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RESPOSTAS FOTOSSINTÉTICAS DE MACROALGAS LÓTICAS EXPOSTAS A MULTIESTRESSORES AMBIENTAIS DE ORIGEM ANTRÓPICA

LUCAS KORTZ VILAS BOAS

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Vegetal).

RIO CLARO – SP
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TÍTULO DA TESE: **RESPOSTAS FOTOSSINTÉTICAS DE MACROALGAS LÓTICAS EXPOSTAS A MULTIESTRESSORES AMBIENTAIS DE ORIGEM ANTRÓPICA**

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RESUMO

Algas bentônicas são importantes contribuintes para a energia autóctone em ambientes lóticos, além de contribuir para o enriquecimento e estruturação do habitat aquático. De maneira geral, os ambientes aquáticos continentais são historicamente degradados por atividades humanas, das quais, uma das principais é a ocupação e uso de áreas circunvizinhas para o plantio, em larga escala, de espécies de interesse agrícola. A expansão de áreas de agricultura sobre áreas adjacentes a ambientes aquáticos continentais pode afetar tais ambientes de diversas maneiras, entre elas, contaminando-os com pesticidas capazes de agir em organismos não-alvo. Além disso, o aquecimento global, exacerbado pela atividade antrópica, pode afetar a biota dos ambientes aquáticos, influenciando nas características físico-químicas da água e afetando na fisiologia dos organismos. Tais condições constituem um ambiente multi-estressor para os organismos lóticos, especificamente para produtores primários, como as macrófitas e as algas bentônicas. Neste contexto, para se compreender os reais impactos ecológicos desta natureza é fundamental que se avalie os efeitos, diretos e indiretos, de agentes multi-estressores sobre a biota lótica, especialmente, como já dito, sobre os produtores primários, uma vez que estes são, em regra, a base das teias tróficas nestes ecossistemas. Assim, considerando esta lógica, o presente trabalho teve como objetivo avaliar a fotossíntese de espécies de algas bentônicas lóticas após exposição ao herbicida tebuthiuron, um herbicida comumente utilizado em culturas de cana-de-açúcar no Brasil, em conjunto com o aumento de temperatura previsto pelo Painel Intergovernamental de Mudanças Climáticas (IPCC). Três concentrações de tebuthiuron (0,05mg/L, 0,6mg/L e 1,2mg/L) e um controle sem adição do herbicida foram utilizados em conjunto com três temperaturas (21,3 ºC, 23,9 ºC e 26 ºC), calculados a partir das projeções de aumento de temperatura propostas para dois cenários, RCP 4.5 e RCP 8.5, do IPCC. Duas espécies de Chlorophyta (*Nitella microcarpa* var. *wrightii* e *Oedogonium* sp.) e uma espécie de Rhodophyta (*Batrachospermum helminthosum*) foram testadas. As técnicas de fluorescência da clorofila e da evolução do oxigênio dissolvido foram utilizadas. Todas as espécies foram afetadas negativamente pela exposição ao tebuthiuron, com diminuição de seu rendimento quântico efetivo (Y(II)). No entanto, as duas espécies de algas verdes mostraram respostas negativas apenas nas concentrações acima de 0.6 mg/L, enquanto *B. helminthosum* apresentou uma maior sensibilidade, com efeito negativo aparente mesmo na concentração mais baixa. Considerando a temperatura, *N. microcarpa* não mostrou efeitos negativos expressivos relacionados a esse parâmetro, provavelmente por se tratar de uma espécie de alga verde comumente encontrada em locais com alta irradiação e, consequentemente, temperatura mais elevada. *Oedogonium* sp., por outro lado, apesar de descrita como uma alga com altos níveis de crescimento em uma grande amplitude de temperaturas, teve sua taxa de fotossíntese líquida (NPR) reduzida tanto no cenário RCP 4.5 quanto no RCP 8.5. *B. helminthosum*, uma espécie comumente descrita como aclimatada a locais de baixa irradiância e consequentemente a menores temperaturas, apresentou sinais de estresse no cenário RCP 8.5, porém teve sua NPR aumentada no cenário RCP 4.5. Alguns efeitos sinérgicos foram observados entre a exposição ao tebuthiuron e ao aumento de temperatura, como em *N. microcarpa* em que uma diminuição mais expressiva da taxa de transporte de elétrons (ETR) foi causada pelo tebuthiuron no cenário RCP 8.5 e em *B. helminthosum* com sua NPR reduzida em maior intensidade pelo tebuthiuron nos dois cenários de temperatura testados. A capacidade de ação do tebuthiuron nesses organismos não-alvo se dá justamente pelo seu modo de ação no fotossistema II, interrompendo o fluxo de elétrons próximo à plastoquinona B. Considerando a elevada toxicidade ambiental do tebuthiuron, seu uso descontrolado e o concomitante aumento de temperatura causado pelo aquecimento global, os resultados do presente estudo sugerem que alguns dos cenários multi-estressores testados podem gerar grandes impactos à comunidade de organismos fotossintetizantes de água doce, possivelmente impondo, inclusive, severos efeitos negativos nos demais níveis tróficos, dado os reflexos de um potencial "efeito cascata".

Palavras-chave: temperatura, IPCC, pesticidas, tebuthiuron, fluorescência, ecotoxicologia.

ABSTRACT

Benthic algae are important contributors to the autochthonous energy input in lotic environments, in addition to contributing to the richness of the habitat. In general, freshwater environments are historically degraded by human activities, with practices such as the occupation of neighboring areas for the large scale cultivation of species of agronomic interest standing out. The expansion of agricultural areas to zones surrounding freshwater ecosystems may affect these environments in several ways, among them, with the contamination with pesticides, capable of acting on non-target organisms. In addition, global warming, increased by anthropogenic activities, may affect freshwater organisms by altering the physicochemical properties of water and influencing their physiology. Such conditions constitute a multi-stressor environment for lotic organisms, specifically for primary producers, such as macrophytes and benthic algae. In this context, in order to understand the ecological impacts in such circumstances, it is crucial to assess the direct and indirect effects of multi-stressor environments on lotic organisms, especially on primary producers, considering their role in the basis of the food web. Therefore, this study aimed to evaluate the photosynthesis of lotic benthic algae species after exposure to different concentrations of the herbicide tebuthiuron, an herbicide commonly used in sugarcane crops in Brazil, along with the temperature increase predicted by the Intergovernmental Panel on Climate Change (IPCC). Three concentrations of tebuthiuron (0.05mg/L, 0.6mg/L and 1.2mg/L) and a control without herbicide were used in conjunction with three temperatures (21.3 ºC, 23.9 ºC and 26 ºC), calculated from projections of temperature increase proposed by the IPCC RCP 4.5 and RCP 8.5 scenarios. Two species of Chlorophyta (*Nitella microcarpa* var. *wrightii* and *Oedogonium* sp.) and one species of Rhodophyta (*Batrachospermum helminthosum*) were tested. The chlorophyll fluorescence and dissolved oxygen evolution techniques were used. All species were negatively affected by exposure to tebuthiuron, with a decrease in their effective quantum yield (Y(II)) when exposed to the herbicide. However, the two species of green algae showed negative responses only at concentrations higher than 0.6 mg/L, while *B. helminthosum* displayed greater sensitivity, with an apparent negative effect even at the lowest concentration. Considering the temperature, *N. microcarpa* did not show significant negative effects related to this parameter, probably related to being commonly found in places with high irradiation and, consequently, higher temperature, as most green algae. *Oedogonium* sp., on the other hand, despite being described as an alga with high growth levels over a wide temperature range, had its net photosynthetic rate (NPR) reduced in both RCP 4.5 and RCP 8.5 scenarios. *B. helminthosum*, a species commonly described as adapted to low irradiance environments and, consequently, lower temperatures, showed signs of stress in the RCP 8.5 scenario, but had its NPR increased in the RCP 4.5 scenario. Some synergistic effects were observed between the exposure to tebuthiuron and temperature, with *N. microcarpa* showing a higher decrease in its electron transport rate (ETR) caused by tebuthiuron in the scenario RCP 8.5 and *B. helminthosum* with its NPR reduced in a greater intensity by tebuthiuron in the two tested temperature scenarios. Tebuthiuron's ability to act on these non-target organisms is precisely due to its mode of action in photosystem II, blocking the electron flow close to the site of plastoquinone B. Considering its high environmental toxicity, its unrestrained use simultaneous with the increase in temperature caused by global warming may generate great impacts on the community of freshwater photosynthetic organisms, possibly affecting other trophic levels due to a potential “cascade effect”.

Keywords: temperature, IPCC, pesticides, tebuthiuron, fluorescence, ecotoxicology.

SUMÁRIO

INTRODUÇÃO GERAL

As flutuações de temperatura no sistema climático terrestre são influenciadas por diversos fatores e podem ser interpretadas através do conceito de "forçamento radiativo", definido como uma mudança no balanço de energia do sistema climático da Terra devido à uma perturbação (HANSEN, SATO e RUEDY, 1997; IACONO et al., 2008; MYHRE et al., 2013). Um forçamento radiativo positivo significa que tal perturbação acarreta um incremento no aporte de energia do sistema outrora em equilíbrio, causando consequentemente um aquecimento da temperatura terrestre até que essa energia em excesso causada pela perturbação seja dissipada. Da mesma maneira, um forçamento radiativo negativo acarretaria um resfriamento do clima, diminuindo a temperatura terrestre até que o estado volte ao equilíbrio (RAMASWAMY et al., 2019).

A influência antrópica no planeta provocou aumentos drásticos na concentração de gases do efeito estufa (GHGs, em inglês) na nossa atmosfera, de maneira que, baseado em estudos paleoclimáticos, as concentrações atuais de dióxido de carbono (CO_2), óxido nitroso (N_2O) e metano (CH_4) são definidas como as maiores em cerca de 800.000 anos (MASSON-DELMOTTE et al., 2013). As altas concentrações de GHGs de origem antrópica proporcionaram um aumento no aporte de energia inserida no sistema climático do nosso planeta, alterando o fluxo de energia entre a superfície do planeta e a troposfera e, consequentemente, aumentando o forçamento radiativo (MYHRE et al., 2013).

Além dos GHGs, substâncias de origem antropogênica definidas como agentes de forçamento climático de curto-prazo (SLCFs, em inglês) também contribuem significativamente para alterações no fluxo de energia entre a superfície do planeta e a troposfera (ZHAO, STEVENSON e BOLLASINA, 2019). Tais substâncias, como aerosóis, ozônio e seus gases precursores, além de outros poluentes atmosféricos têm uma duração relativamente curta na atmosfera (MYHRE et al., 2013), no entanto, são capazes de influenciar e/ou perturbar significativamente o sistema climático durante várias décadas ou séculos, especialmente em escalas regionais (ZHAO, STEVENSON e BOLLASINA, 2019).

Novos estudos utilizando dados paleoclimáticos compreendendo 12 mil anos da temperatura global mostraram que o último pico de temperatura média global foi há cerca de 6,5 mil anos, com uma temperatura 0,7 ºC mais quente que a temperatura média do século XIX. Após esse período, o clima terrestre iniciou uma tendência de esfriamento de aproximadamente 0,08ºC a cada mil anos (KAUFMAN et al., 2020). Contudo, desde o advento das atividades industriais do início do século XIX, e em um período de menos de 150 anos (de 1880 a 2012), a temperatura média global já apresentou um aumento de cerca de 0,85ºC (HANSEN et al.,

2010; HARTMANN et al., 2013), atingindo valores médios mais elevados que o período de aquecimento observado há 6,5 mil anos. Dessa maneira, mesmo considerando variações em escalas de tempo menores, já é possível observar que as atividades antrópicas foram capazes de anular a tendência natural de resfriamento da temperatura média global no sistema climático terrestre e revertê-la para uma tendência de aquecimento (KAUFMAN et al., 2020).

Além do aumento de temperatura observado no último século, diversos parâmetros climáticos também sofreram alterações. Alterações na precipitação e em ciclos hidrológicos (MA et al., 2018; SNYDER et al., 2019), na frequência de eventos climáticos extremos (ZANOCCO et al., 2018), na composição da criosfera (ADLER et al., 2019) e em diversos ciclos biogeoquímicos (STOCKER et al., 2013) já foram reportadas ou são estimadas para a próxima década, como efeito das mudanças climáticas de origem antrópica (CIAIS et al., 2013; HARTMANN et al., 2013). Alterações em tais parâmetros climáticos, que se somam às alterações de temperatura, podem, por sua vez, influenciar na eficiência da dissipação ou acúmulo de energia necessário para que o sistema climático retorne ao equilíbrio, gerando um sistema de feedback que pode amplificar o efeito da perturbação inicial (RAMASWAMY et al., 2019).

Por conta da relação entre tolerância térmica e diversos padrões fisiológicos, alterações de temperatura são capazes de influenciar diretamente a abundância, a distribuição e o comportamento dos organismos (PARMESAN e YOHE, 2003; SUNDAY, BATES e DULVY, 2012). Corroborando a relevância ecológica destas relações, diversos estudos realizados nas últimas décadas têm mostrado que praticamente toda a superfície terrestre sofreu um aumento significativo de temperatura, com alguns locais apresentando um aumento de até 2,5ºC (HARTMANN et al., 2013), e que, concomitantemente, há sérias evidências de alterações na biodiversidade relacionadas justamente a estes aumentos de temperatura. Neste contexto, por exemplo, já se observa que certas espécies de insetos têm mudado seus padrões de distribuição, migrando em direção aos polos (PARMESAN et al., 1999), enquanto espécies de plantas típicas de vegetação de altitude têm se deslocado para regiões de altitudes maiores (KELLY e GOULDEN, 2008). Diversos estudos também têm registrado alterações na fenologia de espécies que incluem, por exemplo: pássaros antecipando o seu período de migração (INOUYE et al., 2000; GIENAPP, LEIMU e MERILÄ, 2007), borboletas aparecendo com antecedência e com picos populacionais alterados (ROY e SPARKS, 2000), mamíferos saindo mais cedo da hibernação (INOUYE et al., 2000) e alterações na floração de algumas espécies de plantas (FITTER e FITTER, 2002).

Além de influenciar padrões de distribuição e/ou fenologia, as respostas fisiológicas dos organismos têm sido diretamente relacionadas à determinados eventos de extinção associados às mudanças de temperatura, tanto em se tratando de extinções de populações (SINERVO et al., 2010) como de espécies individualmente (POUNDS et al., 2006). De fato, ao comparar as taxas de extinção da biodiversidade em massa no passado da Terra com as extinções causadas devido às pressões de origem humana, os dados sugerem que uma sexta onda de extinção em massa, neste caso, de origem antrópica, está em curso (CEBALLOS et al., 2015). Em sua maioria, os casos já reportados nesta nova onda de extinções são de espécies ectotérmicas. Em um cenário de aumento de temperatura, espécies ectotérmicas apresentam diminuição significativa na eficiência dos ajustes em processos de termorregulação que, consequentemente, ampliam os seus riscos de extinção (HUEY, LOSOS e MORITZ, 2010). Stuart et al. (2004) estimaram que um grande número de espécies de anfíbios já deve estar, possivelmente, extinto, enquanto outros autores apontam para outras extinções similarmente preocupantes para espécies e populações de lagartos (SINERVO et al., 2010).

Projeções para a quantidade de extinções relacionadas às mudanças climáticas indicam que esses números tendem a aumentar. Em um estudo utilizando espécies endêmicas de várias regiões da Terra, estimou-se que 37% delas estariam destinadas à extinção já em 2050 (THOMAS et al., 2004). Em adição, há trabalhos que sugerem que, dependendo do nível de aumento da temperatura atmosférica terrestre nas próximas décadas e da capacidade de dispersão dos organismos, uma a cada seis espécies poderá estar extinta no futuro (URBAN, 2015).

Apesar da nitidez do efeito de mudanças de temperatura na distribuição, fenologia ou desaparecimento de espécies, é importante salientar que, em escala ecossistêmica, os efeitos se tornam muito mais complexos, considerando as intrincadas interações entre indivíduos, espécies, populações e comunidades (PEÑUELAS, FILELLA e COMAS, 2002). Alterações na fenologia ou distribuição de uma espécie presente em níveis inferiores da teia trófica podem modificar o estoque de recurso alimentar para espécies em níveis tróficos superiores, influenciando, em cascata, toda estrutura do sistema (MCMAHON e HAYS, 2006). O ciclo de reprodução de espécies que necessitam de polinizadores também pode ser afetado (SHERRY et al., 2006), e mudanças na temperatura podem facilitar a entrada de espécies invasoras (ALEXANDER, DIEZ e LEVINE, 2015) ou de parasitas e doenças (POUNDS et al., 2006). Neste contexto, a quantidade de potenciais efeitos, considerando a enorme complexidade dos ecossistemas, sugere que as mudanças climáticas globais emergem como uma força demasiado

importante na modificação do funcionamento de ecossistemas como os conhecemos (PEÑUELAS, FILELLA e COMAS, 2002).

