys cause Ni pollution (García-García et al., 2018). Ni has already been quantified in polluted sites at concentrations from 2.2 mg L^{-1} (Sanliyuksel Yucel et al., 2016) raising environmental concerns about its effects as high concentrations of Ni in the aquatic ecosystem can be toxic to organisms (Wang et al., 2020). Some effects of Ni are cell density inhibition, ROS increase, change in the maximum rate of relative electron transport (Yong et al., 2019); pigment concentration reduction and enzymatic activity inhibition (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx) in microalgae (Martínez-Ruiz and Martínez-Jerónimo, 2015). Ni also affected the reproduction and the size of males of *Danio rerio* (Alsop et al., 2014); and alterations in plant metabolism by inhibiting enzymatic activity, chlorophyll synthesis and electron transport (Sreekanth et al., 2013).

Algae have an essential environmental role as they are the main producers in the aquatic food chain, helping to maintain the structure and function and balance of ecosystems (Guo et al., 2020; Moreira et al., 2020). The green alga, *Raphidocelis subcapitata*, used as a test organism in this study, is globally distributed and widely used in ecotoxicological tests, as it has a fast life cycle, high sensitivity to xenobiotics and an easy cultivation in the laboratory. It is used in studies of the effects of metals on these organisms (Guo et al., 2020; Machado et al., 2015; Machado and Soares, 2014). There is a lack of knowledge about the combined effects of Co and Ni on microalgae. Some authors assessed the effects of Co-Ni mixtures to other organisms, observing antagonistic effects for the oligochaete Enchytraeus crypticus (He et al., 2015), synergistic effects on Escherichia coli (Barabasz et al., 1990) and antagonistic, additive and synergistic effects, varying according to metal concentration, on Pseudomonas fluorescens (Nweke et al., 2018). Therefore, this research aims to evaluate the effects of isolated and combined effects of Co and Ni on density, photosynthesis, carbohydrates, cell size and complexity, chlorophyll *a* fluorescence (Chl *a*) and ROS production of the freshwater microalga *R. subcapitata*.

2. Material and methods

2.1. Algae culture and toxicity tests

The microalga *R. subcapitata* was obtained from the Department of Ecology and Evolutionary Biology (DEBE, Universidade Federal de São Carlos - UFSCar, São Carlos - SP, Brazil). It was cultivated in CHU12 medium (Chu, 1942) (Table S1. Supplementary material) previously autoclaved (121°C, 1 atm above standard pressure, 20 min) with pH adjusted to 7 ± 0.05. The algae cultures were maintained at 25 ± 1°C, with a photoperiod of 12h/12h (light/dark), light intensity of ≅ 130 µmol photon m^{-2} s^{-1} (LED light) and periodic manual shaking.

Toxicity tests followed the same culture conditions. *R. subcapitata* in the exponential growth phase was inoculated at 5×10^4 cells mL^{-1}, in polycarbonate Erlenmeyer flasks (capacity 500mL) containing 200 mL of the test solution. The freshwater chlorophycea, *R. subcapitata*, were exposed in triplicate to different concentrations of the metals Co (0.10; 0.25; 0.50 and 0.75 mg L^{-1}) and Ni (0.10; 0.15; 0.25 and 0.35mg L^{-1}), isolated and combined forms, during 96 h. Test concentrations were defined in tests performed previously. The control group had no metal addition. Regarding mixture concentrations, we chose a partial fixed-ratio design (Cassee et al., 1998) to avoid treatments with high metal concentrations that could lead to mortality of algal cells, and the 23 combinations are described in Table 1. Stock solutions of Co and Ni were obtained from Titrisol standard solutions of $CoCl_2$ (1000 mg Cd L^{-1}) and $NiCl_2$ (1000 mg Ni L^{-1}) (Merck, Germany).

Table 1. Metal concentrations (mg L^{-1}) used in toxicity tests. Concentrations of Co1 to Co4 and Ni1 to Ni4 refer to isolated tests, while M1 to M23 refer to mixture tests and C = control group.

	Metal concentration (mg L^{-1})	
Treatment	Co	Ni
C	0	0
Co1	0.10	0
Co2	0.25	0
Co3	0.50	0
Co4	0.75	0
Ni1	0	0.10
Ni2	0	0.15
Ni3	0	0.25
Ni4	0	0.35
M1	0.063	0.063
M2	0.063	0.094
M3	0.063	0.156
M4	0.063	0.219
M5	0.125	0.031
M6	0.125	0.063
M7	0.125	0.125
M8	0.125	0.188
M9	0.188	0.031
M10	0.188	0.094
M11	0.188	0.156
M12	0.250	0.063
M13	0.250	0.125
M14	0.250	0.25
M15	0.313	0.031
M16	0.313	0.094
M17	0.375	0.063
M18	0.375	0.188
M19	0.375	0.25
M20	0.438	0.031
M21	0.500	0.125
M22	0.500	0.188
M23	0.500	0.25

2.2. Metal determination

Metals were determined in stock solutions by inductively coupled plasma optical emission spectrometry (ICP OES, iCAP 7000 - Thermo Fischer Scientific, Madison, WI, USA). The results are presented in Table S2 (Supplementary material). Nominal concentrations did not vary more than 20% from actual concentrations (OECD, 2002), thus the nominal concentrations were maintained. Limits of detection were 0.0001 mg Co L^{-1} and 0.005 mg Ni L^{-1}, while quantification limits were 0.010 mg Co L^{-1} and 0.100 mg Ni L^{-1}.

2.3. Cytometer analysis

To analyze cell density, the samples were fixed with formaldehyde buffered with borax (1% final concentration), left in the dark for 10 minutes and frozen in liquid nitrogen. Relative ROS assessment was done according to procedures recommended by Hong et al. (2009). Samples (495 µL) were collected and 5µL of DCFH-DA (2′,7′-Dichlorofluorescein diacetate, CAS number 2044-85-1, Sigma Aldrich) was added obtaining a final concentration of 10 µM DCFH-DA. Then, the samples were incubated for 1 h in the dark and analyzed by flow cytometry.

Cell density and relative ROS were analyzed in FACSCalibur flow cytometer (Becton & Dickinson Franklin Lakes, NJ, U.S.A.) equipped with a 15 mW Argon-ion laser (emission 488 nm). In each sample, we added 10 µL of 6 µm fluorescent beads (Fluoresbrite carboxylate microspheres; Polysciences, Inc., Warrington, PA, USA.) as a standard.

Cell density was determined according to Sarmento et al. (2008) procedures, using SSC-H (lateral dispersion) versus FL3-H (red fluorescence) parameters. FSC-H, SSC-H and FL3-H parameters correspond to the cell size, cellular complexity and chlorophyll *a*(Chl *a*) fluorescence, respectively, and were calculated as reported in Mansano et al. (2017). ROS were determined using FL3-H versus FL1-H (green fluorescence) parameters and calculated by the equations (1 and 2) described by Hong et al. (2009). Then, the obtained data were analyzed by FlowJo software, version V10.0 (Treestar.com, USA).

$\text{FL1-H}_{\text{relative}} = \log(\text{FL1-H}_{\text{samples}}) / \log(\text{FL1-H}_{\text{beads}})$ (1)

$\text{ROS}_{\text{relative}}(\%) = (\text{FL1-H}_{\text{relative[treatments]}} / \text{FL1-H}_{\text{relative[control group]}}) \text{ X } 100$ (2)

2.4. Total carbohydrates

Total carbohydrates were measured according to the methodology proposed by Liu et al. (1973) with the phenol-sulfuric acid reaction. The analysis was performed using a spectrophotometer (HACH DR 5000; HACH Company, Loveland, CO, EUA) at a wavelength of 485nm. Total carbohydrate values were calculated using a calibration curve (Fig S1, Supplemental material), made with dextrose anhydrous (Mallinckrodt Chemicals, USA) standard in 7 concentrations (5, 10, 20, 40, 100, 150 and 200 µg mL^{-1}).

2.4. Photosynthetic activity

The assessments of the maximum quantum yield (Φ_M) and the efficiency of the oxygen evolving complex (OEC) were performed using pulse amplitude modulated fluorometer (PHYTO-PAM® Fluorometer Analyzer, Heinz Walz, Germany). Samples (3 mL) were collected and kept in the dark for 15 min. Minimum fluorescence (F_0), maximum fluorescence (F_m) and Φ_M values (Genty et al., 1989) were provided by Phyto-PAM; and the efficiency of OEC was calculated by the ratio F_0/F_v (where $F_v = F_m – F_0$) (Kriedemann et al., 1985).

2.5. Statistical analysis

For statistically significant differences, normal data were analyzed by ANOVA (one-way analysis of variance) and Dunnett's post-hoc tests, while non-normal data were evaluated using Kruskal-Wallis test and Dunn's post hoc test. Statistical analyses were performed with SigmaPlot 12.0 software and significant differences were considered when $p < 0.05$. The inhibitory concentration (IC_{50}-96h) was calculated by nonlinear regression, with a sigmoidal three-parameter logistic curve in the SigmaPlot 12.0 software. ROS, SSC-H and FL3-H mixture data were normalized using log transformation.

Data from the cell density obtained in mixture toxicity tests were analyzed by CA (concentration addition) and IA (independent action) models using the MIXTOX tool (Jonker et al., 2005). Then, for both models, CA and IA, the deviations S/A (synergism/antagonism); DR (dose ratio-dependent) and DL (dose level-dependent) were modeled by adding the parameters “a” and ‘b”. In S/A deviation, the parameter “a” becomes negative to synergistic interactions and positive to antagonistic. In DR, the addition of a second parameter, b_{DR}, can indicate if the deviation from the reference model is controlled by the mixture composition. In DL, the “a” parameter indicates the deviation, $a > 0$ = antagonism; while $a < 0$ = synergism, and the b_{DL} parameter indicates at what dose level the deviation changes. More information on these deviation functions are available in Jonker et al. (2005). At the end of the analysis, the best fit was chosen by the maximum likelihood method and the best deviation was statistically identified.

3. Results and Discussion

3.1. Single toxicity: algal growth

Our data showed that Co treatments inhibited the cell density of *R. subcapitata* after 72h of exposure at 0.50 and 0.75 mg Co L^{-1}; while at 96h, cell density was inhibited at 0.25; 0.50 and 0.75 mg Co L^{-1} (Fig. 1A). Ni treatments increased cell density after 72h of exposure at 0.10 mg L^{-1} and inhibition after 96h at the highest concentration tested (0.35 mg L^{-1}) (Fig.1B). Other studies also reported that Co decreased cell density to several algae species such as *Chlamydomonas reinhardtii* – 20 mg L^{-1} (Lustigman et al., 1995); *Monoraphidium minutum* (1, 2 and 3 mg L^{-1} of Co^{2+}), *Nitzchia perminuta* (2.5, 3.5 and 5 mg L^{-1} of Co^{2+}) (El-Sheekh et al., 2003) and *Chlorella vulgaris* – 0.6 mg L^{-1} of Co (Afkar et al., 2010). Therefore, even though Co is an essential metal, in high concentrations it can be toxic to microalgae, and the level of toxicity may vary according to the species (Ghafari et al., 2018).

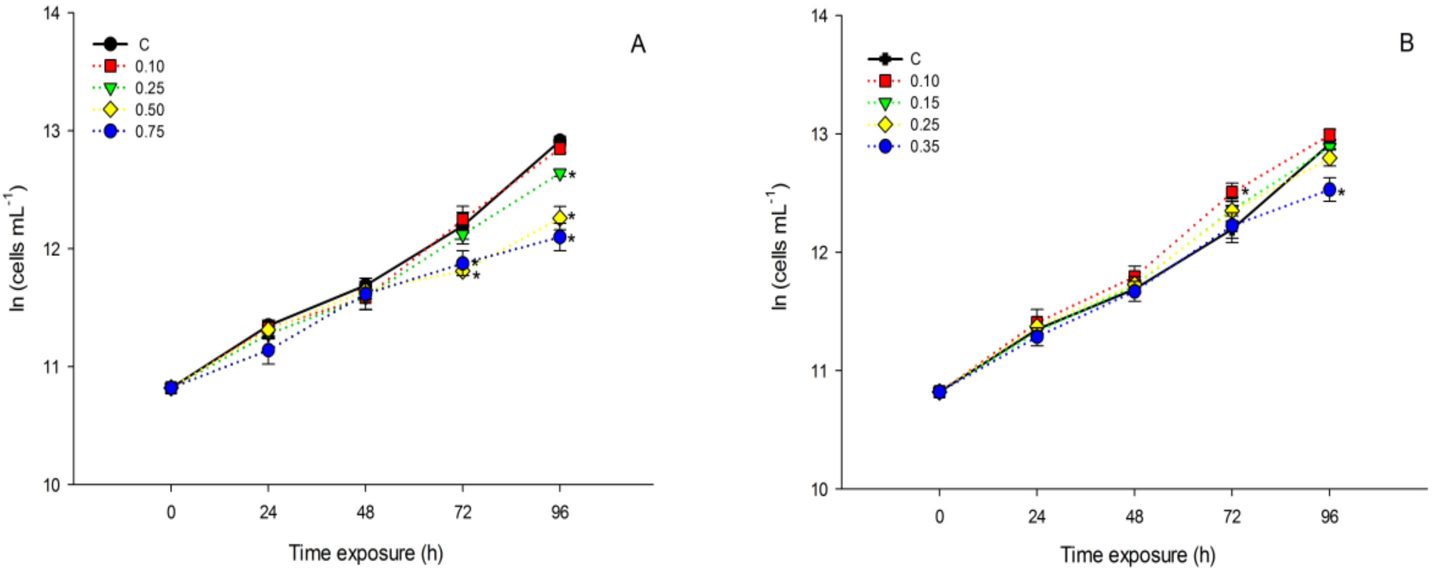


Fig.1. Cell density of *Raphidocelis subcapitata* ((ln(cells mL^{-1})) exposed to Co (A) and Ni (B) during 96h of exposure. The asterisk indicates a significant difference from the control group ($p<$ 0.05). Metal concentrations are expressed in mg L^{-1}; C = control group.

Previous studies have also observed a drop in cell growth of the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, *Dunaliella bioculata* and *Scenedesmus vacuolatus* exposed to Ni (1, 5 and 10 mg L^{-1}) (Haiduc et al., 2009). In addition, Osman et al. (2004) obtained results similar to our results, as at low concentrations, the metal stimulated growth while at high concentrations it decreased the cell growth to *Nitzschia perminuta* (0.5 and 2 mg L^{-1}) and *Scenedesmus obliquus* (0.1 and 1 mg L^{-1}). The metal is able to enter the cell through different pathways, such as specific transporters or receptor channels and ion exchange causing toxic effects if present in concentrations above those necessary (Martínez-Ruiz and Martínez-Jerónimo, 2015).

3.2. Mixtures

3.2.1. Growth

The results obtained from binary mixtures of Co and Ni in the cellular density of *R. subcapitata* were presented in Table 2 (data from concentration addition - CA model) and

Table S3 of Supplementary Material (data from independent action - IA model). In this study, we used the CA and the IA models to analyze the results. The CA model admits that the individual compounds have the same mechanism of action, acting on the same biological target, and consequently the biological response is potential to the respective toxicities of the compounds. While the IA model admits that the isolated compounds have different mechanisms of action, therefore, their effects are independent from each other. Then, when the chemical mechanism of action is unknown, both models are tested and the one that best fits the data is used to explain the data (Mansano et al., 2017). Furthermore, Jonker et al. (2005) concluded that there may be patterns to which data deviate from the CA and IA models, i.e. no deviation, synergism or antagonism (S/A), dose level-dependent (DL) deviation, and dose ratio-dependent (DR).

Table 2. Summary of the modeled data from reference model concentration addition (CA) to cell density of *Raphidocelis subcapitata* exposed to mixtures of Co and Ni during 96 h.