Desafortunadamente, as mudanças climáticas ocorrem concomitantemente com a contínua modificação de paisagens naturais por outras tantas influências humanas, o que gera, em última análise, ambientes multi-estressores para os organismos (FORISTER et al., 2010; HOFFMANN e SGRÒ, 2011). A urbanização, a fragmentação de habitats, a inserção de espécies não nativas, a poluição e a agricultura intensiva são, por exemplo, fatores importantes a serem considerados nas discussões sobre o manejo e a conservação de espécies (CZECH e KRAUSMAN, 1997; JANTZ et al., 2015).

Essas múltiplas pressões antrópicas podem ser um grande problema para, por exemplo, espécies com longos ciclos de reprodução ou com uma diversidade genética reduzida, uma vez que tais espécies têm capacidade de ajuste menos eficiente, correndo, então, grave risco de extinção. Os riscos produzidos por ambientes multi-estressores aumentam quando se considera que as mudanças climáticas e demais estressores antrópicos têm produzido modificações ambientais drásticas em um intervalo de tempo muito curto (HOFFMANN e SGRÒ, 2011). Além disso, estima-se que as reservas ecológicas atuais não seriam suficientemente capazes de manter a biodiversidade ameaçada e correm sério risco de desaparecer (HELLER e ZAVALETA, 2009).

A maior razão para a destruição de habitats naturais nos últimos séculos foi a criação de áreas para pastagem e agricultura (ELLIS et al., 2010), e com o aumento da população humana nos próximos anos, a criação de áreas agricultu̇ráveis pode aumentar cerca de 18% em comparação ao início dos anos 2000 (TILMAN et al., 2001). A transformação de áreas para agricultura próximo a reservas ecológicas é um dos fatores que mais ameaçam a capacidade das mesmas em manter a biodiversidade (HAMILTON et al., 2015), uma vez que, além da conversão da paisagem natural, a prática da agricultura pode influenciar na eutrofização de habitats, devido à quantidade de fertilizantes utilizados, além de gerar impactos pela utilização de pesticidas (TILMAN et al., 2001).

Os pesticidas estão entre os químicos mais utilizados globalmente, com mais de 3 milhões de toneladas aplicados anualmente ao redor do mundo (PIMENTEL, 2005). Devido à resistência adquirida por algumas pragas, muitos produtores passaram a aplicar uma maior quantidade ou a combinar pesticidas diferentes, indicando que o volume de uso destas substâncias tende a aumentar (GLIESSMAN, 2015). Apenas no Brasil, a quantidade de pesticidas vendida no ano de 2014 foi de 508 mil toneladas, sendo que 58% deste montante foram da classe dos herbicidas (IBGE, 2014).

Além de afetar a composição da biota, modificar a relação entre diferentes espécies e alterar o comportamento de alguns organismos não-alvo presentes em áreas agricultáveis (RELYEA e EDWARDS, 2010; MARTINOU, SERAPHIDES e STAVRINIDES, 2013), alguns pesticidas são persistentes e possuem grande chance de carreamento (OLIVEIRA et al., 2016), podendo afetar organismos presentes em ambientes além do local de aplicação. Entre os ambientes que podem ser fortemente afetados por pesticidas carreados para além dos limites das áreas de sua aplicação estão os ambientes aquáticos continentais (PETERS, BUNDSCHUH e SCHAFER, 2013).

Um herbicida comumente utilizado no Brasil, principalmente em culturas de cana-de-açúcar, é o tebuthiuron. Classificado como um agrotóxico "relativamente não seletivo", é um composto da classe dos derivados de uréia e é principalmente aplicado no solo, sendo absorvido pela raiz das plantas e levado até as folhas (RYBERG et al., 2010) onde inibe a fotossíntese, uma vez que impede o fluxo de elétrons no fotossistema II (HATZIOS, PENNER e BELL, 1980). Devido à sua pequena adsorção ao sedimento e alta resistência à degradação química e biológica, é uma substância altamente persistente, podendo permanecer por anos no local de aplicação dependendo do tipo de solo (GOMES et al., 2006). Por conta destas características, o tebuthiuron é considerado como substância com alto potencial de contaminação ambiental, uma vez que pode ser carreado para águas superficiais e/ou subterrâneas (US EPA, 1994).

Nos últimos anos, com a eliminação da prática da queimada da palha de cana-de-açúcar após a colheita, devido à Lei Estadual nº 11.241 (São Paulo, 2002), ocorreu um aumento na quantidade utilizada desse herbicida no estado de São Paulo. Uma vez que a presença de uma espessa camada de palha nos campos dificulta a chegada desse agrotóxico ao solo, maiores quantidades têm sido utilizadas na tentativa de mantê-lo eficiente na eliminação de pragas (TOFOLI et al., 2009). Neste cenário, a contaminação de águas superficiais pode ocorrer devido ao deslocamento da substância no momento da aplicação, a ocorrência de precipitações após a aplicação ou o cuidado impróprio ao descartar resíduos ou lavar equipamentos (CAUX et al., 1997). Em um trabalho realizado com espécies de organismos fotossintetizantes e animais de ambientes aquáticos na Austrália, Van Dam et al. (2004) verificaram que o tebuthiuron é altamente tóxico para a comunidade fitoplanctônica e de macrófitas aquáticas, demonstrando pequeno efeito nos invertebrados e vertebrados aquáticos.

Em testes aplicando concentrações ambientais esperadas (5,8 mg/L) de acordo com o Ministério do Meio Ambiente do Canadá (PETERSON et al., 1994), o tebuthiuron causou uma inibição de mais de 70% na fixação de carbono na maioria das espécies de microalgas e de macrófitas testadas. Após mensurar a quantidade residual de tebuthiuron próxima a plantações

de cana-de-açúcar na China, verificou-se que as concentrações encontradas em diversos corpos d'água (0,007 a 0,022 mg/L) foram tóxicas para as algas (QIAN et al., 2017).

Mesmo em concentrações relativamente pequenas (0,012 a 0,094 mg/L), o tebuthiuron foi capaz de afetar significativamente o rendimento quântico fotossintético (Y(II)) de microalgas marinhas, inibindo mais de 50% do Y(II) já nos primeiros minutos de exposição (MAGNUSSON et al., 2010). Do mesmo modo, estudos mostram que o tebuthiuron também é capaz de afetar negativamente a comunidade de diatomáceas marinhas como um todo (WOOD et al., 2016). A alta toxicidade desse herbicida para organismos fotossintetizantes, mesmo em concentrações pequenas, se dá devido ao seu mecanismo de ação, uma vez que age inibindo o transporte de elétrons no fotossistema II, um processo crucial no metabolismo de tais organismos (QIAN et al., 2017).

Fica claro, portanto, que, por já serem, em sua maioria, áreas extremamente deterioradas devido à ocupação antrópica, os ambientes aquáticos continentais e sua biodiversidade se encontram sob um risco ainda maior dos impactos das mudanças climáticas e da contínua expansão de áreas agrícolas (WOODWARD, PERKINS e BROWN, 2010). Os impactos destas substâncias nos ecossistemas lóticos, em especial, dada a sua característica hierárquica e unidirecional, podem causar efeitos ramificados, uma vez que qualquer poluente pode ser carreado, à jusante, por longas distâncias ao longo do perfil longitudinal do rio (MALMQVIST et al., 2008).

Nos rios e riachos tropicais, devido à grande diversidade de características físicas e químicas e da biota desses ecossistemas, é difícil reconhecer claramente um padrão do tipo de energia efetivamente responsável pela sustentação de suas teias tróficas. Entretanto, sabe-se que existem rios e riachos sustentados tanto por energia autóctone quanto alóctone (LAU, LEUNG e DUDGEON, 2008).

Considerando o componente autóctone da energia que sustenta a teia trófica em rios e riachos, as macroalgas bentônicas compõem uma comunidade que contribui imensamente para a produtividade primária de riachos, sendo identificadas como produtores dominantes em vários ambientes (STEVENSON, BOTHWELL e LOWE, 1996; ARSCOTT, BOWDEN e FINLAY, 1998, BRANCO et al., 2017). Além disso, as macroalgas são fonte de alimentos mais importantes que detritos para alguns organismos consumidores (MAYER e LIKENS, 1987), e contribuem para o aumento da diversidade e riqueza do habitat, uma vez que aumentam a heterogeneidade espacial (DOWNES et al., 2000). Algas bentônicas também são consideradas como sendo bioindicadores ideais para ecossistemas lóticos por serem aderidas ao substrato e, portanto, receberem integralmente os efeitos das mudanças no ambiente (BELLINGER e

SIGEE, 2015). Neste sentido, estudos recentes têm mostrado que estas algas podem ser fortemente afetadas pela presença de herbicidas no ambiente aquático (p.ex., OLIVEIRA, VILAS BOAS e BRANCO, 2016).

Considerando todo o contexto exposto, é bastante razoável supor que estudos que avaliem os efeitos de multi-estressores ambientais, tais como as mudanças climáticas associadas aos efeitos de pesticidas sobre espécies-chave, como são as macroalgas para os ecossistemas lóticos de baixas ordens, sejam fundamentais para ampliar o conhecimento dos potenciais impactos dos mesmos sobre a estrutura e o funcionamento de sistemas naturais. Ou seja, parece claro que estudos que se proponham a compreender os impactos desses estressores em nível de espécie são importantes na tomada de decisões de conservação (WILLIS et al., 2015).

Neste contexto, o presente estudo se propôs a avaliar a resposta fotossintética de espécies de macroalgas lóticas sob exposição concomitante a diferentes concentrações do herbicida tebuthiuron e diferentes condições de aumento de temperatura relacionadas aos cenários de aquecimento global propostos pelo painel intergovernamental de mudanças climáticas (IPCC). Através das técnicas de fluorescência da clorofila *a* e evolução de oxigênio dissolvido, procurou-se elucidar se tais variáveis afetam a fotossíntese desses organismos, bem como observar se há alguma interação entre os estressores.

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CAPÍTULO 1

Effect of tebuthiuron and temperature increase related to climate change on the photosynthesis of *Nitella microcarpa* var. *wrightii* (Charophyceae).

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ABSTRACT

Charales is an important order of freshwater algae, closely related to embryophytes and relevant primary producer in the aquatic food web, capable of increasing water transparency and habitat heterogeneity. Anthropogenic activities such as use of herbicides in agricultural areas and the temperature increase related to climate change can combine into a multi-stressor environment and potentially influence the photosynthetic performance of such organisms. Using chlorophyll *a* fluorescence analysis, we evaluated the photosynthesis of *Nitella microcarpa* var. *wrightii* after 7 days exposure to multi-stressor scenarios composed by different nominal concentrations of the herbicide tebuthiuron (0.05 mg/L; 0.6 mg/L and 1.2 mg/L), and two temperature increase scenarios related to projections by the Intergovernmental Panel on Climate Change (RCP 4.5 and RCP 8.5). The chlorophyll *a* fluorescence parameters were significantly different in the multi-stressor treatments. Very evident negative effects were found in treatments with nominal concentrations of 0.6 mg/L tebuthiuron or higher, with significant reductions in all measured fluorescence parameters. In addition, tests showed that while tebuthiuron by itself reduced the electron transport rate (ETR) of this species in all tested concentrations, the temperature increase in the RCP 8.5 scenario significantly strengthened the action of the herbicide. Our study highlights that unrestrained usage of pesticides such as tebuthiuron could not only jeopardize the physiological performance of *N. microcarpa* but also negatively influence their carbon fixation and contribution to primary productivity in aquatic ecosystems, especially in scenarios where the temperature increase due to climate change lessens the ability of these organisms to withstand other stressors.

KEYWORDS

Charales; Chorophyll a Fluorescence; Herbicide; IPCC; Multi-stressor; Sugarcane Cultivation Scenario.

INTRODUCTION

Historically, the expansion of areas for agricultural activities is one of the main drivers of habitat destruction, endangerment of species and extinction (MAXWELL et al. 2016; NEWBOLD et al. 2018). Agricultural land use can double the amount of sediment in bodies of water, increasing eutrophication (TILMAN et al. 2001) and influencing the nutrient ratio and form (i.e. dissolved or particulate) in rivers and streams throughout their entire length, even at river-sea deltas (SCHMIDT et al. 2018, MANNING et al. 2020). In addition, agricultural land use significantly increases the amount of pesticides that end up in bodies of water (SZÖCS et al. 2017). All these changes in the environment directly affect aquatic organisms.

In Brazil, one widely used pesticide, especially in sugarcane (*Saccharum officinarum* L.) crops, is tebuthiuron (1-(5-tert-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea). Tebuthiuron is a non-selective phenyl-urea herbicide that acts by inhibiting the electron transport of photosynthesis probably, as other phenylurea herbicides such as diuron, by binding to the D1 protein (LIU 2010). It is mainly applied directly at the soil, where it is absorbed by the roots or leaves of early weed shoots and inhibits their growth. In 2002, the Brazilian federal government issued new regulations for sugarcane crops attempting to reduce and fully ban the practice of burning in sugarcane agricultural fields by 2017. Such a ban, however, has caused an increase in the use of herbicides in sugarcane crops (TONIÊTO et al. 2016) since, with the cane straw layer covering the soil, the current practice consists of applying greater amounts of herbicide in order to effectively inhibit weed growth.

Tebuthiuron has high leaching potential, and it has been detected at small concentrations (0,09µg/L) in Brazilian groundwaters in the past (GOMES et al. 2001). Its soil transport is possibly related to the microbial activity in early layers of soil, with higher activity in tropical soils diminishing its potential for groundwater contamination (CERDEIRA et al. 2007; RACKE et al. 1997). In lakes and streams throughout the world, reported tebuthiuron concentrations range from 0,007mg to 0,094mg/L in countries such as China and Australia, where, even at such low concentrations, adverse effects were observed both to marine and freshwater algae (MAGNUSSON et al. 2010; QIAN et al. 2017).

While tebuthiuron alone is toxic to some aquatic organisms, many other anthropogenic activities affect biodiversity along with the habitat contamination and destruction by agricultural activities. Climate change, for instance, has been a frequent subject in scientific discussion during the last decades. The increase in atmospheric temperature is one segment of many variables of anthropogenic climate change (HARTMANN et al. 2013). Future temperature projections by the IPCC show, with very high certainty, that even in mitigation

scenarios, global mean temperature will continue to increase in the following decades (IPCC 2013; COLLINS et al. 2013).

Due to the variation in their thermal tolerances, changes in temperature directly influence the distribution of organisms. With higher energy demands for thermoregulation, species significantly lose fitness, increasing their risk of extinction, especially if ectothermic (HUEY et al. 2010; STUART et al. 2004). Reported impacts on biodiversity due to temperature increase range from physiological or behavioral changes to the extinction of species (SINERVO et al. 2010; SUNDAY et al. 2012). The temperature increase coupled with the destruction of habitats and other anthropogenic activities constitutes a multi-stressor environment for biodiversity (FORISTER et al. 2010; HOFFMANN AND SGRÒ 2011). The risks of multi-stressor environments are even greater when considering that climate change and other anthropic stressors have produced drastic environmental changes in a very short time and measuring the effects of such impacts can become much more complex when considering the biotic interactions between populations (PEÑUELAS et al. 2002).

Charales, or stoneworts, a monophyletic order of freshwater green algae, has long been considered one of the closest evolutionary relatives of embryophytes (land plants) (BOWER 1908). Whether Charales or Coleochaetales, two orders of Charophycean algae, is the sister group of embryophytes is still a subject of much debate (MISHLER AND CHURCHILL 1985; KAROL et al. 2001; MCCOURT, DELWICHE and KAROL 2004), in any case Charales retain many similarities to land plants and remains a major group in understanding the evolutionary history of plants as a whole.