Concentration addition (CA)				
	CA	S/A	**DR**	DL
Max	4.35	4.43	4.25	4.60
β Cd	1.17	1.23	1.38	0.10
β Ni	3.17	2.83	4.20	1.82
IC_{50} for Cd	0.29	0.42	0.38	0.38
IC_{50} for Ni	0.31	0.39	0.40	0.40
A	-	-2.23	-4.25	0.01
$b_{DR/DL}$	-	-	4.73	262.67
SS	9.74	5.93	4.45	6.77
r^2	0.74	0.84	0.88	0.82
χ^2 or F test	19.56	15.88	25.1	11.64
Df	-	1	2	2
p (χ^2/F)	1.16×10^{-7}	6.75×10^{-5}	3.6×10^{-6}	0.003

Data obtained using Jonker et al. (2005) methodology. Max = maximum response value, β = slope response of isolated compounds, IC_{50} = median growth inhibition concentration; a and $b_{DR/DL}$ = function parameters; SS = sum of the squared residuals; r^2 = regression coefficient; χ^2 or F test = statistical test; df = degrees of freedom; *p*(χ^2/F) = level of significance of statistical test; CA = concentration addition model; IA = independent action model; S/A = synergism/antagonism deviation; DR = dose ratio-dependent deviation; DL = dose level-dependent deviation. DR is in bold because it was the deviation that best fit the data.

From our data, both models (CA and IA) were statistically significant, but the model that best fit the Co-Ni data was CA (Table 2). Results from IA model yielded a sum of the squared residual (SS) value of 9.26 ($p = 5.91 \times 10^{-8}$, $r^2 = 0.76$; Supplementary Material, Table S3). After adding the parameter “a”, the SS value was 6.13 ($p=2.81 \times 10^{-4}$; $r^2 = 0.84$). By adding the parameter $b_{DR/DL,}$ the model did not calculate the results of SS, r^2 and p. Data from the CA model yielded an SS of 9.74 ($p = 1.16 \times 10^{-7}$; $r^2 = 0.74$). By adding the parameter “a”, the SS value decreased to 5.93 ($p = 6.75 \times 10^{-5}$; $r^2 = 0.84$). When the parameters b_{DR} and b_{DL} were added, the SS values were 4.45 ($p = 3.6 \times 10^{-6}$; $r^2 = 0.88$) and 6.77 ($p = 0.003$; $r^2 = 0.82$), respectively. Thus, based on these results and on the mixtures' isolobogram (Fig. 2), the CA model and DR deviation best fit our data as they presented the significant p value, the smallest SS value and the largest r^2 value. The DR deviation indicates that there was synergism at low doses of Co and high doses of Ni; while antagonism occurred at high doses of Co and low doses of Ni ($a < 0$ and $b > 0$).

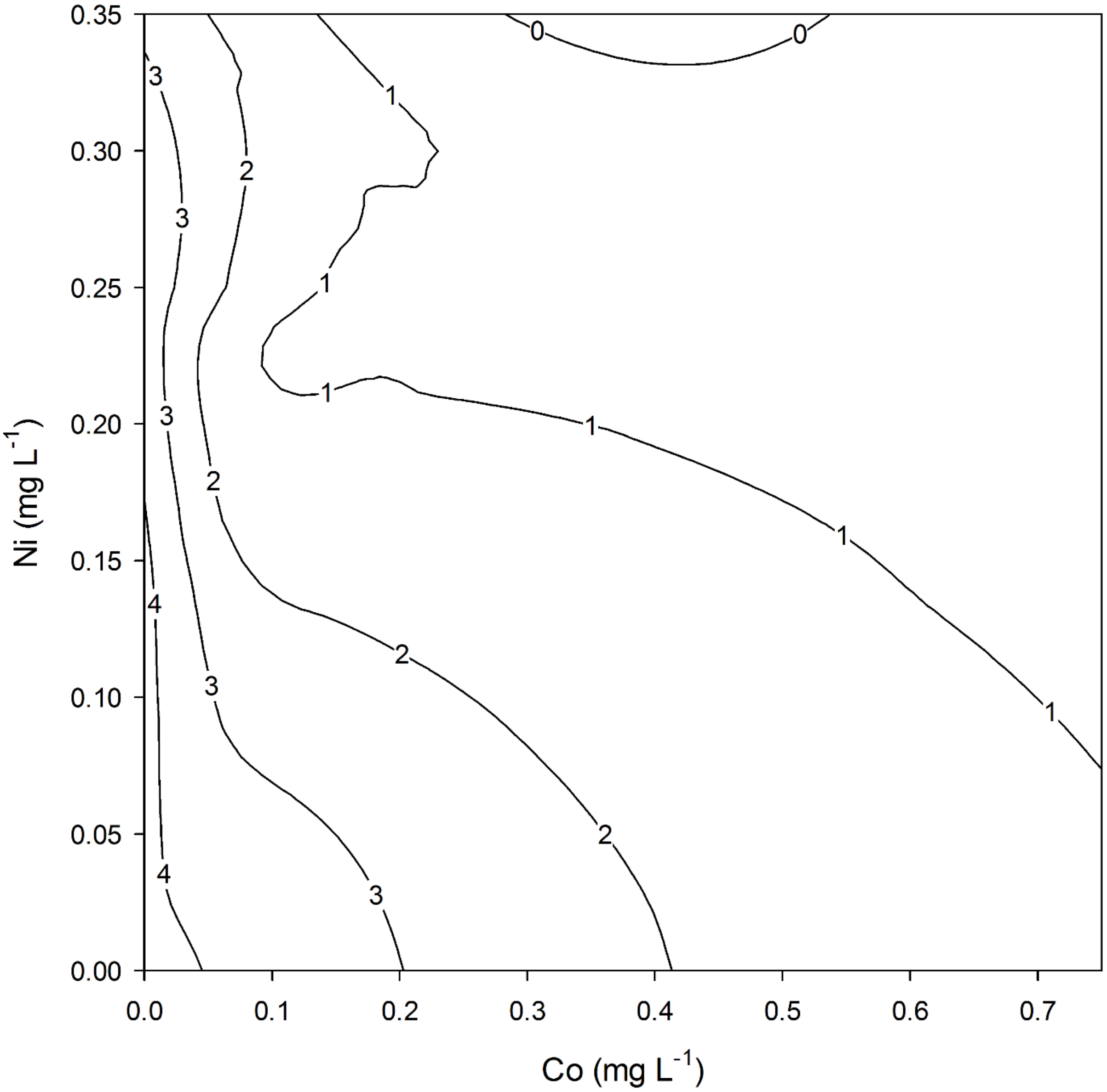


Fig. 2. Isobologram of the cobalt (Co) and nickel (Ni) mixtures, based on their effects on the cellular density of *Raphidocelis subcapitata*, following the reference model of concentration addition (CA) and dose-ratio dependent (DR) deviation. The concave shape represents synergism while the convex shape shows antagonism (Alloatti et al., 2013).

The mixture between Co and Ni in this study showed synergistic effects at low concentrations of Co and high concentrations of Ni (around 0.25 mg L^{-1}). These results are a matter of concern given the concentrations already recorded for both metals in the environment. It is known that Co and Ni are widely used in industrial processes, due to their high durability and corrosion resistance, consequently, their demands increase the waste generation, such as urban and industrial effluents, chemical and especially mining waste (Alves et al., 2022). In the environment, metals have already been quantified in different concentrations: *e.g.*, 0.01 mg Co L^{-1} - Paraopeba River (Thompson et al., 2020); 0.25 mg Co L^{-1} - Água Boa stream (Dourado et al., 2017) and 2.39 mg Co L^{-1} – Tanjero River (Aziz et al., 2012); while for Ni, 0.014 mg Ni L^{-1} - Paraopeba River (Thompson et al., 2020); 0.30 mg Ni L^{-1} - Água Boa stream (Dourado et al., 2017) and 1.08 mg Ni L^{-1} - Doce River (de Carvalho et al., 2017).

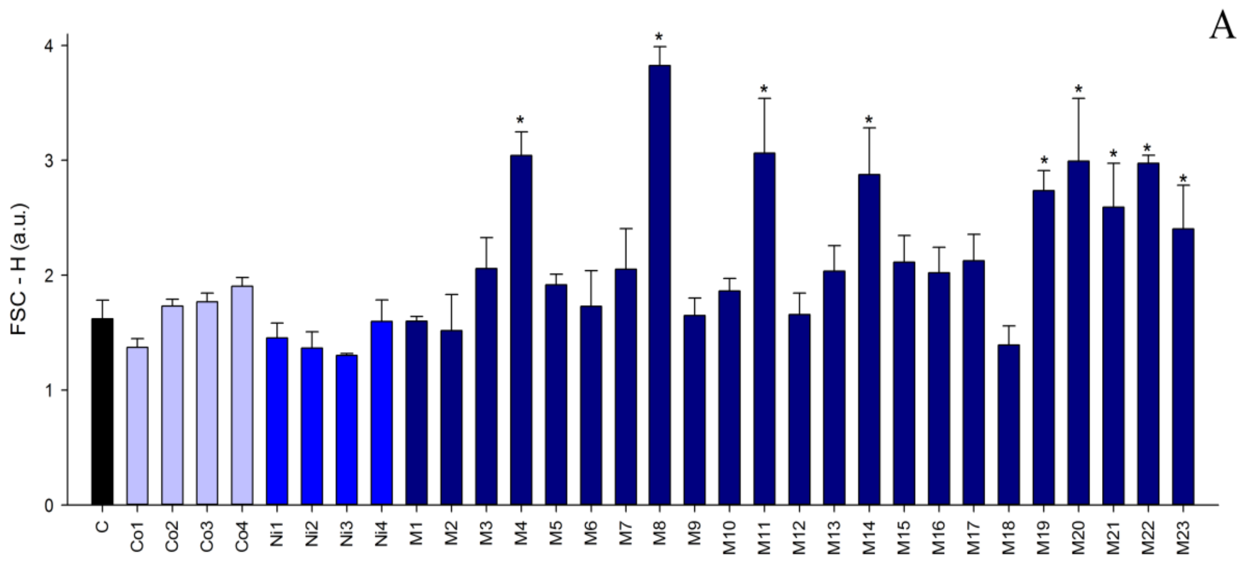
Polechońska and Samecka-Cymerman (2018) evaluated the effects of Co and Ni on *Hydrocharis morsus-ranae* (plant). The authors observed a lower decrease in biomass in plants exposed to Ni compared to plants exposed to Co and Ni, indicating that Ni toxicity can increase with other metals, such as Co, Zn, Mn and Mo. The mixture of metals can change the toxicity of the compounds, i.e., they show different effects than their isolated forms (Batool and Javed, 2015).

As far as we know, this is the first study that investigates the effects of Co and Ni mixtures in multiple endpoints to *R. subcapitata*. Therefore, we reinforce the importance of investigating the effects of metallic mixtures on organisms as their interaction can cause metabolic changes, interfere with transport, bioavailability, binding and excretion (Cedergreen, 2014). Furthermore, our results indicated synergistic and antagonistic effects in the Co-Ni mixture, varying according to the mixture composition (CA model and DR deviation). The synergistic effect can occur when one metal facilitates the absorption of another, and exposure to metals can increase membrane permeability, leading to increased cellular uptake and toxicity; while antagonism can occur when metals compete for the same transport site in the membrane (Franklin et al., 2002; Starodub et al., 1987), which may explain the effect observed in this study as the Co and Ni metals are divalent and may have competed for transport.

3.2.2. Morphology and Chlorophyll *a* (Chl *a*) fluorescence

Co exposure led to an increase in SSC-H (cellular complexity) at 0.50 and 0.75 mg Co L^{-1} (Fig. 3B); a decrease in FL3-H (Chl *a* fluorescence) at 0.10 mg Co L^{-1} (Fig. 3C); and did not significantly change the FSC-H (cell size) parameter (Fig. 3A). Ni exposure decreased the Chl *a* fluorescence (FL3-H) in all concentrations tested (Fig. 3C); while cell size (FSC-H) (Fig. 3A) and cell complexity (SSC-H) (Fig. 3B) were not affected significantly.

Metal mixture of Co and Ni led to an increase in cell size (Fig. 3A) in the treatments: M4; M8; M11; M14; M19; M21; M22 and M23. The cellular complexity (Fig. 3B) did not change significantly in metal combinations M1 to M12. The increase was significant in combinations M13 to M23, except for M17.On the other hand, Chl *a* fluorescence (Fig. 3C) suffered significant increases and decreases. Treatments that decreased fluorescence were M1 to M8, and M17; and treatments that led to an increase in Chl *a* fluorescence were M13 to M16; M18; M19; M21 and M22.


A
FSC - H (a.u.)
0
1
2
3
4
C
Co1
Co2
Co3
Co4
Ni1
Ni2
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Ni4
M1
M2
M3
M4
M5
M6
M7
M8
M9
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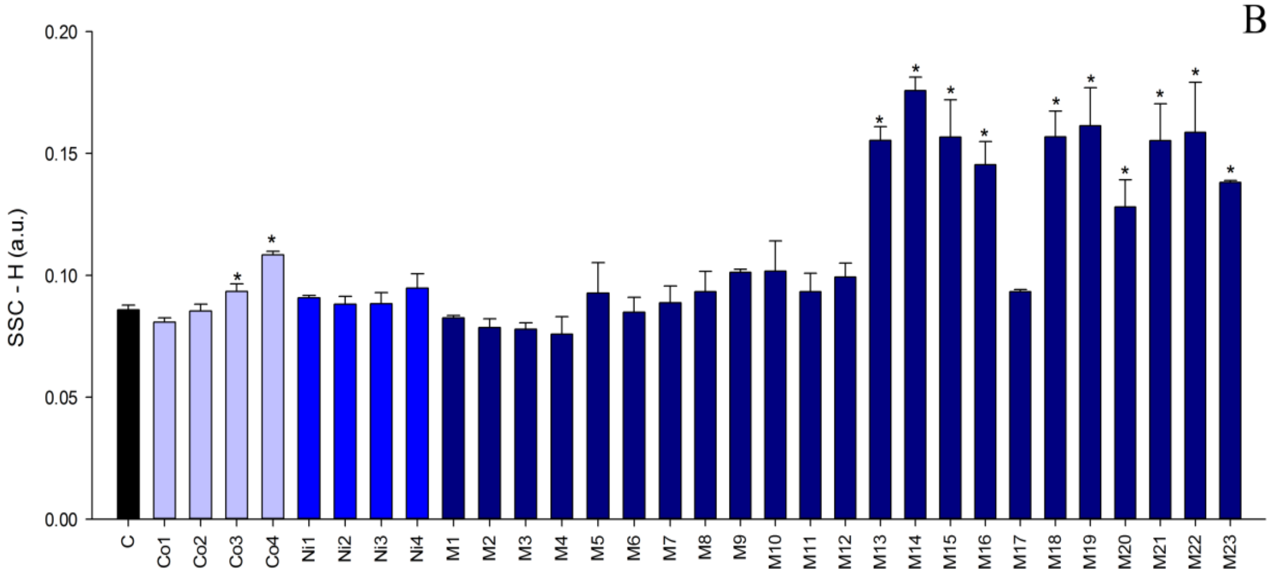
B
SSC - H (a.u.)
0.00
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C
Co1
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Ni3
Ni4
M1
M2
M3
M4
M5
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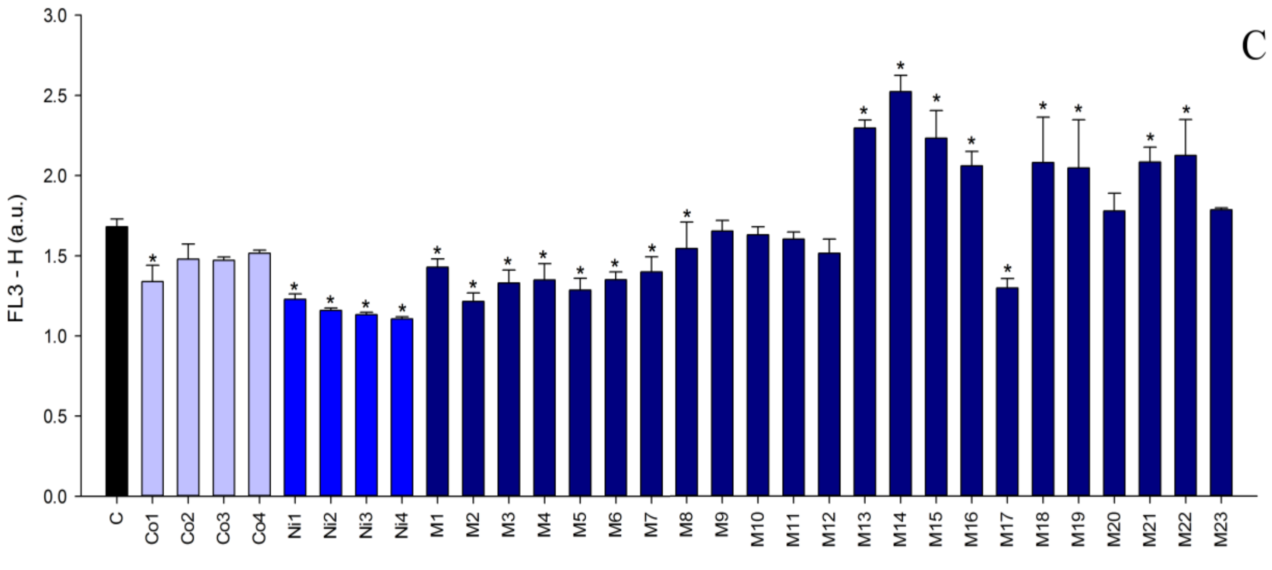
C
FL3 - H (a.u.)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
C
Co1
Co2
Co3
Co4
Ni1
Ni2
Ni3
Ni4
M1
M2
M3
M4
M5
M6
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M9
M10
M11
M12
M13
M14
M15
M16
M17
M18
M19
M20
M21
M22
M23
*