In aquatic habitats, these organisms influence nutrient cycling and greatly contribute to the autochthonous energy input for the food web (VAN DONK and VAN DE BUND 2002). Their presence also prevents the resuspension of sediments, reducing turbidity and increasing water transparency, sometimes even more strongly than angiosperms (NÖGES et al. 2003; BLINDOW, HARGEBY and HILT 2014). Moreover, Charalean algae increase habitat heterogeneity, possibly raising the abundance of zooplankton due to the higher number of refuge spots from predation (BOLDUC et al. 2016) and offer a high surface for the growth of periphyton, which in turn favors the development of macroinvertebrates (DECLERCK et al. 2011; BLINDOW et al. 2014).

Studying the effects of multi-stressor conditions, such as tebuthiuron exposure and temperature increase related to climate change scenarios, can shed some light on how freshwater organisms are affected by anthropogenic activities, while also helping in understanding how they could be affected in the future. Since Charalean algae highly contribute

to the autochthonous energy production, to the stability of freshwater ecosystems as a whole and tebuthiuron targets the photosynthesis of embryophytes, a close relative of these algae, evaluating their photosynthetic performance under such conditions seems relevant.

In the present paper, the photosynthetic performance of a species of Charalean algae, *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves was evaluated after being submitted to different nominal concentrations of tebuthiuron along with temperature increases related to the IPCC scenarios projections. With previous studies showing the negative effect of diverse herbicides on the photosynthesis of Charalean algae, we hypothesize that tebuthiuron also negatively affects the photosynthetic performance of this species, especially in higher nominal concentrations. In addition, due to their natural occurrence in highly irradiated, and consequently warmer, areas, the temperature increase may not affect the photosynthetic performance of these organisms by itself; however, in treatments combining higher nominal concentrations of tebuthiuron and higher increases in temperature, the negative response to tebuthiuron may be exacerbated due to the higher energy demand for thermoregulation.

MATERIAL AND METHODS

Collection and preparation of algal specimens

The samples of the Charalean species *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves (hereafter called *N. microcarpa*) used in the experiments were collected in the Pari River, located in the western region of the state of São Paulo, Brazil (22°38'33.0"S 50°12'14.8"W). The stream was classified as an opened stream, with sunlight reaching the rocky substrate. After collection, algal specimens were taken to the laboratory in transparent vials containing river water. In the laboratory, in order to remove possible sediments and epiphytes associated with the stem, the specimens were cleaned with hard bristle brushes and jets of distilled water under a stereoscopic microscope. After cleaning, specimens were weighed and separated into 60 samples of 150mg (± 10mg) (n = 5 for each multi-stressor treatment).

Following the procedures described by Oliveira et al. (2016), samples were then transferred to 150 ml erlenmeyer flasks containing 100 ml of Bold's basal medium (BBM) (WATANABE 2005) and acclimated under the experimental conditions. Then, Charalean specimens were kept in B.O.D. incubators (Nova Ética, model 411 / FDP355) under constant temperature determined for each experimental treatment (described below), constant irradiance (140 $\mu mol.m^{-2}s^{-1}$) and 12h / 12h photoperiod (light / dark cycle). After a 24-hour acclimation period, samples belonging to treatments with the presence of herbicides had their BBM replaced

by BBM with the addition of active ingredient according to the nominal concentrations established for each treatment. In order to avoid nutrient depletion and to keep the nominal concentrations of active ingredient constant during the days of exposure, the media of the samples were renewed on the third day and fifth day, following the recommendations of the 221 chemistry test guide from the Organization for Economic Cooperation and Development (OECD 2006) and the procedures described by Oliveira et al. (2016). The samples remained under such culture conditions for a total of 7 days, with experimental analyses being carried on the 7th day of exposure.

Determination of temperature scenarios

The temperature scenarios were calculated by adding the maximum values of the range projected for the tropical region by the IPCC (COLLINS et al. 2013) to a mean of measured temperatures (MMT) of streams in the basin where samples were collected. The temperature of streams (Table 1) were measured during winter season (June 2019) using a multiparameter probe (HORIBA U-50) and the resulting mean was of 21.6ºC. In order to obtain the highest temperature in streams, measurements were taken between 15:00h and 17:00h, since the peak temperature for streams in this region during winter has been shown to occur at 16:00h (NECCHI et al. 1996). While higher temperatures are found during the summer, in tropical regions most algae thrive during winter, especially due to the lower rainfall regime (BRANCO and PEREIRA 2002), therefore, testing temperatures from this season is more appropriate.

Following Vilas Boas et al. (2019), the two IPCC scenarios used were RCP 4.5, where a stabilization in the release of carbon into the atmosphere would occur, and RCP 8.5, where large emissions of carbon would continue to occur. The maximum projected values of temperature increase in these two scenarios are 2.3ºC and 4.4ºC for RCP 4.5 and RCP 8.5 respectively (COLLINS et al. 2013). While the MMT (21.6ºC) was used as control, treatments RCP 4.5 and RCP 8.5 had the experimental temperatures of 23.9ºC and 26ºC respectively.

Determination of tebuthiuron nominal concentrations

Three treatments with different nominal concentrations of tebuthiuron were used, in addition to the control (C), without herbicide. Experimental solutions were prepared by dilution with distilled water, considering the concentration of active ingredient in the commercial formulation Combine®500 SC. The treatment with the lowest nominal concentration (T1) corresponded to the maximum nominal concentration allowed in bodies of water by the North

American Environmental Protection Agency: 0.05mg of active ingredient per liter (U.S. ENVIRONMENTAL PROTECTION AGENCY 1988). The second treatment (T2) used the recommended value for the application of tebuthiuron in sugarcane crops in clay soils: 0.6mg/L (Combine®500 SC). Finally, the third treatment (T3) simulated a 'worst case scenario', with twice the recommended value for application on clay soils (1.2mg/L). Therefore, the tested nominal concentrations of tebuthiuron were, from the lowest to the highest: 0.05 mg/L, 0.6 mg/L and 1.2 mg/L.

Multi-stressor treatments

Considering the temperature scenarios and nominal concentrations of tebuthiuron, treatments with combinations of the two stressors were made, with three temperature scenarios combined with three nominal concentrations of tebuthiuron and control (0.0 mg/L), thus totaling 12 multi-stressors treatments. Treatments were defined as (see the topics above Determination of temperature scenarios and Determination of tebuthiuron nominal concentration for acronyms): MMT Control , MMT T1, MMT T2 and MMT T3; RCP 4.5 Control, RCP 4,5 T1; RCP 4.5 T2 and RCP 4.5 T3; and RCP 8.5 Control, RCP 8.5 T1, RCP 8.5 T2 and RCP 8.5 T3.

Experimental analyses

The photosynthetic performance of *Nitella microcarpa* was evaluated through chlorophyll *a* fluorescence (BAKER 2008; VILAS BOAS et al. 2019). The analysis of photosynthetic responses using the chlorophyll *a* fluorescence were performed using a Diving-PAM fluorometer (Walz, Effeltrich, Germany). After a period of 30 minutes of acclimatization in the dark, the samples were positioned on the optical fiber of the equipment and through the "Induction Curve" function (SCHREIBER et al. 1995), 12 pulses of saturating light (2000µmol photons.m^2.s^{-1}) lasting 0.8s were applied at 15s intervals (OLIVEIRA et al. 2016). Based on the data produced by these curves, the following photosynthetic parameters of each species submitted to each treatment were evaluated (KLUGHAMMER and SCHREIBER 2008; Oliveira et al. 2016): i) effective quantum yield of photosystem II ("Y(II)"); ii) quantum yield of regulated non-photochemical energy loss in photosystem II ("Y(NPQ)"); iii) quantum yield of non-regulated non-photochemical energy loss in photosystem II ("Y (NO)") and iv) electron transport rate (ETR).

Statistical Analyses

The differences in the photosynthetic responses of specimens were evaluated by the two-way ANOVA test. For construction of these tests, Y(II), Y(NO), Y(NPQ) and ETR were used as dependent variables, while tebuthiuron treatments and temperature scenarios were used as independent variables. These tests were conducted with the aid of the statistical software IBM© SPSS© Statistics (IBM Corp. Released 2019).

RESULTS

The two-way ANOVA tests indicated that the exposure to different nominal concentrations of tebuthiuron affected all fluorescence parameters, while the temperature scenarios alone did not affect the evaluated parameters and the interaction between the two factors affected the values of electron transport rate (ETR).

In this regard, as shown by the similar drop in all lines in fig. 1, *N. microcarpa* showed statistically significant reductions ($p < 0.001$, $F = 9.677$) on the quantum photosynthetic yield (Y(II)) in all temperature scenarios for treatments T2 (-85%, -74% and -59% for MMT, RCP 4.5 and RCP 8.5 respectively) and T3 (-91%, -87% and -59%) in comparison to Control. No significant differences for Y(II) were observed between Control and T1 (fig. 1).

In addition, the values of Y(NO) were significantly higher ($p < 0.001$, $F = 9.875$) in treatments T2 (+40%, +57% and +4%) and T3 (+46%, +66% and +2%) than those of Control, as shown by the slight incline in all lines in fig. 2, while no statistically significant difference was observed between the values of Y(NO) for T1 and Control.

For Y(NPQ), the values reported in T3 (-83%, -74% and +8% for MMT, RCP 4.5 and RCP 8.5 respectively) were significantly different ($p < 0.05$, $F = 5.225$) than those of Control. Moreover, no statistically significant difference was found between Y(NPQ) values of Control, T1 and T2 (fig. 3).

The ETR values dropped significantly with the increase in nominal concentrations of tebuthiuron ($p < 0.001$, $F = 96.641$). T1 presented statistically significant differences from Control, T2 and T3, with values 42%, 44% and 67% lower than control for MMT, RCP 4.5 and RCP 8.5 respectively. T2 and T3 presented statistically significant differences from control and T1 as well, with T2 presenting values 84%, 82% and 85% lower than control for MMT, RCP 4.5 and RCP 8.5 respectively and T3 with reductions of 79%, 88% and 94% for the same scenarios in comparison to control (fig. 4). Considering the temperature scenarios, the statistical tests showed a significant influence of the interaction between the temperature scenarios factor

and the nominal concentrations of tebuthiuron ($p < 0.01$, $F = 3.621$), with higher percentages of ETR reduction in the RCP 8.5 scenario in comparison to the MMT and the RCP 4.5 scenario.

DISCUSSION

Considering both stressing factors, the exposure to tebuthiuron was the factor that significantly affected all measured photosynthetic parameters of *N. microcarpa*, while the interaction between stressors only affected the electron transport rate (ETR).

The negative effect of tebuthiuron exposure was very evident, especially in treatments with nominal concentrations equal to or higher than 0.6mg/L, regardless of the temperature scenarios. Although not a target species of this herbicide, the mode of action of tebuthiuron is capable of diminishing the photosynthetic performance of *N. microcarpa* if found in concentrations recommended for field application or higher. This species of Charophycean algae has been reported as susceptible to the action of other herbicides, such as glyphosate and its degradation products, being affected differently by the product form and length of exposure (OLIVEIRA et al. 2016).

The exposure to 0.6 mg/L or higher nominal concentrations of tebuthiuron lowered the effective quantum yield (Y(II)) of this species, reducing its capacity of converting photons into electrons (TAIT et al. 2017). At the same time, the increase in the non-regulated non-photochemical energy loss in photosystem II (Y(NO)) indicates a stressful situation that could result in damage to the photosynthetic apparatus (KLUGHAMMER and SCHREIBER 2008). Furthermore, generally, the significantly lower Y(NPQ) values in T3 indicate that, at the highest nominal concentration, the negative effect is even more significant. Y(NPQ) represents a form of controlled energy release that demonstrates an attempt to acclimatize or to release energy without damaging the photosynthetic apparatus (ZHAO et al. 2017). The decrease in this parameter therefore would indicate the loss of this controlled path of energy release for this species.

While concentrations of this magnitude (> 0.6 mg/L) in natural aquatic ecosystems may seem high from a certain standpoint, the mode of application as a pre-emergence herbicide in clear soil, its relative persistence, high water solubility and mobility into run-off water all contribute for higher concentrations of tebuthiuron in runoff and consequently aquatic environments (CAUX et al 1997; VAN DAM et al. 2004). Experiments in Australia measuring tebuthiuron in surface runoff in clear fields after natural rainfall showed that 1170 days after a single application (2.5 kg/ha) and 2426mm of accumulated rain, 1% of the applied concentration (25 g/ha) was lost to runoff (THORNTON and ELLEDGE 2016). In Brazil,

where the amount of herbicides applied in sugarcane has risen after the ban of straw burning (TONIÊTO et al. 2016) and the rainfall regime is high, it doesn't seem too farfetched to expect that concentrations close to or higher than 0.6 mg/L could occur in aquatic ecosystems close to sugarcane crops.

The electron transport rate parameter, however, showed a significant negative effect of tebuthiuron even in the lowest nominal concentration (0.05mg/L). Tebuthiuron, as other phenylurea herbicides, acts by binding into D1 protein, in the Q_B binding domain. With tebuthiuron bonded to D1, the electron transfer in photosystem II does not fully carry out, causing a charge recombination that can result in the formation of chlorophylls triplet state, which, in turn, can react with oxygen and create singlet oxygen, capable of damaging photosystem II (FUFEZAN et al. 2002; KRIEGER-LISZKAY 2004). In addition, the low ETR values could represent less electrons reaching photosystem I and being available for reducing NADP+. Although direct calculations of carbon fixation values using ETR measurements depend on many factors (BAKER and OXBOROUGH 2004; FIGUEROA et al. 2013), these results indicate that exposure to nominal concentrations of tebuthiuron of 0.05mg/L and especially 0.6mg/L or higher not only causes physiological stress to this species, but could also impact in their role as primary producers in the ecosystem by decreasing their carbon fixation.

One interesting remark is that, even though the temperature value of the RCP 8.5 scenario (26ºC) falls out of the reported optima (20~25ºC) for *Nitella* species (VIEIRA JR. and NECCHI JR. 2003), the temperature effect alone did not show any significant effect on the photosynthetic performance of *N. microcarpa*. However, the negative effects of the exposure to tebuthiuron on the ETR values were significantly higher in the scenario with the highest temperature. While the exposure in the MMT scenario to the nominal concentrations of 0.05mg/L, 0.6mg/L and 1.2mg/L lowered the ETR of *N. microcarpa* by 42%, 84% and 79% respectively in comparison to control, the percentages of reduction were of 67%, 85% and 94%, in the RCP 8.5 scenario. Such results corroborate our hypothesis that the increase in temperature could lower the capacity of this species to withstand the stress of other factors, such as pesticides.

In conclusion, lotic macroalgae are important components of the aquatic communities and are susceptible to various stressors in the aquatic ecosystem. Our paper contributes to the knowledge that the use of pesticides is an important variable in studies about the ecology of such organisms. The unrestrained use of such substances can not only negatively affect the physiology of a single species but also possibly influence its contribution to primary

productivity in these environments, especially in multi-stressor scenarios of climate change and temperature increase.

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TABLE AND FIGURES

Table 1. Temperature values (ºC) and GPS coordinates of 10 streams in the Cervo river microbasin, used to determine de mean of measured temperatures (MMT) of treatments and calculate the experimental temperatures.