Fig. 3. Mean values of cell size (FSC-H) (A), cellular complexity (SSC-H) (B) and chlorophyll *a* fluorescence (FL3-H) (C) of *R. subcapitata* exposed to different concentrations of Co and Ni, isolated (Co1-Co4 and Ni1-Ni4) and combined (M1-M23) after 96h of exposure. Asterisks (*) indicate significantly different values ($p<0.05$) when compared to the control group (C).

In previous studies, where microalgae were exposed to metals, an increase was observed in the cell size: Mn (0.4 and 0.8 mg L^{-1}) and Cr (0.2 mg L^{-1}) (Alho et al., 2022); Cu (1 mg L^{-1}) (Franqueira et al., 2000); Zn (0.0052-0.03 mg L^{-1}) and Al (0.4-1.0 mg L^{-1}) (Gebara et al., 2020). This increase may indicate damage to the cell division process, and is related to the uncoupling of cell growth and cell division (the cell is not able to complete the division) (Gebara et al., 2020; Mansano et al., 2017). Furthermore, metals can change membrane permeability, and the increase in cell size can occur due to increased permeability to Na^+ (Franqueira et al., 2000). Our results suggest that the mixed metals interfered with the cell division process, which caused an increase in the cell size in some treatments. Therefore, we highlight the difference between the effect of single and combined metals, as noted by Batool and Javed (2015). The authors pointed out that all combinations of metals Co, Cr and Pb showed synergistic interactions, that is, the effects of the mixture of metals were more toxic than the effect of isolated metals.

Cell complexity may be related to the internalization of metals by the microalgae (Gebara et al., 2020). Under stress conditions, the microalgae may have this action as a defense mechanism, to minimize or prevent the toxic effects of metals (Alho et al., 2022). Exposure to Zn, Al and Mn increased cell complexity for *R. subcapitata* cells (Alho et al., 2022; Gebara et al., 2020). Our data suggest that Co activated this defense mechanism in treatments of 0.50 and 0.75 mg Co L^{-1}; as well as some treatments of the mixture, in which we observed an increase in cell complexity. Exposure to isolated Ni did not alter this parameter, which may indicate that the microalgae activated another defense mechanism, such as extracellular adsorption (Juarez et al., 2008).

Chl *a* fluorescence is a good indicator of the physiological status of microalgae, as it provides information on energy in photosynthesis (absorption, distribution and utilization) (Franqueira et al., 2000). Therefore, the decrease in fluorescence may indicate some damage in the photosynthetic process, such as the inhibition of electron flow in the reaction center of photosystem II (PSII) on the donor side (Alho et al., 2022; Franqueira et al., 2000). However, the increase in fluorescence may suggest an attempt to maximize light capture, or an inhibition of electron transport may occur, but on the acceptor side (Alho et al., 2022; Franqueira et al., 2000). This parameter indicated that the microalgae suffered a decrease in chl *a* fluorescence, for both metals, in all tested concentrations, suggesting damage in the photosynthetic process. The results of the mixture of metals showed a decrease, but also an increase in fluorescence in combinations with higher concentrations of Co and Ni, suggesting an attempt to increase light capture.

3.2.3. ROS production

We observed an increase in ROS production at 0.50 mg Co L^{-1}, while in Ni exposure, there was a decrease in the ROS at 0.35 mg L^{-1} (Fig. 4). In mixtures, we observed increased ROS production at M3, M4, M8, M11, M13, M14, M16, M17 and M19, and the ROS production increased with increasing Ni concentration in metallic combinations. In addition to that, only at M12 did the ROS production decrease.

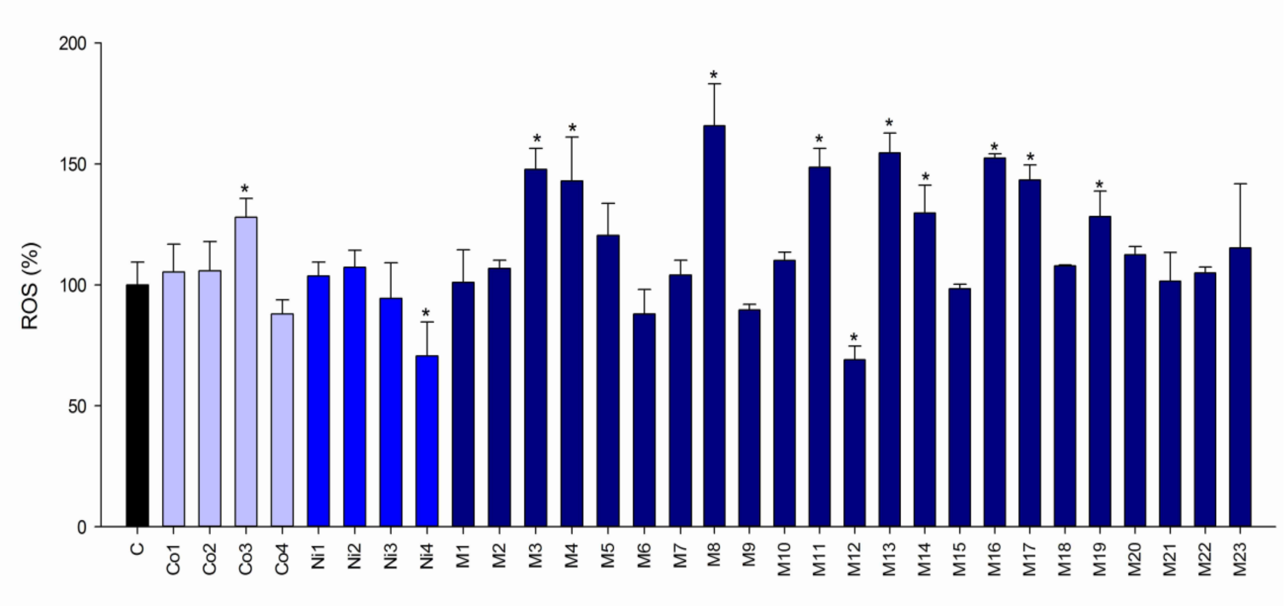

Fig. 4. Cellular reactive oxygen species (ROS) produced by *R. subcapitata* exposed to Co (Co1-Co4) and Ni (Ni1-Ni4), isolated and combined (M1-M23), at 96h. Asterisks represent significant differences ($p < 0.05$) from the control group (C). Metal concentrations are described in Table 1.

ROS generation by aerobic organisms occurs during metabolic processes such as photosynthesis and electron transport, in which mitochondria, chloroplasts and peroxisomes are natural production sites of ROS, as well as detoxification. Enzymatic activity and also non-enzymatic defenses are essential to maintain the antioxidant defense system of aerobic organisms, controlling ROS levels. Any imbalance in these defense mechanisms leads to ROS production to generate oxidative stress (Szivák et al., 2009).

Environmental stress conditions such as UV radiation, high light intensity and exposure to contaminants such as metals and herbicides, can increase ROS production in cells, causing serious injury or cell death (Mei et al., 2007). Metals have the ability to inactivate antioxidant enzymes by binding to thiol groups or replacing ions, generating high levels of free radicals (Szivák et al., 2009). High ROS production can be highly harmful to organisms, in addition to causing damage to the photosynthetic apparatus and cell death (Pinto et al., 2003; Sharma et al., 2012). Co is capable of inducing oxidative stress through its reaction with hydrogen peroxide to produce hydroxyl radicals (Mei et al., 2007) and Ni is able to increase ROS production causing oxidative stress in aquatic organisms (Brix et al., 2017).

The increase in ROS by exposure to Co and also in the Co-Ni mixture suggests that metals led cells to oxidative stress. On the other hand, we observed a decrease in ROS in exposure to Ni (Ni4), suggesting that *R. subcapitata* activated its antioxidant mechanisms and these were able to decrease the content of intracellular ROS (Alho et al., 2022), which possibly occurred at M12.

3.2.4. Total carbohydrates

Total carbohydrates significantly decreased exposure to both metals, isolated, at all concentrations tested (Fig 5). In the metal mixtures, carbohydrate synthesis decreased in M1; M2; M5; M9; M12; M17 and M20. On the other hand, the synthesis was increased in treatments M8; M11; M14; M15; M16; M18; M19; M22 and M23. Low concentrations of Ni combined with different concentrations of Co showed a predominant decrease in carbohydrate synthesis. Significant increases occurred in combinations in which Co and Ni concentrations were intermediate or high. We highlight this last effect as it differs from the effect observed in the exposure of isolated metals.

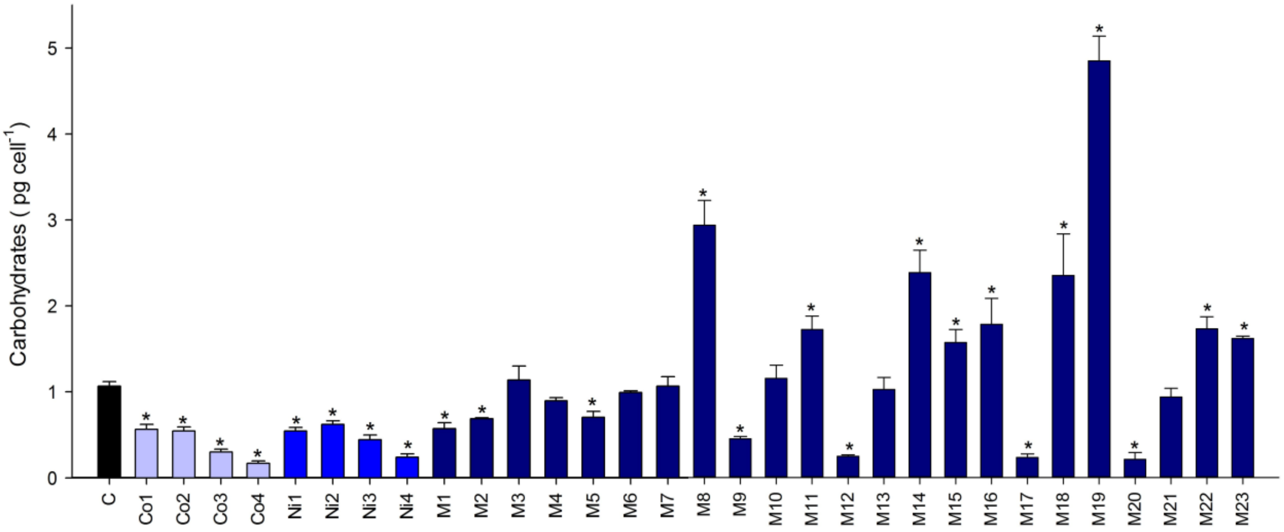

Fig. 5. Total carbohydrates (pg cell^{-1}) of *R. subcapitata* exposed to different concentrations of Co and Ni, isolated (Co1-Co4 and Ni1-Ni4) and combined (M1-M23), after 96h of exposure. Asterisks (*) indicate a significantly different value ($p<0.05$) when compared to the control group (C).

Carbohydrates are important macromolecules for energy storage, they are present in the structure of the cell wall and are intermediaries in the respiratory and photosynthetic pathways (Martínez-Ruiz and Martínez-Jerónimo, 2015). Carbohydrates are important as alternative sources of energy, raw material and food storage material, which are used for structural modifications that adapt cells under conditions of metal exposure or other stress conditions (Chia et al., 2015).

Carbohydrate synthesis can be altered by environmental stress and also by metals (Fawzy et al., 2020; Rocha et al., 2018). Alho et al. (2019) observed an increase in carbohydrate synthesis in *R. subcapitata* exposed to Cd, suggesting that the increase in carbohydrate synthesis may function as a detoxification mechanism due to the interaction that occurs between metal cations and the negative charge of these biomolecules.

On the other hand, some authors have reported a decrease in the synthesis of this macromolecule (Elsalhin et al., 2016; Fawzy et al., 2020; Martínez-Ruiz and Martínez-Jerónimo, 2015). Martínez-Ruiz and Martínez-Jerónimo (2015) observed changes in the external cellular structure of *Ankistrodesmus falcatus*, as well as a decrease in carbohydrate synthesis due to Ni exposure (0.005, 0.008 and 0.017 mg L^{-1}). The authors suggested that these results may be directly related as the cell wall provides protection to the cells, but at high metal concentrations it cannot fulfill this function as the metal possibly altered the structure of the cell wall. Furthermore, the decrease in the biomolecule may also indicate that there had been membrane depolarization.

Elsalhin et al. (2016) reported a decrease in carbohydrates in *Spirulina platensis* by exposure to Co (2.5 and 3 mg L^{-1}), suggesting that the reduction in synthesis may have been responsible for the inhibitory effect on photosynthetic activity. Fawzy et al. (2020) exposed *Synechocystis pevalekii* and *Scenedesmus bernardii* to Co, and reported the decrease in carbohydrates, highlighting the ability of metals to alter the metabolism of the organisms. Our results indicate that isolated metals and some mixture combinations decreased total carbohydrates, suggesting that there was probably an inhibitory effect on the synthesis process of this macromolecule. Otherwise, the increased synthesis in combinations of Co-Ni with intermediate or high concentrations suggests an attempt to minimize the damage caused, activating this detoxification mechanism or storing these molecules as an alternative energy reserve.

3.2.5. Phyto-PAM analyses

Our results showed that isolated Co exposure decreased Φ_M at 0.50 and 0.75 mg Co L^{-1} (Fig. 6A), while the F_0/F_v ratio was altered in all the tested Co concentrations (Fig. 6B). Ni exposure did not affect photosynthetic parameters Φ_M (Fig. 6A) and F_0/F_v (Fig. 6B). Metal mixtures affected these endpoints at the same combinations: M16, M17, M21, M22 and M23.