Stream	***Latitude***	***Longitude***	***Temperature***
01	22° 35' 22.9344" S	50° 24' 59.9436" W	22.08 ºC
02	22° 35' 33.3096" S	50° 25' 23.0232" W	22.34 ºC
03	22° 35' 22.2" S	50° 24' 45.3456" W	20.40 ºC
04	22° 34' 29.5932" S	50° 25' 6.6828" W	21.77 ºC
05	22° 38' 26.304" S	50° 20' 26.736" W	21.70 ºC
06	22° 38' 23.784" S	50° 19' 5.5092" W	21.21 ºC
07	22° 38' 31.2216" S	50° 12' 13.9824" W	20.37 ºC
08	22° 36' 38.1456" S	50° 27' 59.9292" W	21.68 ºC
09	22° 40' 12.6048" S	50° 28' 55.4088" W	22.23 ºC
10	22° 45' 29.0952" S	50° 31' 36.8112" W	22.07 ºC
MMT:			***21.6ºC***

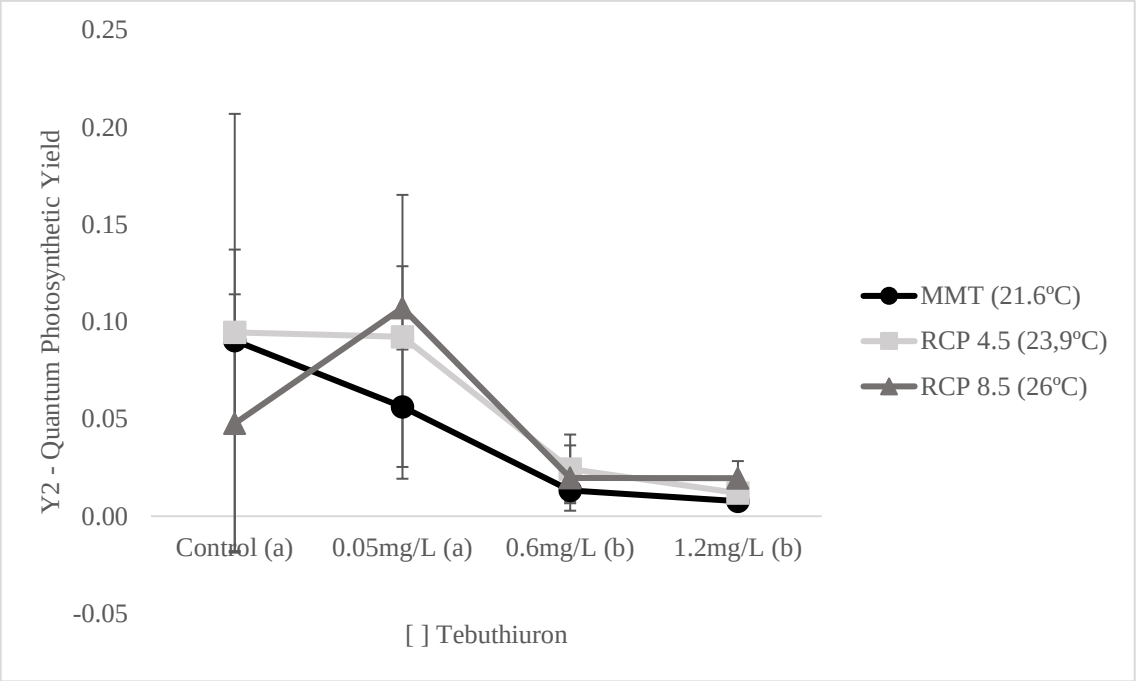

Figure 1 – Y(II) (effective quantum yield of photosystem II) mean values (n = 5) and standard deviations of *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

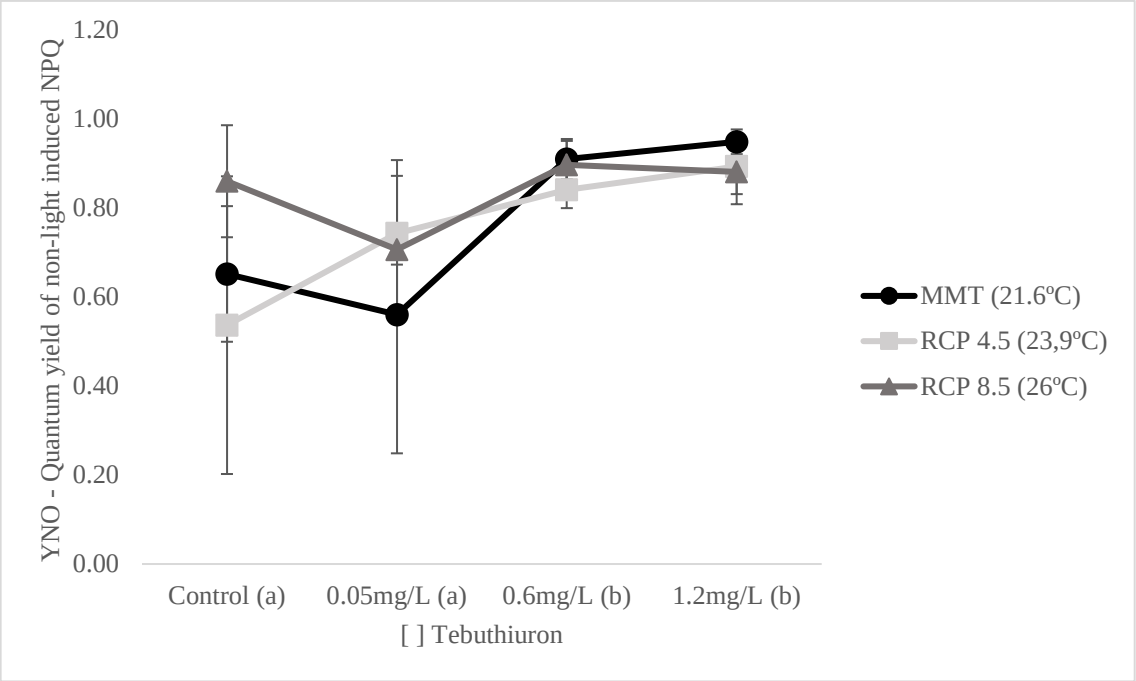

Fig. 2. Y(NO) (non-regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

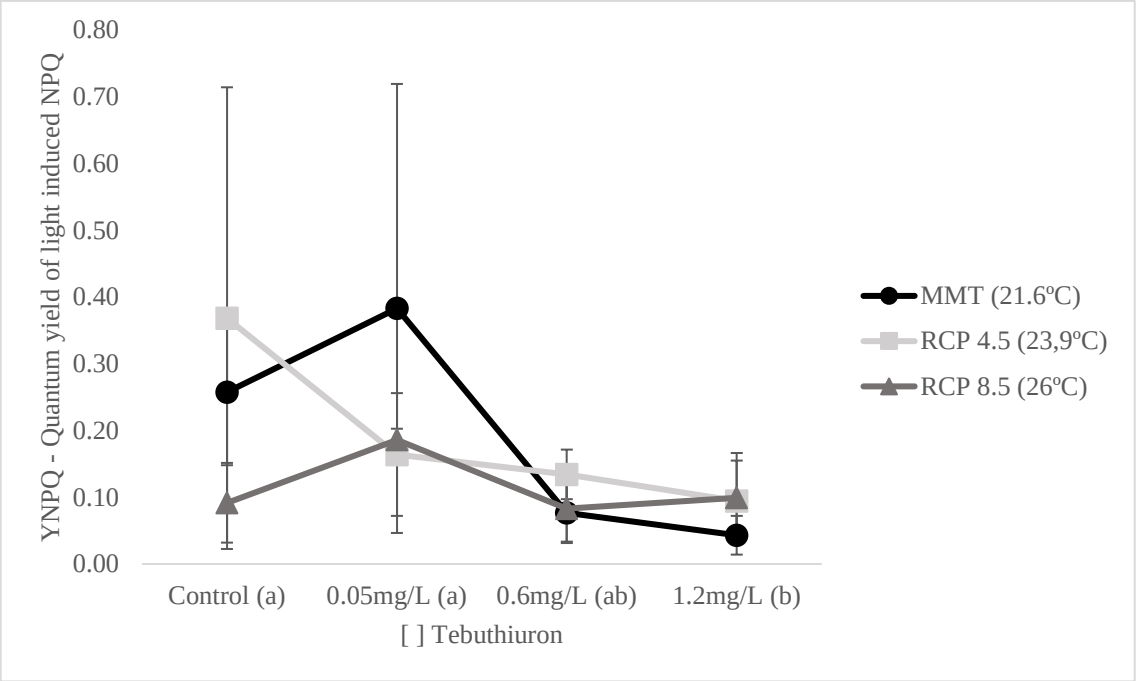

Fig. 3. Y(NPQ) (regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

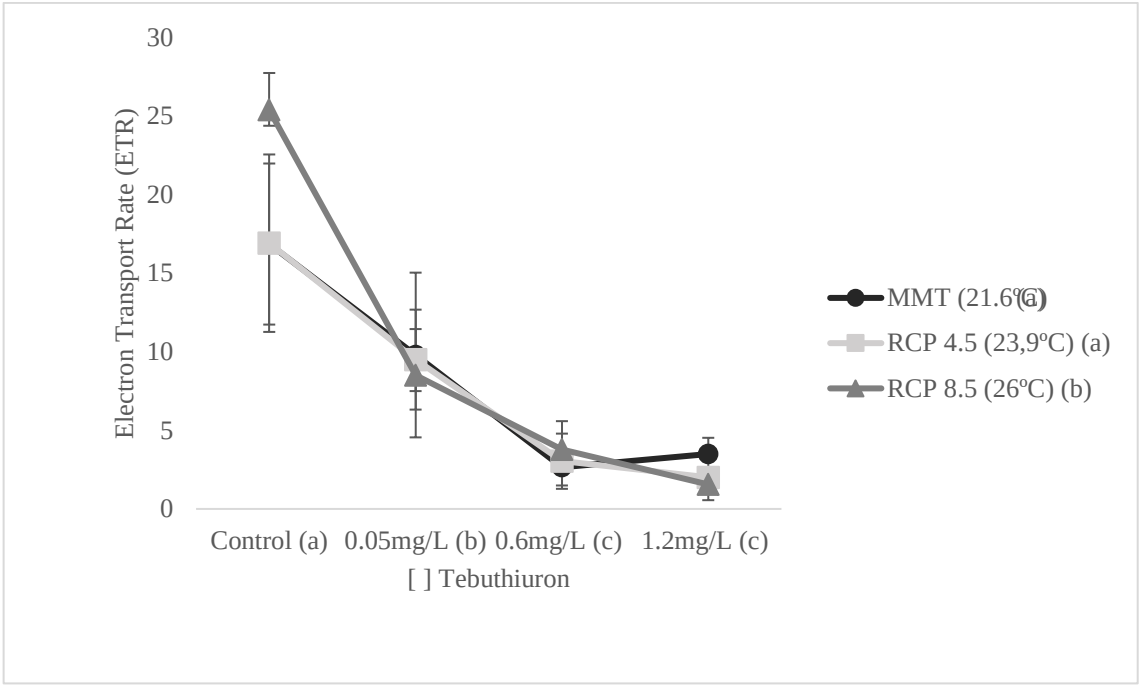

Fig. 4. ETR (electron transport rate) mean values (n = 5) and standard deviations of *Nitella microcarpa* var. *wrightii* H.Groves & J.Groves after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

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CAPÍTULO 2

In a multi-stressor scenario, exposure to the herbicide tebuthiuron and temperature increase related to climate change can individually impair the photosynthesis of *Oedogonium* sp.

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ABSTRACT

Freshwater habitats are among the most degraded natural environments, with organisms in such habitats usually living in multi-stressor conditions. Green macroalgae are important primary producers in the freshwater environment and negative effects on their photosynthesis may influence other trophic levels. In this study, we tested the photosynthetic performance of *Oedogonium* sp. after 7-day exposure to multi-stressor conditions composed by nominal concentrations of tebuthiuron, a commonly used herbicide in sugarcane crop fields in Brazil, and the temperature increase related to projections of anthropogenic climate change. For the control, samples of *Oedogonium* sp. were experimentally maintained in a culture medium without the herbicide and under the mean of measured temperatures (MMT) of the streams of the river microbasin where specimens of this filamentous green alga were collected (i.e., 21.6 ºC). Treatments were composed by combining nominal concentrations (0.05 mg/L, 0.6 mg/L and 1.2 mg/L) of tebuthiuron, with temperature increases of +2.3 ºC and +3.4 ºC, values projected by the Intergovernmental Panel on Climate Change for the scenarios RCP 4.5 and RCP 8.5, respectively. Chlorophyll *a* fluorescence analysis showed that the exposure to concentrations of tebuthiuron of 0.6 mg/L or higher, regardless of temperature, negatively affected the photosynthetic performance of the alga, showing diminished capacity of light-conversion (i.e., reduction in Y(II)) associated to an indication of possible damage to the photosynthetic apparatus (i.e., increase in Y(NO)). In the oxygen evolution curve analyses, the net photosynthetic rate of *Oedogonium* sp. dropped significantly in the RCP 4.5 and RCP 8.5 scenarios in comparison to MMT (i.e., control group), with decreases ranging from 13% to 70% among treatments. Despite these results make clear the occurrence of negative effects of exposure to both stressors individually, no joint effect of the stressors was observed. Although no interaction was observed in the multi-stressor scenarios, since *Oedogonium* is a widely distributed green alga and an important primary producer, the negative effects recorded for individual stressors on its photosynthesis may ultimately affect other trophic levels. In a broader sense, considering that the stressors tested in the present study can affect different photosynthetic parameters of the algal model (i.e., *Oedogonium* sp.), the eventual fulfillment of IPCC projections along with continuous usage of chemical compounds such as tebuthiuron will likely compose a potentially tough scenario for photosynthetic metabolism of freshwater green algal species.

KEYWORDS

Chorophyll *a* Fluorescence; ; IPCC; ; Sugarcane crops; Green algae; Chlorophyta

INTRODUCTION

Countless anthropogenic stressors regularly affect organisms in the natural environments. The large array of industrially developed chemical compounds that end up in diverse ecosystems and the associated human activities that promote habitat destruction constitute a multi-stressor setting for biodiversity as a whole (SMITH et al. 2015). Among the most degraded natural environments are freshwater ecosystems. Historically, human expansion has explored and degraded inland aquatic habitats, with many still affected nowadays (MALMQVIST et al. 2008).

Such stressful conditions affect important physiological processes, among them photosynthesis, which not only endangers the well-being of primary producers but also generate impacts across multiple levels of organization, possibly generating trophic cascade effects within these ecosystems (WOODWARD et al. 2010). Although the freshwater trophic web receives great amounts of energy from external sources (allochtonous energy), primary producers (autochthonous energy) have a crucial role in the environment and are able to sustain many freshwater communities, especially in tropical regions (LAU et al. 2008; NERES-LIMA et al. 2017).

Green macroalgae represent one of the larger groups of primary producers, especially in high-irradiated lotic environments (BRANCO et al. 2017). Along with being a great food source, these organisms also serve as habitat and as a refuge for predation for aquatic invertebrates (BOLDUC et al. 2016). Among green algae, the genus *Oedogonium* Link ex Hirn stands out for its worldwide distribution, its great biotechnological potential, with applications for bioenergy by biofuel production and for its reported usage in bioremediation (LAWTON et al. 2014; ADEGOKE et al. 2018; ROBERTS et al. 2018). This genus has more than 650 described species, although many of them probably badly defined, since species identification in *Oedogonium* and delimitation of Oedogoniales is a long-standing problem (ALBERGHINA et al. 2006; GUIRY and GUIRY 2021).

There has been a significant increase in the registration of new pesticides in Brazil in the past few years, along with an increase in sales of previously registered products, resulting in a higher risk of contamination of freshwater environments by pesticides, especially those close to agricultural areas. A substantial part of Brazilian agriculture is sugarcane farming, and Brazil is one of the largest producers of sugarcane in the world, with an estimated harvest of 665 million tons of sugarcane for the year 2020 (CONAB, 2020). Sugarcane planting usually occurs twice a year and after the ban of the practice of burning the remaining sugarcane straw on crop fields before planting, farmers have increased the volume of herbicides applied on the

fields, for the chemicals to pass through the straw layer and reach the soil (TONIÊTO et al. 2016).

One commonly used herbicide in sugarcane farming in Brazil is tebuthiuron (1-(5-tert-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea). This herbicide belongs to the phenyl-urea class of pesticides and acts by inhibiting the electron transport in photosystem II, preventing the photosynthesis of early weed shoots after absorption by the root (LIU, 2010). Tebuthiuron has high leaching potential and some studies have reported its presence in groundwater in Brazil (e.g. GOMES et al. 2001). The World Health Organization classifies tebuthiuron as moderately toxic (WORLD HEALTH ORGANIZATION, 2019) while international authorities such as the European Union have limited its usage and classifies it as very toxic, especially to aquatic life (EUROPEAN CHEMICALS AGENCY, 2021).

Along with the exposure to anthropogenic chemical compounds, organisms must withstand the stress of anthropogenic climate change. In freshwater environments, climate change can mainly affect freshwater organisms by changing water temperature and the rainfall regime (ENGELMAN et al. 2008). Being already heavily degraded environments due to human settling and exploration, freshwater ecosystems are highly susceptible to biodiversity loss caused by climate change and synergistic human activities (DUDGEON, 2019).

The Intergovernmental Panel on Climate Change (IPCC, 2013) proposed, in its 5th Assessment Report, four Representative Concentration Pathways (RCPs) scenarios. RCP 2.6, where strong mitigation policies would take place, RCP 4.5 and RCP 6.0, intermediate scenarios where greenhouse gases emissions would stabilize at their current level without many efforts to constrain emissions, and RCP 8.5, where global greenhouse gases emissions would continue to increase. In any of the four RCPs, projections indicate that global mean temperature will increase by at least 2ºC in the following decades.

Temperature is highly relevant to biodiversity, being a strong driver of carbon flow in some freshwater environments (BELLE et al. 2018), capable of changing habitat suitability (MAROTZKE et al. 2017), and influencing the life history, interaction and persistence of individuals within populations (KNOUFT and FICKLIN 2017). Considering the relevance of global warming and its impact on biodiversity, the concomitant stressful action of anthropogenic substances such as herbicides in the natural environment and the importance of benthic algal communities, it is now imperative to analyze the photosynthetic performance of algal species subjected to such multi-stressor conditions. Ultimately, these effects could cause significant changes not only in the performance of a single species, but also in the functioning

of the ecosystem as a whole, especially in those environments where these organisms are responsible for a large part of primary production.

In this chapter, we tested the effect of simultaneous exposure to different tebuthiuron concentrations and temperature increase related to climate change scenarios on *Oedogonium* sp., a globally distributed algal species. Since the photosynthesis process of *Oedogonium* sp. is similar to that of the target organisms of tebuthiuron, we hypothesize that the alga will be negatively affected by the herbicide. In addition, as a genus of green algae globally distributed and commonly found in environments with higher solar irradiance, the temperature factor by itself may not cause significant responses, however, due to the energy spent in thermoregulation processes, the temperature increase can escalate the negative responses to the herbicide.

MATERIAL AND METHODS

Sampling and preparation of algal samples

Specimens of *Oedogonium* sp. Link ex Hirn were collected from the Pari river, located in the Cervo river microbasin, in the western region of the state of São Paulo, Brazil (22°38'33.0"S 50°12'14.8"W) and taken to the laboratory in transparent vials containing river water. Manual cleaning of samples was performed using a stereoscopic microscope, jets of distilled water, hard bristle brushes and forceps in order to remove sediment, possible epiphytes and invertebrates. 60 samples of 150mg (± 10mg) were prepared after weighing in an analytical scale (n = 5 for each multi-stressor treatment).

Samples were acclimated for 24 hours under the experimental conditions in 150ml erlenmeyer flasks containting 100ml of Bold's basal medium (BBM) (Watanabe 2005) inside B.O.D. incubators (Nova Ética, model 411 / FDP355) under constant irradiance (140 µmol.m-2s-1), 12h / 12h photoperiod (light / dark cycle) and constant temperature determined for each experimental treatment (see the topic Determination of temperature scenarios).

After acclimation, the BBM of samples was replaced with BBM containing the active ingredient of tebuthiuron in accordance with each multi-stressor treatment. Photosynthetic performance analyses were performed after a 7-day period. Following Oliveira et al. (2016) and adapting the recommendations of the 221 chemistry test guide by the Organization for Economic Cooperation and Development (OECD, 2006), the medium of all samples was renewed on the third and fifth day in order to avoid nutrient depletion and to keep a constant nominal concentration of the active ingredient.

Determination of temperature scenarios

First of all, for determining the control temperature, we calculated the mean of measured temperatures (MMT) of the streams in the river microbasin, Cervo river microbasin, where samples of *Oedogonium* sp. Link ex Hirn were collected. While in tropical regions the highest stream temperatures are reached in the summer, the seasonality of freshwater algae in such regions is mostly related to the rainfall regime (BRANCO and PEREIRA 2002), with larger populations occurring in winter. During winter in tropical regions, the highest stream temperatures are found at 16:00h, therefore, temperature measurements were taken between 15:00h and 17:00h during winter (June 2019) using a multiparameter probe (HORIBA U-50), with a resulting mean of 21.6ºC (Table 1).

Calculations of experimental temperatures were performed as described by Vilas Boas et al. (2019) and considering two scenarios of the Intergovernmental Panel on Climate Change (IPCC): RCP 4.5 and RCP 8.5. In general terms, in scenario RCP 4.5, the IPCC projects the stabilization of emissions into the atmosphere, while in scenario RCP 8.5 it is considered that an increase in emissions would occur (IPCC, 2013). Thus, to obtain the experimental temperatures, the maximum projected values (COLLINS et al. 2013) of 2.3ºC (RCP 4.5) and 4.4º C (RCP 8.5) were added to the MMT. While the MMT (21.6ºC) was used as control, the experimental scenarios RCP 4.5 and RCP 8.5 had the experimental temperatures of 23.9ºC and 26ºC, respectively.

Determination of nominal concentrations of tebuthiuron

The tested nominal concentrations of tebuthiuron were, from lowest to highest: 0.05 mg/L (T1), 0.6 mg/L (T2) and 1.2 mg/L (T3). T1 concentration corresponds to the maximum concentration allowed by the North American Environmental Protection Agency in water bodies: 0.05 mg of active ingredient per liter (U.S. Environmental Protection Agency, 1988). The second treatment (T2) corresponds to the recommended dosage of application of tebuthiuron for sugarcane crops on clay soils: 0.6 mg/L (Combine®500 SC). Finally, the third treatment (T3) corresponds to a 'worst case scenario', with twice the recommended dosage of application on clay soils (1.2 mg/L). A control (C) without the addition of herbicide was also performed.

Multi-stressor treatments

Combinations of the two stressors were made as follows: MMT plus two temperature scenarios were combined with 3 concentrations of tebuthiuron, in addition to the control without herbicide, totaling 12 multi-stressor treatments. The 12 multi-stressor treatments were coded

by the following acronyms: MMT Control, MMT T1, MMT T2 and MMT T3; RCP 4.5 Control, RCP 4,5 T1; RCP 4.5 T2 and RCP 4.5 T3; and RCP 8.5 Control, RCP 8.5 T1, RCP 8.5 T2 and, finally, RCP 8.5 T3 (see the topics Determination of temperature scenarios and Determination of nominal concentrations of tebuthiuron for acronyms).

Experimental analyses

The photosynthetic performance of *Oedogonium* sp. was evaluated through the chlorophyll *a* fluorescence and dissolved oxygen evolution techniques (NECCHI JR and ZUCCHI 2001; VIEIRA JR and NECCHI JR 2003; BRANCO et al. 2017; VILAS BOAS et al. 2019). Specimens were analyzed after 7 days exposure to the multi-stressor treatments.

Using a Diving-PAM fluorometer (Walz, Effeltrich, Germany), the following photosynthetic chlorophyll *a* parameters were measured: i) Y(II) - effective quantum yield of photosystem II; ii) Y(NPQ) - quantum yield of regulated non-photochemical energy loss in photosystem II and iii) Y(NO) - quantum yield of non-regulated non-photochemical energy loss in photosystem II. Measurements were taken through the "Induction Curve" function (SCHREIBER et al. 1995), with 12 pulses of saturating light (2,000 µmol photons.m^2.s^{-1}) lasting 0.8 s applied at 15 s intervals at samples after a 30 min period of acclimatization in the dark (VILAS BOAS et al., 2019).

The measurements of dissolved oxygen were carried out with the aid of an oxygen meter, equipped with a self-stirring probe (brand YSI, model 5100). Based on the variation of the initial and final dissolved oxygen concentrations after one hour incubation in their respective treatments (LITTLER and ARNOLD 1985; THOMAS 1988), net photosynthetic rate and dark respiration rates were calculated in clear and dark glass bottles, respectively.

Formulas for the calculations were, as follows: NP = [(F) - (I)] * V / IT / DW and DR = [(I) - (F)] * V / IT / DW where NP: net photosynthetic rate; DR: dark respiration rate; (F): final concentration of dissolved oxygen after the incubation period; (I): initial concentration of dissolved oxygen before the incubation period; V: volume (liters) of medium in the bottle; IT: incubation time; DW: dry weight.

Statistical Analyses

Statistical tests were performed in the statistical software IBM© SPSS© Statistics (IBM 2019). Possible differences in photosynthetic parameters (both from chlorophyll *a* fluorescence and from the dissolved oxygen evolution) among treatments and control group were identified by means of a two-way ANOVA tests. For these tests, the photosynthetic parameters were used as

dependent variables, while the factors of multi-stressor treatments (concentrations of tebuthiuron and temperature scenarios) were used as independent variables.

RESULTS

The two-way ANOVA tests revealed that different factors affected different photosynthetic parameters. The chlorophyll *a* fluorescence results were affected by the nominal tebuthiuron concentrations, while the parameters obtained by the oxygen evolution technique were affected by the temperature scenarios. No evidence of an interaction between factors was found.

The photosynthetic yield (Y(II)) of *Oedogonium* sp. significantly dropped ($p < 0.001$, $F = 11.29$) in treatments with nominal concentrations of 0.6 mg/L of tebuthiuron or higher in comparison to control (-77%, -66% and -60% for T2 in the MMT, RCP 4.5 and RCP 8.5 respectively and -86%, -83% and -81% for T3 in the MMT, RCP 4.5 and RCP 8.5, respectively) (fig 1.). At the same time, the values of Y(NO) were significantly higher ($p < 0.001$, $F = 10.98$) in T2 (+79%, +27% and +26% for MMT, RCP 4.5 and RCP 8.5) and T3 (+104%, +34% and +53% for MMT, RCP 4.5 and RCP 8.5) in comparison to control (fig. 2). No significant differences were found between any treatments for Y(NPQ) (fig. 3).

Considering the dissolved oxygen evolution analysis, the net photosynthetic rates (NPR) of *Oedogonium* sp. were significantly reduced ($p < 0.001$, $F = 23.09$) as the temperature of the scenarios increased, showing an inverse relation. MMT presented the highest values of NPR, with RCP 4.5 (-54% in Control, -63% in T1, -70% in T2 and -57% in T3) and RCP 8.5 (-42% in Control, -36% in T1, -44% in T2 and -13% in T3) being significantly lower from MMT (fig. 4). The dark respiration rate was significantly lower ($p < 0.001$, $F = 52.15$) in the RCP 8.5 scenario compared to the MMT (-82% in Control, -81% in T1, -89% in T2 and -93% in T3) (fig. 5).

DISCUSSION

Chlorophyll *a* fluorescence analyses showed that the exposure to nominal concentrations of tebuthiuron of 0.6 mg/L or higher negatively affected the photosynthetic performance of *Oedogonium* sp. The lower values of photosynthetic yield (Y(II)) in T2 and T3 suggest that the capacity of light energy conversion of *Oedogonium* sp. is severely diminished in such concentrations (TAIT et al. 2017) at the same time, higher Y(NO) values indicate a release of excess energy through paths that could induce damage to the photosynthetic apparatus of this filamentous green algae via photooxidative stress (KLUGHAMMER and SCHREIBER 2008).

The photooxidative stress in plants and algae is caused by singlet oxygen species ($^1O_2$) (KRIEGER-LISZKAY 2005). After a charge recombination in PSII occurs, either due to photoinhibition or due to the action of pesticides that bind to photosystem II (PSII), there is a formation of chlorophyll triplet state which, in the presence of O_2, can react to form $^1O_2$ (FUFEZAN et al. 2002). $^1O_2$ is very reactive and rapidly reacts with target molecules, causing cellular damage that ultimately can lead to cell death (Triantaphylidès et al. 2008).

A study using a species of diatom as model, *Chaetoceros muelleri*, has shown similar results after exposure to lower concentrations of tebuthiuron (THOMAS et al. 2020). In that case, the authors note that the effect of tebuthiuron was lowering the photosynthetic yield and hampering the electron flow in photosystem II, which not only inhibited the photosynthesis of the diatom, but also produced damage to the photosystem, which, in turn, decreased the algae's growth rate. Since *Chaetoceros muelleri* is a unicellular species, the negative effects (i.e., cell death due to the photooxidative stress) caused by tebuthiuron are more pronounced and dramatic than in a filamentous species like *Oedogonium* sp.

While the exposure to tebuthiuron produced significant negative effects on the chlorophyll *a* fluorescence parameters in our tests, with potential damage due to photooxidative stress, no differences were detected by the dissolved oxygen analyses. The lack of significant differences in oxygen concentrations might be due to the mode of action of this herbicide. As other PSII inhibitor herbicides, tebuthiuron acts by blocking the electron flow in photosynthesis, diminishing the production of ATP and NADPH and ultimately hampering the carbon assimilation by the organism (DUKE and DAYAN 2018). These so-called "dark-reactions" occur in the Calvin cycle, after the oxidization of water, therefore, while the release of O_2 is still occurring in the culture medium, the algae is not capable of completing its carboxylation cycle (BUKHOV 2004).

The temperature increases related to the IPCC scenarios did not significantly affect any of the measured chlorophyll *a* fluorescence parameters and contrary to our hypothesis, did not show any interaction with the tebuthiuron nominal concentrations. However, a significant effect of this factor was observed in the net photosynthetic rate of *Oedogonium* sp.

Values of net photosynthetic rate of *Oedogonium* sp. dropped significantly in both temperature scenarios, confirming that the fulfillment of the IPCC scenarios RCP 4.5 and RCP 8.5 may negatively affect the primary productivity of this organism. Considering that species of *Oedogonium* are very relevant primary producers in tropical lotic ecosystems, this impact can possibly spread to further levels of organization, influencing the trophic webs that are energetically supported by this filamentous green algae. In general, *Oedogonium* species have

been reported as possessing high growth rates in a wide range of temperatures, a characteristic that promotes them as potential candidates for biomass applications, however, changes in temperature still significantly affect their growth rate (LAWTON et al. 2014).

In conclusion, our results showed that our hypotheses were partially confirmed. Even though *Oedogonium* is not the target species of the herbicide, due to the similar photosynthesis pathway that this algae possess to seed plants, the exposure to tebuthiuron in nominal concentrations of 0.6 mg/L or higher significantly hamper its photosynthetic performance. In addition, contrary to our expectations, the temperature increases related to climate change, even in the less severe scenario of the IPCC (RCP 4.5), negatively affected the net photosynthetic rate of this algae. Although no synergy or interaction was observed between exposure to tebuthiuron and increases of temperature related to climatic change, the impact in different measured photosynthetic parameters proves that multi-stressor scenarios, like the one investigated in the present study, constitute a stressfull environment for the species, affecting its photosynthesis and possibly ultimately affecting other trophic levels.

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TABLE AND FIGURES

Table 1. Temperature values (ºC) and GPS coordinates of 10 streams in the Cervo river microbasin, used to determine de mean of measured temperatures (MMT) of treatments and calculate the experimental temperatures.