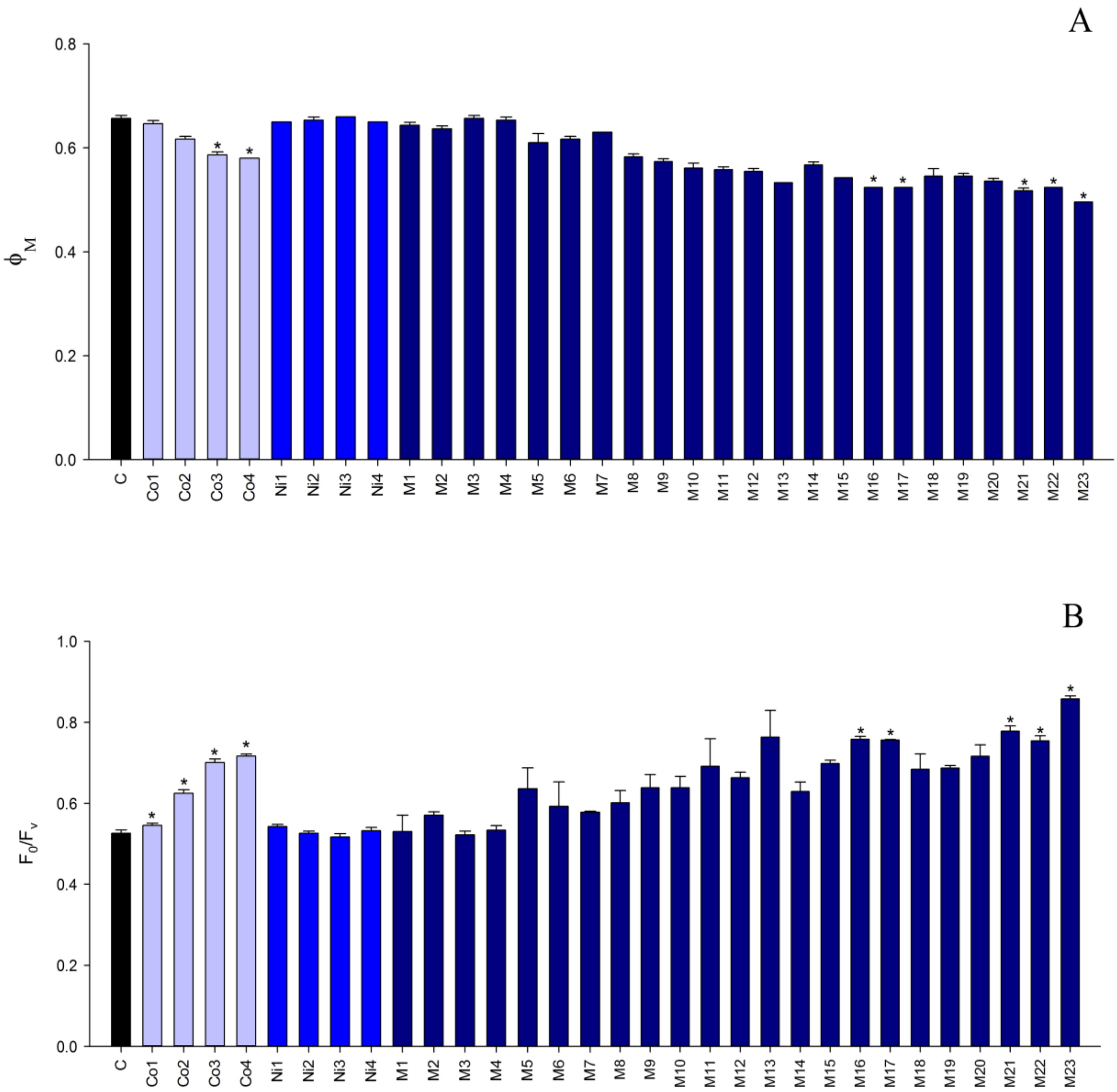

Fig. 6. Maximum quantum yield (Φ_M) (A) and measurement of the efficiency of Oxygen Evolving Complex (F_0/F_V) (B) of *R. subcapitata* exposed to Co (Co1-Co4) and Ni (Ni1-Ni4), isolated and combined (M1-M23), after 96h of treatment. Asterisks (*) means the significant difference ($p < 0.05$) when compared to the control group (C).

Maximum quantum yield (Φ_M) of photosystem II (PSII) indicates the ability of dark-adapted cells to convert light energy into chemical energy (Juneau et al., 2002). The F_0/F_v ratio, in which F_0 indicates the initial fluorescence and F_v the variable fluorescence, points to the efficiency of the oxygen evolution complex (OEC), where water photolysis occurs (breakdown of the molecule and production of oxygen) (Herlory et al., 2013).

Plekhanov and Chemeris (2003) investigated the effects of Zn, Co and Cd on *Chlorella pyrenoidosa* and observed a decrease in photosynthetic activity for exposure to all metals. Isolated Co decreased Φ_M values, which may indicate a partial blockage or a decrease in the transport of electrons from PSII to photosystem I (PSI), which occurs due to the reoxidation of quinone A (Herlory et al., 2013; Mallick and Mohn, 2003). The increase in F_0/F_v with increasing metal concentration suggests that Co may have displaced Mn (manganese), calcium and chloride ions, interfering with the photo-oxidation reaction of water (Alho et al., 2019; Echeveste et al., 2017). El-Sheekh et al. (2003) exposed *Monoraphidium minutum* and *Nitzschia perminuta* to Co at different concentrations. The authors observed a decrease in the amount of O_2 released as the metal concentration increased, interfering with electron transport. Besides that, their results indicated that the site of action of the metal is located on the acceptor side of the PSII (P680) for both algae. Begović et al. (2016) exposed *Lemna minor* to Co and observed the influence of the metal on the electron transport between the OEC and the PSII reaction centers, supporting the hypothesis of displacement of Mn by Co.

Ni is capable of replacing magnesium (in the central atom of chlorophyll) forming an unstable molecule and consequently decreases photosynthetic activity (Baumann et al., 2009). Furthermore, when interacting with the OEC, it can cause the depletion of two polypeptides, leading to the inhibition of electron transport. Therefore, it reaches the OEC, affects the PSII, and may interfere with the entire photosynthetic pathway (Martínez-Ruiz and Martínez-Jerónimo, 2015). However, even though the metal is capable of causing these effects, the concentrations used in this study did not show significant damage to the photosynthetic parameters of *R. subcapitata* exposed to Ni.

The significant effect of the mixture between Co-Ni occurred in the same combinations for Φ_M and F_0/F_v, suggesting that the mixture caused damage to the OEC and consequent interference in the photosynthetic process, leading to a decrease in Φ_M. These changes occurred in the combinations with the highest Co concentrations and intermediate and high Ni concentrations.

4. Conclusion

Our results demonstrated that Co exposure increased ROS and cell complexity, and decreased cell density, Chl *a* fluorescence and total carbohydrates. Photosynthetic parameters were affected by Co, which altered Φ_M and F_0/F_v. On the other hand, Ni exposure did not significantly alter Φ_M and F_0/F_v; however, it decreased the cellular density, Chl *a* fluorescence, ROS and total carbohydrates. Data from Co-Ni mixtures showed that CA model and DR deviation best fit our results, indicating that synergism occurred at low doses of Co and high doses of Ni and antagonism occurred at high doses of Co and low doses of Ni. Metal mixtures caused morphological (increase in cell complexity and size) and physiological changes (increase and decrease in Chl *a* fluorescence), in addition to a decrease in Φ_M and an increase in F_0/F_v. Carbohydrate synthesis and ROS production also increased and decreased significantly. We emphasize the importance of investigating the effects of mixed metals on the metabolism of organisms as the isolated effect may differ from the effect of the mixture, in which it was potentialized or not. The results can also guide future studies related to the mechanism of action of combined metals, and help the development of guidelines and risk analysis. In addition, we emphasize the relevance of studies with microalgae as they are organisms sensitive to contaminants and the basis of the trophic chain. Thus, any damage suffered by these organisms may reach higher trophic levels.

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Supplementary Material

Table S1. CHU 12 culture medium composition (Chu, 1942).