Stream	***Latitude***	***Longitude***	***Temperature***
01	22° 35' 22.9344" S	50° 24' 59.9436" W	22.08 ºC
02	22° 35' 33.3096" S	50° 25' 23.0232" W	22.34 ºC
03	22° 35' 22.2" S	50° 24' 45.3456" W	20.40 ºC
04	22° 34' 29.5932" S	50° 25' 6.6828" W	21.77 ºC
05	22° 38' 26.304" S	50° 20' 26.736" W	21.70 ºC
06	22° 38' 23.784" S	50° 19' 5.5092" W	21.21 ºC
07	22° 38' 31.2216" S	50° 12' 13.9824" W	20.37 ºC
08	22° 36' 38.1456" S	50° 27' 59.9292" W	21.68 ºC
09	22° 40' 12.6048" S	50° 28' 55.4088" W	22.23 ºC
10	22° 45' 29.0952" S	50° 31' 36.8112" W	22.07 ºC
MMT:			***21.6ºC***

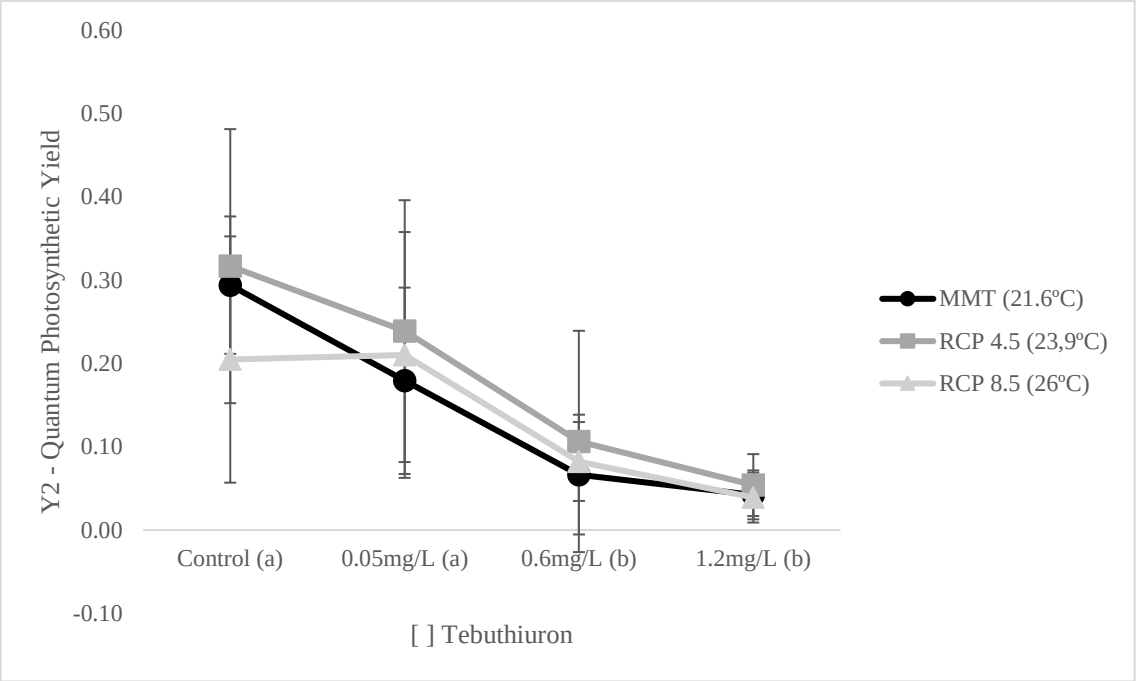

Figure 1 – Y(II) (effective quantum yield of photosystem II) mean values (n = 5) and standard deviations of *Oedogonium* sp. Link ex Hirn after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

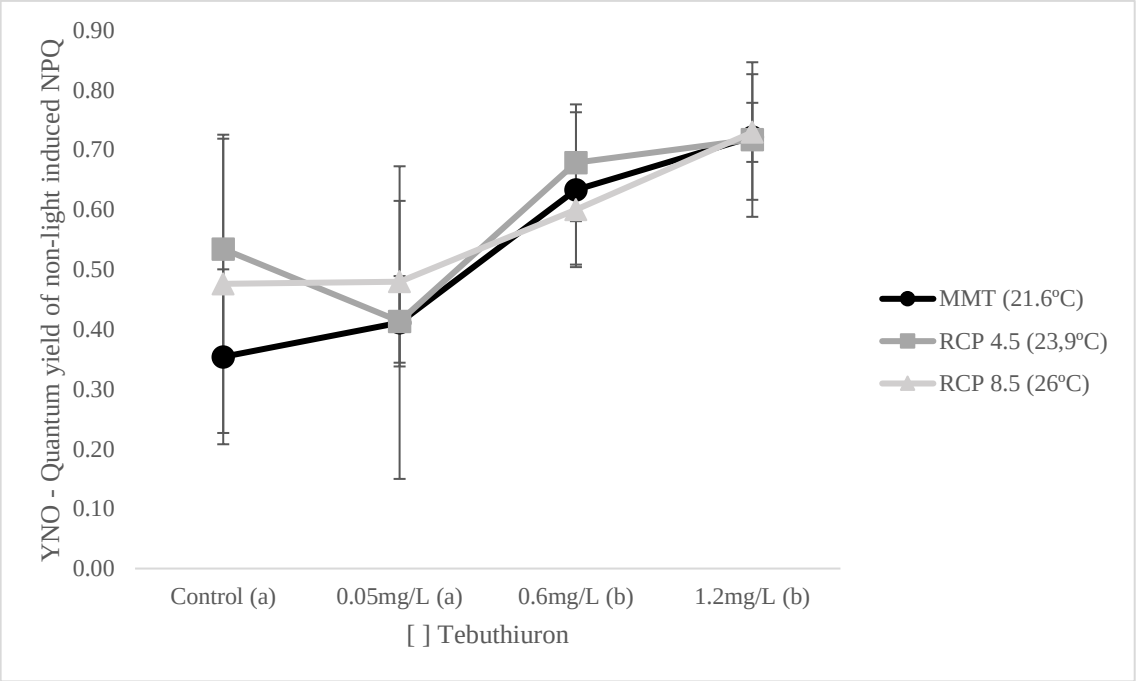

Figure 2 – Y(NO) (non-regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Oedogonium* sp. Link ex Hirn after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

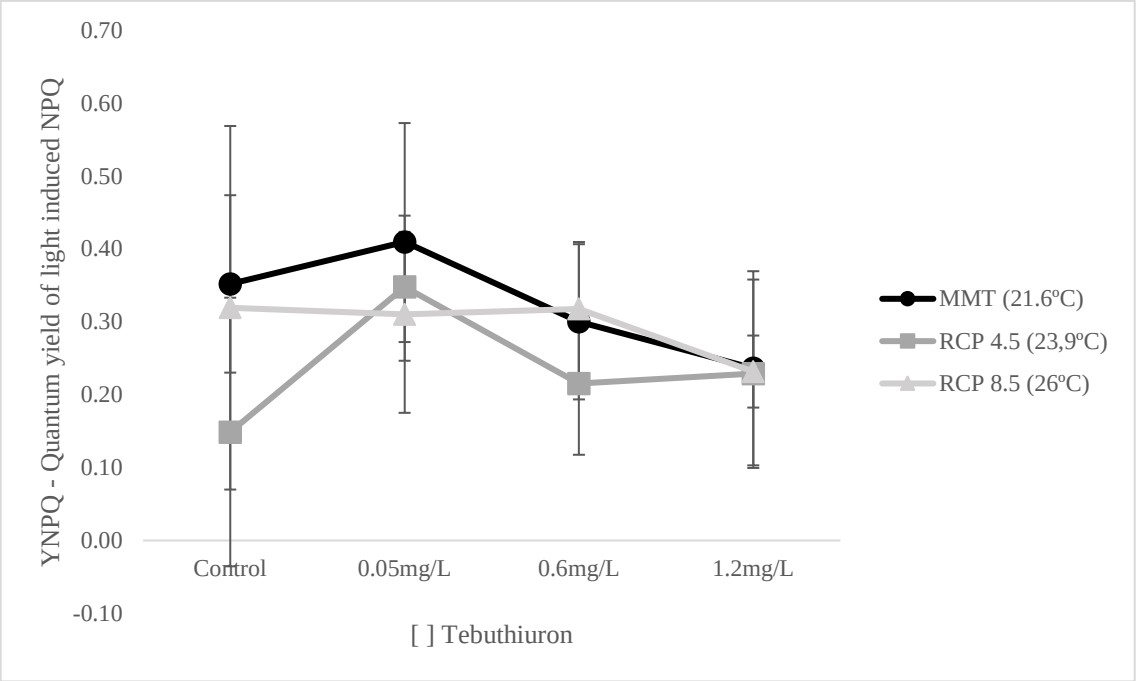

Figure 3 – Y(NPQ) (regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Oedogonium* sp. Link ex Hirn after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios.

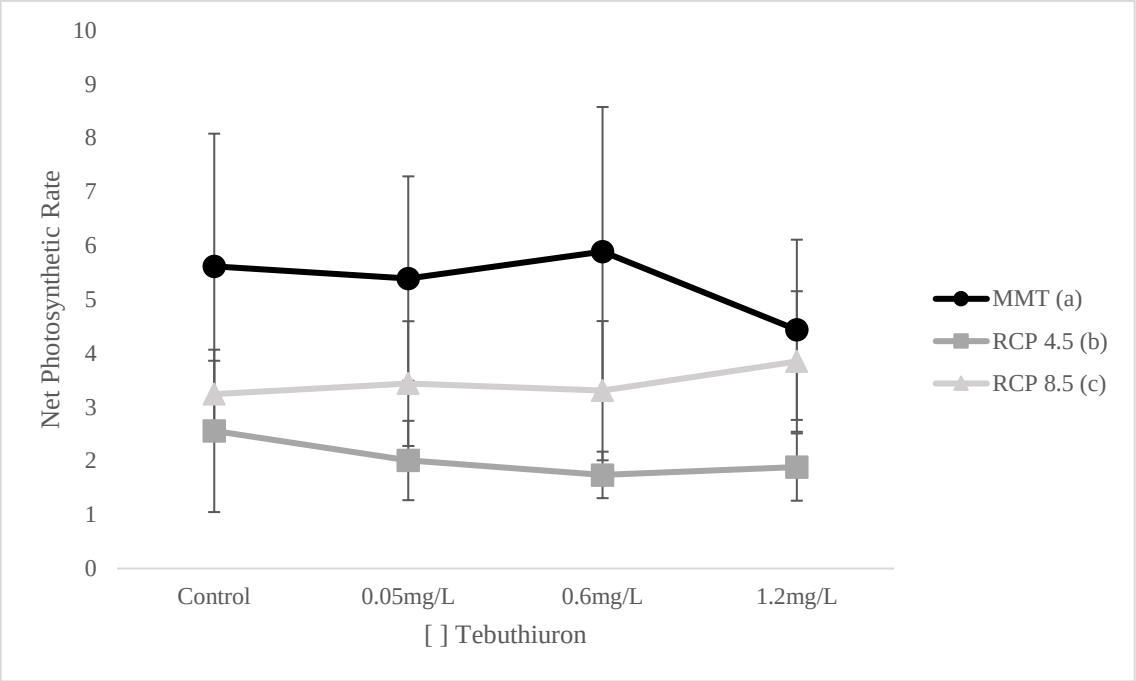

Figure 4 – NPR (net photosynthetic rate – $mgO_2.g^{-1}DW.h^{-1}$) mean values (n = 5) and standard deviations of *Oedogonium* sp. Link ex Hirn after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

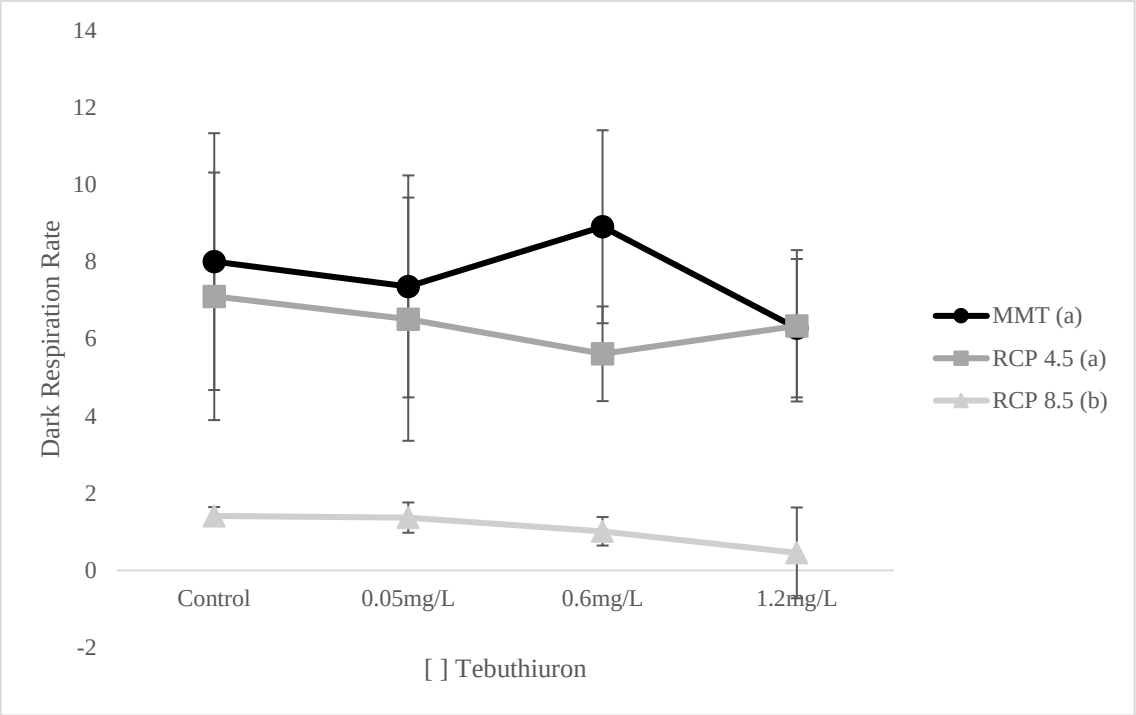

Figure 5 – DRR (dark respiration rate – $mgO_2.g^{-1}DW.h^{-1}$) mean values (n = 5) and standard deviations of *Oedogonium* sp. Link ex Hirn after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

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CAPÍTULO 3

A multi-stressor environment impairs the photosynthetic performance of *Batrachospermum helminthosum* (Rhodophyta).

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ABSTRACT

Freshwater biodiversity is rapidly declining. Agricultural areas increase the amount of pesticides entering freshwater, where non-target species may be affected. Along with contaminants acting on freshwater species, the temperature increases due to anthropogenic climate warming also affects these organisms, constituting a multi-stressor environment. In lotic environments, red algae belonging to Batrachospermales are important contributors to the energy input and richness of the habitat. These algae are generally shade-adapted and present a potential as bioindicators of water quality. Considering the importance of these algae for the lotic food web as a whole, we tested the photosynthetic performance of *Batrachospermum helminthosum* in multi-stressor scenarios characterized by concurrent exposure to the herbicide tebuthiuron, a commonly used pesticide in Brazil, and temperature increases as projected by the Intergovernmental Panel on Climate Change (IPCC). Tests were conducted for 7 days, with three nominal concentrations of tebuthiuron (0.05 mg/L, 0.6 mg/L and 1.2 mg/L) combined with the temperatures of 21.6ºC, 23.9ºC and 26ºC, calculated using a mean of measured temperatures of the stream where algal specimens were sampled and the increases projected by two IPCC scenarios: RCP 4.5 and RCP 8.5. Tebuthiuron caused alarming and widespread negative effects on the photosynthetic response of *B. helminthosum*, since in all temperature scenarios all tested parameters were harmed. In this context, we observed a decrease in photosynthetic yield (Y(II)), regulated energy dissipation (Y(NPQ)), net photosynthetic rate (NPR) and dark respiration rate (DRR), while we recorded an increase in non-regulated energy dissipation (Y(NO)). In addition, we observed that the negative responses were amplified with the increase in the tebuthiuron concentrations, so that the temperature increase projected by RCP 8.5 scenario constituted a very stressful condition for the algae, as highlighted by the increased values of Y(NO). A somewhat unexpected response we found for RCP 4.5 was that, in the absence of tebuthiuron, there was an increase in NPR, showing a slight beneficial effect of temperature increase projected specifically for this IPCC scenario. Thus, this result suggests that although described as shade-adapted algae, this species is capable of withstanding moderate increases in temperature. The high sensitivity of the species to tebuthiuron highlights its potential bioindicator status, since the tebuthiuron concentration advised by the United States Environmental Protection Agency as safe for drinking water (0.05 mg/L) caused a significant decrease in photosynthetic yield. Ultimately, these results show the importance of constant control and management of pesticide usage, especially considering the simultaneous occurrence of global warming.

KEYWORDS

Batrachospermales; red algae; Chorophyll *a* Fluorescence; Herbicide; IPCC; Sugarcane Crops

INTRODUCTION

Freshwater is, perhaps, the most essential nature resource (VÖRÖSMARTY; MCINTYRE, GESSNER, DUDGEON *et al*., 2010). Although lakes, reservoirs and rivers cover only 2.3% of the Earth's surface, these freshwater environments host 9.5% of the Earth's described animal species (REID; CARLSON; CREED; ELIASON *et al.*, 2019). As well as many environments that are being threatened by the human-caused biodiversity crisis (TURVEY; CREES, 2019), the populations of organisms from freshwater declined by 83% from 1970 to 2018, decreasing more than populations of marine and terrestrial species (GROOTEN; ALMOND, 2018).

Historically, freshwater habitats have long been degraded due to human expansion, with the strong increase of urbanization and agricultural practices constituting great threats to these environments (MARTINUZZI; JANUCHOWSKI-HARTLEY; PRACHEIL; MCINTYRE *et al.*, 2014). The expansion of agricultural areas may affect freshwater ecosystems by reducing stream flows or groundwater stores for supporting irrigation (SCANLON; JOLLY; SOPHOCLEOUS; ZHANG, 2007), by reducing riparian habitat (HEARTSILL-SCALLEY; AIDE, 2003) and by increasing the amount of sediments, nutrients and pesticides entering these areas (SZÖCS; BRINKE; KARAOGLAN; SCHÄFER, 2017; TILMAN; FARGIONE; WOLFF; D'ANTONIO *et al.*, 2001).