Reagent	Concentration (g L^{-1})
$Ca(NO_3)_2.4H_2O$	4.3
K_2HPO_4	0.5
$MgSO_4.7H_2O$	7.5
KCl	0.5
Na_2CO_3	2
$FeCl_3.6H2O$	0.05

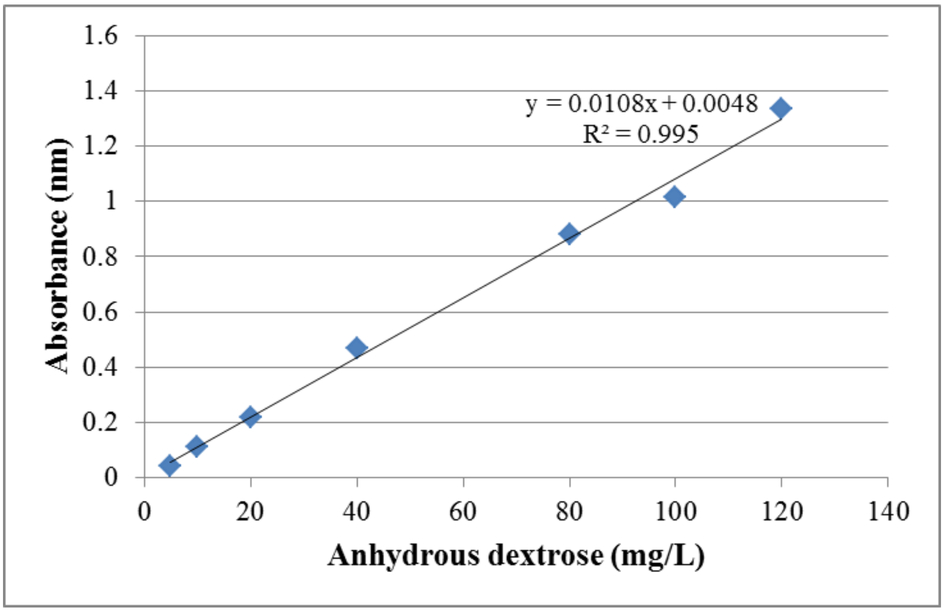
Table S2. Comparison between nominal and measured concentrations of cobalt (Co) and nickel (Ni) in the stock solutions, quantified by inductively coupled plasma optical emission spectrometry (ICP OES, iCAP 7000 - Thermo Fischer Scientific, Madison, WI, EUA).

Metal	**Nominal concentration (mg L^{-1})**	**Measured concentration (mg L^{-1})**
Co	1000.00	834.0 ± 0.217
Ni	1000.00	956.0 ± 0.109

Table S3. Summary of the modeled data from reference model independent action to cell density of *Raphidocelis subcapitata* exposed to mixtures of Co and Ni during 96 h.

	Independent action			
	IA	S/A	DR	DL
Max	5.17	4.30	4.19	4.19
β Cd	0.76	1.61	1.55	1.55
β Ni	1.32	1.02	0.92	0.92
IC_{50} for Cd	0.21	0.39	-0.01	-0.01
IC_{50} for Ni	0.28	1.41	1.37	1.37
a	-	-4.93	-0.03	-0.01
$b_{DR/DL}$	-	-	-0.02	2.00
SS	9.26	6.13	-	-
r^2	0.76	0.84	-	-
X^2 or F test	20.95	13.19	-	-
Df	-	1	-	-
p (X^2/F)	5.91×10^{-8}	2.81×10^{-4}	-	-

Figure S1. Calibration curve to analyze total carbohydrates using anhydrous dextrose.



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Conclusões gerais

Ao final deste trabalho, foram obtidas as seguintes conclusões:

- A microalga *R. subcapitata* foi um organismo-teste apropriado para analisar os efeitos dos metais isolados e combinados.

- A análise de múltiplos *endpoints* é uma ferramenta importante para a compreensão dos mecanismos de ação dos metais.

- As medições realizadas pelo dispositivo Phyto-PAM são vantajosas para investigações em toxicologia aquática, pois são sensíveis e podem ser obtidas rapidamente.

- A espécie *R. subcapitata* teve maior sensibilidade ao Cd, do que ao Co e Ni, em relação à densidade celular.

- A curva de distribuição de sensibilidade (SSD) indicou que, dentre 39 espécies de diversos grupos taxonômicos, *R. subcapitata* foi a décima mais sensível ao Cd.

- A curva de distribuição de sensibilidade (SSD) indicou que, dentre 29 espécies de diversos grupos taxonômicos, *R. subcapitata* foi a segunda mais sensível ao Co.

- O Cd levou ao aumento expressivo da geração de ERO e também dos parâmetros analisados por citometria de fluxo (fluorescência da clorofila *a*, tamanho e complexidade celular).

- O Co foi o metal que mais causou alterações nos parâmetros fotossintéticos, diminuindo o Φ_M e levando ao aumento de F_0/F_V. Além disso, diminuiu a síntese de carboidratos totais (em todas as concentrações testadas) e também a densidade celular.

- O Ni diminuiu a fluorescência da clorofila *a* e a síntese de carboidratos totais em todas as concentrações testadas; alterou a geração de ERO e a densidade celular; aumentou F_0/F_V na maior concentração testada, porém não afetou de maneira significativa o rendimento quântico máximo.

- Os testes de toxicidade com os metais Cd e Co combinados indicaram que essa mistura implicou em interações sinérgicas em altas concentrações de Co e baixas de Cd, e interações antagônicas em baixas concentrações de Co e altas de Cd, previstas pelo modelo de referência de adição de concentração (CA) e desvio dependente da proporção da dose (DR). Os parâmetros fotossintéticos (Φ_M e F_0/F_v) tiveram interações antagônicas.

- Os testes de toxicidade dos metais Cd e Ni em mistura tiveram os dados explicados pelo modelo de ação independente (IA) com desvio dependente do nível da dose (DL), com isso, apresentaram interações antagônicas em baixas doses e sinérgicas em altas doses, com mudança do nível da dose maior que a IC_{50}. Além disso, a combinação dos metais levou ao aumento do tamanho e da complexidade celular; alterou a fluorescência da clorofila *a* e a geração de ERO; contudo, não alterou de maneira significativa o Φ_M e F_0/F_v.

- Os testes de toxicidade com Co e Ni combinados resultou em sinergismo em baixas doses de Co e altas de Ni; e antagonismo em altas doses de Co e baixas de Ni. O modelo que melhor explicou os dados foi o CA com desvio DR. Além disso, a mistura desses metais alterou todos os parâmetros testados (ERO, carboidratos, Φ_M, F_0/F_v, fluorescência da clorofila *a*, tamanho e complexidade celular).

Considerações finais

Os resultados deste trabalho nos permitiram concluir que os três metais estudados causaram danos ao metabolismo da microalga *Raphidocelis subcapitata*. Os metais isolados afetaram a densidade celular, a fluorescência da clorofila *a*, o tamanho e a complexidade celular, além de alterar a geração de ERO, a síntese de carboidratos e, de maneira menos expressiva, os parâmetros fotossintéticos.

A mistura dos metais Cd-Co, Cd-Ni e Co-Ni resultou em interações sinérgicas e antagônicas que variaram de acordo com os desvios que melhor explicaram os dados obtidos. Os parâmetros fotossintéticos tiveram interações antagônicas na mistura metálica composta por Cd-Co. A mistura de Co-Ni diminuiu o Φ_M e aumentou F_0/F_v entretanto, Cd-Ni não alteraram esses parâmetros de maneira significativa.

Ainda que o número de estudos sobre a toxicidade de misturas de poluentes venha aumentando nos últimos anos, é muito importante que esses estudos avancem para que se tenha um direcionamento a respeito das possíveis interações que podem acontecer entre os diversos compostos que podem coocorrer no ambiente em múltiplas concentrações. Os resultados deste trabalho podem orientar os próximos estudos sobre o mecanismo de ação dos metais combinados e também auxiliar novas diretrizes e análises de risco.

Por fim, reforça-se a importância dos estudos sobre a toxicidade de misturas de poluentes sobre o metabolismo de microalgas, já que os efeitos dos compostos combinados podem ser diferentes dos efeitos dos compostos isolados, ou seja, as misturas podem levar a efeitos sinérgicos ou antagônicos. Além da relevância de trabalhos com microalgas, pois são organismos fotoautotróficos e sensíveis aos contaminantes, é também importante estudar os efeitos danosos de xenobióticos em misturas sobre organismos de níveis tróficos superiores, para que se tenha um panorama mais próximo da realidade dos ecossistemas aquáticos.