Specifically considering pesticides, the unrestricted and widespread use of these substances may ultimately lead them into water via leaching, run-off, spray drift or volatilization (CEREJEIRA; VIANA; BATISTA; PEREIRA *et al.*, 2003; PEREIRA; ANTUNES; CASTRO; MARQUES *et al.*, 2009). In water, pesticides may act on non-target species, generating cascade effects and impacts across multiple trophic levels (LU; LI; SHA; WANG *et al.*, 2020; ROGERS; SCHMIDT; DABNEY; HLADIK *et al.*, 2016). In addition, many aquatic sites possess a wide plethora of contaminants acting simultaneously on organisms (HUA; RELYEA, 2014; SMITH; MCINTYRE; HALPERN; COOKE *et al.*, 2015).

Tebuthiuron (1-(5-tert-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) is a phenyl-urea herbicide commonly used in sugarcane crops (QIAN; MATSUMOTO; LIU; LI *et al.*, 2017) which acts inhibiting the electron transport in photosystem II after absorption by the root (LIU, 2010). Although generally applied at the soil, this herbicide has high leaching potential and water bodies adjacent to such crops may be subjected to residues of this herbicide (GOMES; SPADOTTO; LANCHOTTE, 2001; PEREIRA; ANTUNES; CASTRO; MARQUES *et al.*, 2009).

Due to its mode of action as inhibitor of PSII, this herbicide is considered of high environmental risk, capable of acting on any photosynthetic organism (THOMAS; FLORES; KASERZON; REEKS *et al.*, 2020). For that reason, the European Union classifies this contaminant as very toxic, especially to aquatic life (EUROPEAN CHEMICALS AGENCY, 2021). The reported toxic effects of tebuthiuron on non-target freshwater species include, among others, chronic effects to duckweed and green algae (VAN DAM; CAMILLERI; TURLEY; BINET *et al.*, 2004), to diatoms (THOMAS; FLORES; KASERZON; REEKS *et al.*, 2020) and even to fishes (BERNARDES; MASCHIO; OLIVEIRA; ALMEIDA, 2014).

Along with the increasing amounts of contaminants in water and the growing interference in the aquatic ecosystems due to the expansion of agricultural areas, freshwater habitats are threatened by climate change. The scenarios proposed by the Intergovernmental Panel on Climate Change in the fifth assessment report project with very high certainty that the global mean temperature will increase by at least 2ºC in the following decades, even in those scenarios where strong mitigation efforts might occur (COLLINS; KNUTTI; ARBLASTER; DUFRESNE *et al.*, 2013). The increase in temperature may affect freshwater habitats by changing the dynamics and chemical properties of water (NICKUS; BISHOP; ERLANDSSON; EVANS *et al.*, 2010), in addition to affecting the metabolism of organisms and possibly generating cascade effects (WOODWARD; PERKINS; BROWN, 2010).

Temperature is a highly important variable in environments, since it is a limiting factor in the adaptative capacity of species (KNOUFT; FICKLIN, 2017; MAROTZKE; JAKOB; BONY; DIRMEYER *et al.*, 2017) and at the same time a strong driver of carbon flow in freshwater environments (BELLE; MUSAZZI; TÕNNO; POSKA *et al.*, 2018). Considering the carbon flow of freshwater habitats, the food web receives energy inputs from sources in-water (autochthonous) and from the surrounding terrestrial vegetation (allochthonous) (NERES-LIMA; MACHADO-SILVA; BAPTISTA; OLIVEIRA *et al.*, 2017). In temperate lotic ecosystems, allochthonous energy is regarded as the most important energy source for freshwater biodiversity, with the river continuum concept stating that low order streams are strongly influenced by shading of the riparian vegetation, limiting autotrophic production (VANNOTE; MINSHALL; CUMMINS; SEDELL *et al.*, 1980). In tropical regions, however, autochthonous energy may play a more important role in sustaining the aquatic food web, with many studies describing the relevance of benthic algal communities as one of the main primary producers (DOUGLAS; BUNN; DAVIES, 2005; LAU; LEUNG; DUDGEON, 2008; MARCH; PRINGLE, 2003; NERES-LIMA; MACHADO-SILVA; BAPTISTA; OLIVEIRA *et al.*, 2017; BRANCO et al. 2017).

In lotic environments, the benthic algal community (periphyton and macroalgae) is a large contributor to the autochthonous energy input (STEVENSON, 1996, BRANCO et al. 2017). Moreover, these organisms contribute to the species richness, increase habitat complexity, stabilize the substrate, in addition to being considered as an important food source (BATTIN; KAPLAN; DENIS NEWBOLD; HANSEN, 2003; DOWNES; LAKE; SCHREIBER; GLAISTER, 2000; MARCH; PRINGLE, 2003). The majority of benthic algae in lotic environments are green algae (~35%), followed by cyanobacteria (~25%), red algae (~20%), diatoms and yellow-green algae (~21%) (NECCHI JR, 2016).

Most stream red algae (Rhodophyta) present photosynthetic characteristics of shade adapted algae, with higher abundance of these organisms in waters with low irradiation, low flow speed and, in general, sites with low to moderate nutrient levels, possibly serving as indicators of good water quality (BRANCO; RIOLFI; CRULHAS; TONETTO *et al.*, 2017; ELORANTA; KWANDRANS, 2004; NECCHI JÚNIOR; ALVES, 2005). The order Batrachospermales represents the largest taxonomic group of freshwater Rhodophyta worldwide, with the species within this order, mainly species of the genus *Batrachospermum*, significantly contributing to the autochthonous energy input, especially in low-order shaded streams (ENTWISLE; VIS; CHIASSON; NECCHI JR *et al.*, 2009).

Considering the threatening conditions of freshwater habitats worldwide, the relevance of global climate change and the importance of Batrachospermales species for the energy flow and heterogeneity of freshwater lotic habitats, we believe that it is important to analyze the photosynthetic performance of such algal species subjected to multi-stressor conditions related to exposure to herbicides such as tebuthiuron and to the temperature increase. Since species of the genus *Batrachospermum* are among the best represented and distributed Batrachospermales from tropical streams, the effects of such multi-stressor conditions on species specifically of this genus might generate information not only about impacts on the physiological performance of a single species, but might also show how they affect the ecosystem functioning as a whole, especially in shaded lotic environments where these organisms are abundant.

In the present study, the photosynthetic performance of the gametophytes of *Batrachospermum helminthosum*, a species of Batrachospermales, was evaluated after being submitted to different concentrations of tebuthiuron along with temperature increases related to the IPCC scenarios projections. Considering the previous descriptions of the shade-adapted traits of these algae and their higher occurrence in sites with good water quality, we hypothesize that not only temperature increase and the presence of tebuthiuron may individually generate

negative effects on the photosynthetic performance of *B. helminthosum*, but this multi-stressor condition may ultimately amplify the negative effect on this species.

MATERIAL AND METHODS

Sampling and preparation of algal specimens

The samples of *Batrachospermum helminthosum* Skuja were collected from the Pari river, located in the Cervo river microbasin, in the western region of the state of São Paulo, Brazil (22°38'33.0"S 50°12'14.8"W). Algal samples were taken to the laboratory in transparent vials containing river water. Samples were then cleaned in order to remove sediment, possible epiphytes and invertebrates. Cleaning was performed using jets of distilled water and hard bristle brushes under the stereoscopic microscope. After cleaning, samples of 150mg (± 10mg) were prepared, considering five replicates (n = 5) for each multi-stressor treatment. Weighing was performed using an analytical scale (Shimadzu AUW320).

Samples were then subjected to the experimental conditions for 24h for acclimation. The 150 mg samples were transferred to 150 ml erlenmeyer flasks containing 100 ml of Bold's basal medium (BBM) (WATANABE, 2005) and placed inside B.O.D. incubators (Nova Ética, model 411 / FDP355) under constant temperature determined for each experimental treatment (described below), constant irradiance (140 $\mu mol.m^{-2}s^{-1}$) and 12h/12h photoperiod (light/dark cycle).

After the 24-hour acclimation period, the BBM of the samples belonging to treatments with exposure to tebuthiuron were replaced with BBM containing the concentrations of the active ingredient of tebuthiuron as specified in each treatment (see the topic Determination of nominal concentrations of tebuthiuron). The tests of photosynthetic performance were conducted after 7 days exposure to the multi-stressor treatments. Culture media was renewed on the third and fifth days, to avoid nutrient depletion and to keep a constant nominal concentration of the active ingredient (OLIVEIRA; VILAS BOAS; BRANCO, 2016; OECD, 2006).

Determination of temperature scenarios

Calculations of experimental temperatures were performed as described by Vilas Boas et al. (2019), using a mean of measured temperatures (MMT) of the streams in the river microbasin where samples of *B. helminthosum* were obtained as reference value and the temperature increase values of two specific IPCC scenarios. The first scenario used was RCP 4.5, where a stabilization of the carbon emissions is expected, with a projected maximum temperature

increase of 2.3ºC for the tropical region (COLLINS; KNUTTI; ARBLASTER; DUFRESNE *et al.*, 2013). The second scenario used was RCP 8.5, where increases in carbon emissions are expected and the projected maximum temperature increase is of 4.4ºC for the tropical region (COLLINS; KNUTTI; ARBLASTER; DUFRESNE *et al.*, 2013).

The mean of measured temperatures (MMT) for the river microbasin where samples were collected was of 21.6ºC (Table 1). Measurements were performed between 15:00h and 17:00h in June 2019, using a multiparameter probe (HORIBA U-50). In tropical regions, freshwater algae are more abundant during the winter due to lower rainfall regime, and, during winter, the highest stream temperatures are found at 16:00h (BRANCO; PEREIRA, 2002). By adding the maximum increase projected for both IPCC scenarios to MMT, the temperature of the experimental scenarios RCP 4.5 and RCP 8.5 were, then, set to 23.9ºC and 26ºC respectively. In addition, MMT (21.6ºC) was used as control.

Determination of nominal concentrations of tebuthiuron

The tested nominal concentrations of tebuthiuron corresponded, from lowest to highest, to the maximum concentration allowed by the North American Environmental Protection Agency in bodies of water (T1) (U.S. Environmental Protection Agency, 1988); to the recommended dosage of application of tebuthiuron for sugarcane crops (T2) (Combine®500 SC); and to a 'worst case scenario', with twice the recommended dosage of application on clay soils (T3). Nominal concentrations were of 0.05 mg/L (T1), 0.6 mg/L (T2) and 1.2 mg/L (T3), while control (C) had no herbicide.

Multi-stressor treatments

For the multi-stressor treatments, combinations of the temperature scenarios and concentrations of tebuthiuron were made. A total of 12 multi-stressor treatments were performed, coded by the following acronymous: MMT Control, MMT T1, MMT T2 and MMT T3; RCP 4.5 Control, RCP 4.5 T1; RCP 4.5 T2 and RCP 4.5 T3; and RCP 8.5 Control, RCP 8.5 T1, RCP 8.5 T2 and RCP 8.5 T3 (see the topics Determination of temperature scenarios and Determination of nominal concentrations of tebuthiuron for acronyms).

Experimental analyses

Chlorophyll *a* fluorescence analysis and dissolved oxygen analysis were performed to assess the photosynthetic performance of *Batrachospermum helminthosum* (NECCHI JR; ZUCCHI,

2001; VIEIRA JR; NECCHI JR, 2003; VILAS BOAS; OLIVEIRA; NECCHI JR e BRANCO, 2019). Analyses were performed after 7 days exposure to the multi-stressor treatments.

The measured fluorescence parameters were: i) Y(II) - effective quantum yield of photosystem II; ii) Y(NPQ) - quantum yield of regulated non-photochemical energy loss in photosystem II and iii) Y(NO) - quantum yield of non-regulated non-photochemical energy loss in photosystem II. Measurements were taken using a Diving-PAM fluorometer (Walz, Effeltrich, Germany), using the "Induction Curve" function (SCHREIBER; BILGER; NEUBAUER, 1995), with 12 pulses of saturating light (2,000 µmol photons.m^2.s^{-1}) lasting 0.8 s applied at 15 s intervals at samples after a 30 min period of acclimatization in the dark (OLIVEIRA; VILAS BOAS; BRANCO, 2016).

Net photosynthetic rate and dark respiration rate were calculated using the difference of the initial and final oxygen concentrations after one hour incubation in light and dark bottles, respectively (LITTLER; ARNOLD, 1985). Dissolved oxygen was measured using an oxygen meter, equipped with a self-stirring probe (brand YSI, model 5100).

Formulas for the calculations were, as follows: NP = [(F) - (I)] * V / IT / DW and DR = [(I) - (F)] * V / IT / DW where NP: net photosynthetic rate; DR: dark respiration rate; (F): final concentration of dissolved oxygen after the incubation period; (I): initial concentration of dissolved oxygen before the incubation period; V: volume (liters) of medium in the bottle; IT: incubation time; DW: dry weight.

Statistical Analyses

Differences in the values of photosynthetic parameters among the different multi-stressor treatments were identified by two-way ANOVA tests. To perform the two-way ANOVA tests, chlorophyll *a* parameters, net photosynthetic rate and dark respiration rate were used as dependent variables, while the factors of multi-stressor treatments (tebuthiuron concentrations and temperature scenarios) were used as independent variables. The statistical software IBM© SPSS© Statistics (IBM, 2019) was used.

RESULTS

Significant differences among treatments and control group were found in the two-way ANOVA tests for all measured photosynthetic parameters and all tested treatments. Considering the chlorophyll *a* fluorescence parameters, the Y(II) values were significantly lower ($p < 0.001$, $F = 25.11$) in all treatments with exposure to tebuthiuron in comparison to the control. In the MMT scenario, T1, with 0.05 mg/L of tebuthiuron, showed a reduction of 14%

in the Y(II) values in comparison to the control, while the Y(II) values of T2 and T3 Y(II) were 91% and 99% lower than control, respectively (fig. 1). In the other tested temperature scenarios, the Y(II) values were reduced in all treatments in comparison to the control as well. Thus, the reduction in Y(II) in RCP 4.5 scenario was of the order of -57%, -96%, -77% for T1, T2 and T3, respectively, while in RCP 8.5 was of the order of -78%, -87% and -92% for T1, T2 and T3, respectively. No significant effect of the temperature factor was observed for the Y(II) parameter (fig. 1).

The Y(NPQ) parameter was affected only by the tebuthiuron concentrations, with no effect of the temperature scenarios detected in the two-way ANOVA tests. Y(NPQ) values were significantly lower ($p < 0.001$, $F = 13.91$) in the T1, T2 and T3 treatments in comparison to the control (-29%, -56%, -62% respectively for the MMT, -19%, -25% and -58% respectively for RCP 4.5 and -39%, -54% and -69% for RCP 8.5) (fig. 2). For Y(NO), the three treatments with exposure to tebuthiuron showed a significant increase ($p < 0.001$, $F = 34.84$) in the values of this parameter. In the MMT scenario, T1, T2 and T3 showed Y(NO) values 22%, 57% and 62% higher than control, respectively (fig. 3). For the RCP 4.5 scenario, Y(NO) was 19%, 28% and 41% higher than control for T1, T2 and T3 respectively. Lastly, in the RCP 8.5 scenario, Y(NO) values were 38%, 57% and 56% higher than control, respectively. The values of Y(II), Y(NPQ) and Y(NO) in treatments T2 and T3 were also significantly different from the values of these parameters in T1.

For the oxygen evolution analysis, the two-way ANOVA tests showed that the net photosynthetic rate (NPR) was significantly affected by the tebuthiuron ($p < 0.05$, $F = 3.11$) and temperature ($p < 0.05$, $F = 3.91$) factors and by the interaction ($p < 0.01$, $F = 4.09$) between these two factors. The NPR of *B. helminthosum* was significantly reduced with the interaction of the temperature factor with the tebuthiuron concentrations, especially in the RCP 4.5 scenario with T2 (with a reduction of 79% in comparison to the same treatment in the MMT) and with T3 (-66% in comparison to the same treatment in the MMT). The dark respiration rate (DRR) was significantly different ($p < 0.001$, $F = 5.67$) in all treatments in comparison to control. In the MMT, DRR values were 73%, 82% and 91% lower than control for T1, T2 and T3, respectively (fig. 5). For the RCP 4.5 scenario, values were 74%, 95% and 95% lower than control for T1, T2 and T3, respectively. In the RCP 8.5 scenario, T1 showed an increase in respiration rate of 8% in comparison to control, while T2 and T3 showed reductions of 12% and 55% respectively (fig. 5).

DISCUSSION

The chlorophyll *a* fluorescence analysis showed clear signs of the negative effects of tebuthiuron on the photosynthetic performance of *B. helminthosum*. The Y(II) parameter represents the efficiency of conversion of light energy to chemical energy by the photosynthetic organism, with higher values meaning beneficial effects, i.e.: higher efficiency of photoconversion (TAIT; HAWES; SCHIEL, 2017). The excess of the light energy received by the organism is usually dissipated via two pathways, represented by the Y(NPQ) and Y(NO) parameters. While Y(NPQ) represents a controlled pathway of energy dissipation and increased values may show signs of acclimation (ZHAO; CHEN; FENG; ZHANG *et al.*, 2017), Y(NO) represents a harmful pathway, which may lead to the formation of reactive oxygen species and possibly damage to the photosynthetic apparatus, with increases in the values of this parameter showing signs of stress (HIDEG; MAJER, 2010; KLUGHAMMER; SCHREIBER, 2008).

The exposure to the tebuthiuron nominal concentration of 0.05 mg/L (T1) significantly lowered the efficiency of photoconversion (i.e., Y(II)) of *B. helminthosum* in comparison to control, while simultaneously decreasing the Y(NPQ) values and increasing Y(NO) (figs. 1-3). DRR was also significantly lowered with the addition of tebuthiuron to the medium (fig. 5). In addition, the T2 and T3 treatment showed further reductions of Y(II), Y(NPQ), DRR and increases in Y(NO), demonstrating a dose-dependent effect, where higher nominal concentrations of tebuthiuron may develop more severe effects on this species of red algae.

Considering the temperature scenarios, only the Y(NO) and NPR showed significant differences related to this factor. In general, the results showed that the smaller increase in temperature generated in scenario RCP 4.5 would not be prejudicial to this species, since no differences were observed for most parameters. In fact, in the absence of tebuthiuron, the increase of 2.3ºC resulted in a consequent increase in the NPR values as well (fig. 4).

For the RCP 8.5 scenario, however, the Y(NO) values were significantly higher than those of the MMT treatment, showing that an increase of 4.4ºC in temperature would constitute a stressful environment for this species (fig. 3). Even though few parameters were affected by temperature and no statistical difference was found between RCP 8.5 and MMT in the net photosynthetic rate, the Y(NO) result showed an important negative response to this temperature scenario. Similar results were found by VILAS BOAS; OLIVEIRA; NECCHI JR e BRANCO (2019), with the gametophyte of other species of Batrachospermales (*Sirodotia delicatula*) showing increased Y(NO) values when subjected to experimental temperatures above 27ºC.

Regarding the interaction between factors in the NPR analysis, it is interesting to note that, while the RCP 4.5 scenario had higher NPR values than MMT when no tebuthiuron was present, the addition of tebuthiuron caused a significant drop in this parameter, reaching values below those of the MMT for the T2 and T3 treatments. This response may show that, while tebuthiuron is toxic in any scenario, in association with the temperature increase the negative effects of this herbicide can be even more intense on the photosynthetic performance of this red alga. Such response confirms our hypothesis and highlights the aggravated effect of a multi-stressor scenario.

An important remark is that the lifetime advisory concentration of tebuthiuron issued by the United States Environmental Protection Agency (US-EPA) (i.e., 0.05 mg/L) produced significant negative effects on the photosynthesis of *B. helminthosum*, suggesting that, although concentrations up this limit may be considered safe for drinking water to humans, such concentrations may produce environmental risks, especially when considering water bodies where this species, and probably other lotic Batrachospermales, is the main base of the food web.

The sensitivity of this species to low concentrations of tebuthiuron also highlights the potential status of this species as a bioindicator of good water quality (STANCHEVA; SHEATH, 2016). Although we cannot state that the effect produced by the lowest concentration would eradicate the alga from sites with tebuthiuron, higher concentrations caused severe damage to the species, with reductions of 50-99% in the effective quantum yield (Y(II)), therefore, this organism might disappear from sites with water containing concentrations of this pesticide of 0.6 mg/L or higher.

Ultimately, these results show the importance of proper control of pesticide usage, especially when reference values defined as safe for human consumption might develop negative effects on non-target organisms. The current climate change may also cause unforeseen effects when organisms are subjected to multi-stressor scenarios, highlighting the importance of updated guidelines and constant studies on this subject.

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TABLE AND FIGURES

Table 1. Temperature values (ºC) and GPS coordinates of 10 streams in the Cervo river microbasin, used to determine de mean of measured temperatures (MMT) of treatments and calculate the experimental temperatures.

Stream	***Latitude***	***Longitude***	***Temperature***
01	22° 35' 22.9344" S	50° 24' 59.9436" W	22.08 ºC
02	22° 35' 33.3096" S	50° 25' 23.0232" W	22.34 ºC
03	22° 35' 22.2" S	50° 24' 45.3456" W	20.40 ºC
04	22° 34' 29.5932" S	50° 25' 6.6828" W	21.77 ºC
05	22° 38' 26.304" S	50° 20' 26.736" W	21.70 ºC
06	22° 38' 23.784" S	50° 19' 5.5092" W	21.21 ºC
07	22° 38' 31.2216" S	50° 12' 13.9824" W	20.37 ºC
08	22° 36' 38.1456" S	50° 27' 59.9292" W	21.68 ºC
09	22° 40' 12.6048" S	50° 28' 55.4088" W	22.23 ºC
10	22° 45' 29.0952" S	50° 31' 36.8112" W	22.07 ºC
MMT:			***21.6ºC***

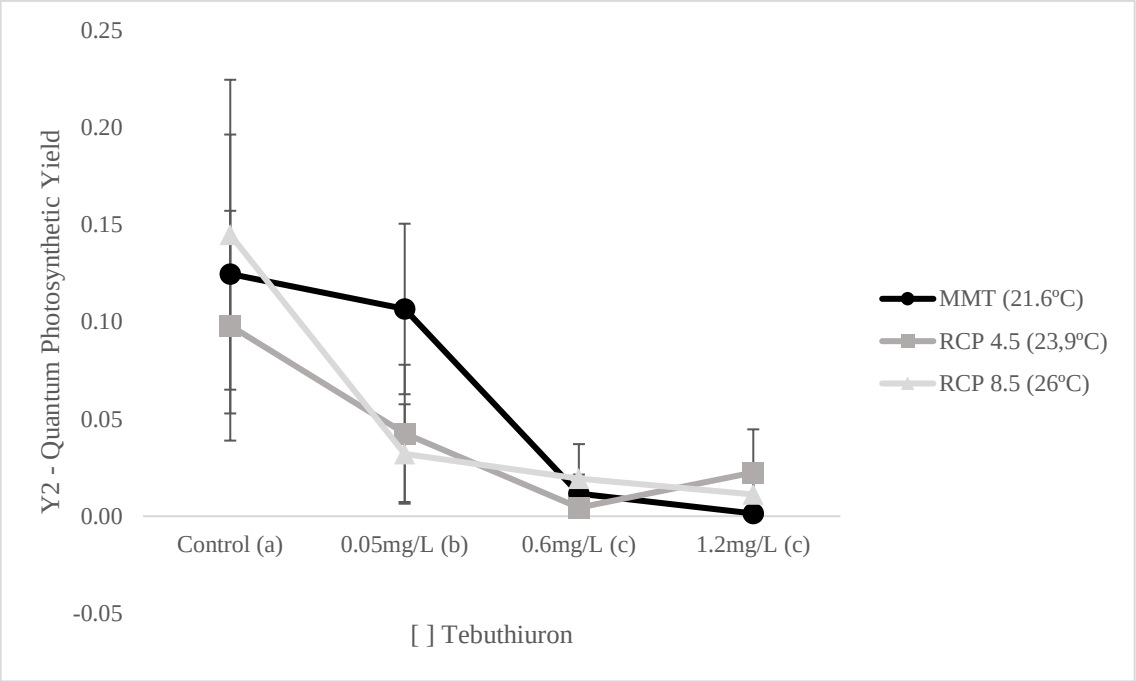

Figure 1 – Y(II) (effective quantum yield of photosystem II) mean values (n = 5) and standard deviations of *Batrachospermum helminthosum* Skuja after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

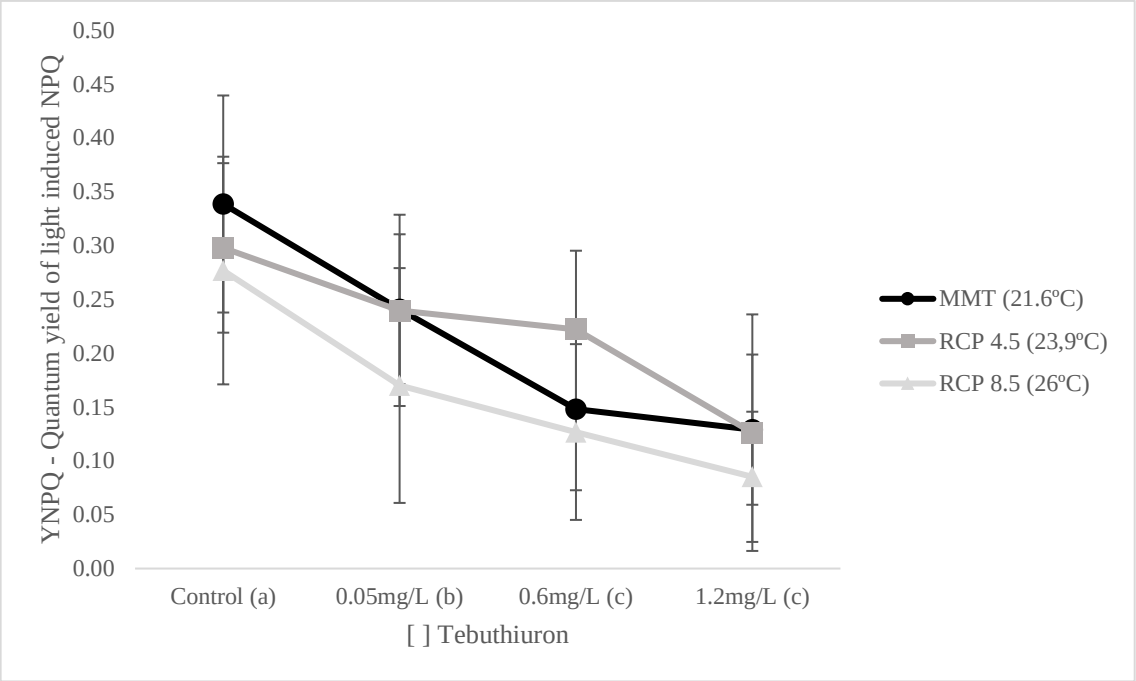

Figure 2 – Y(NPQ) (regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Batrachospermum helminthosum* Skuja after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

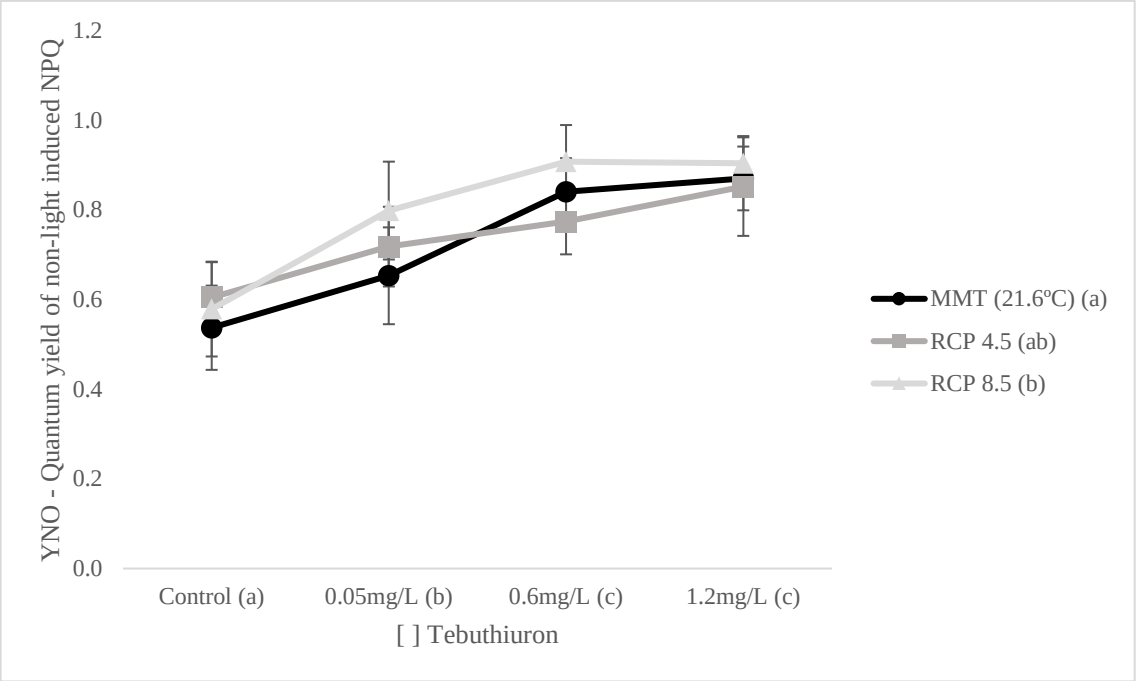

Figure 3 – Y(NO) (non-regulated non-photochemical energy loss in photosystem II) mean values (n = 5) and standard deviations of *Batrachospermum helminthosum* Skuja after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

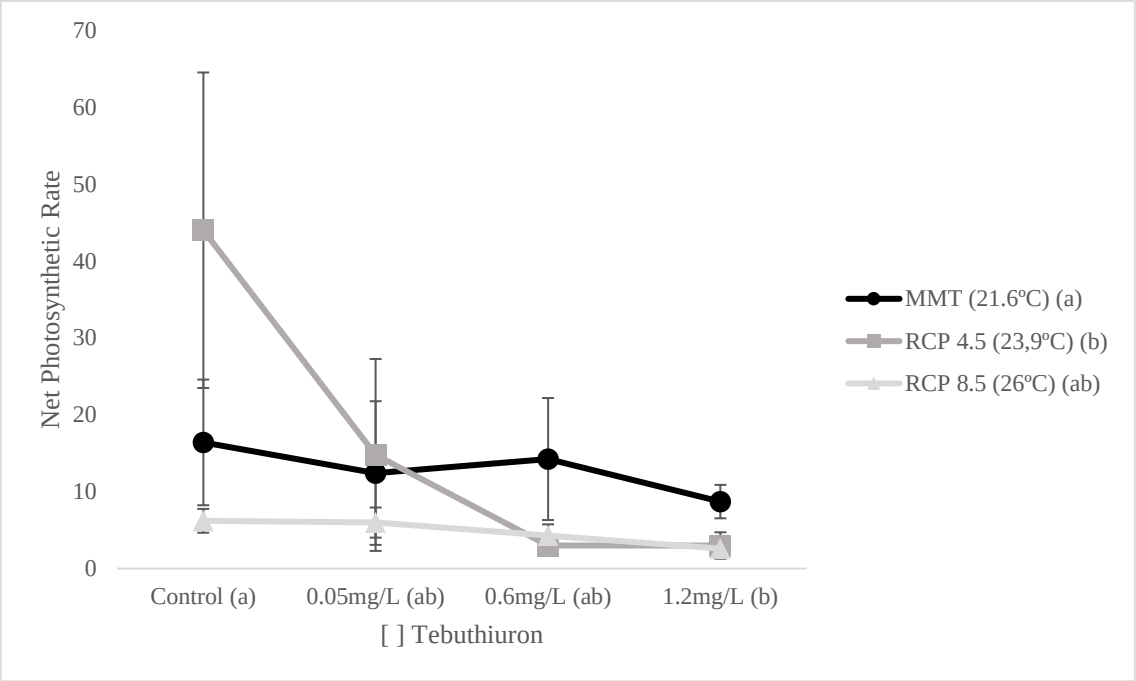

Figure 4 – NPR (net photosynthetic rate – $mgO_2.g^{-1}DW.h^{-1}$) mean values (n = 5) and standard deviations of *Batrachospermum helminthosum* Skuja after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

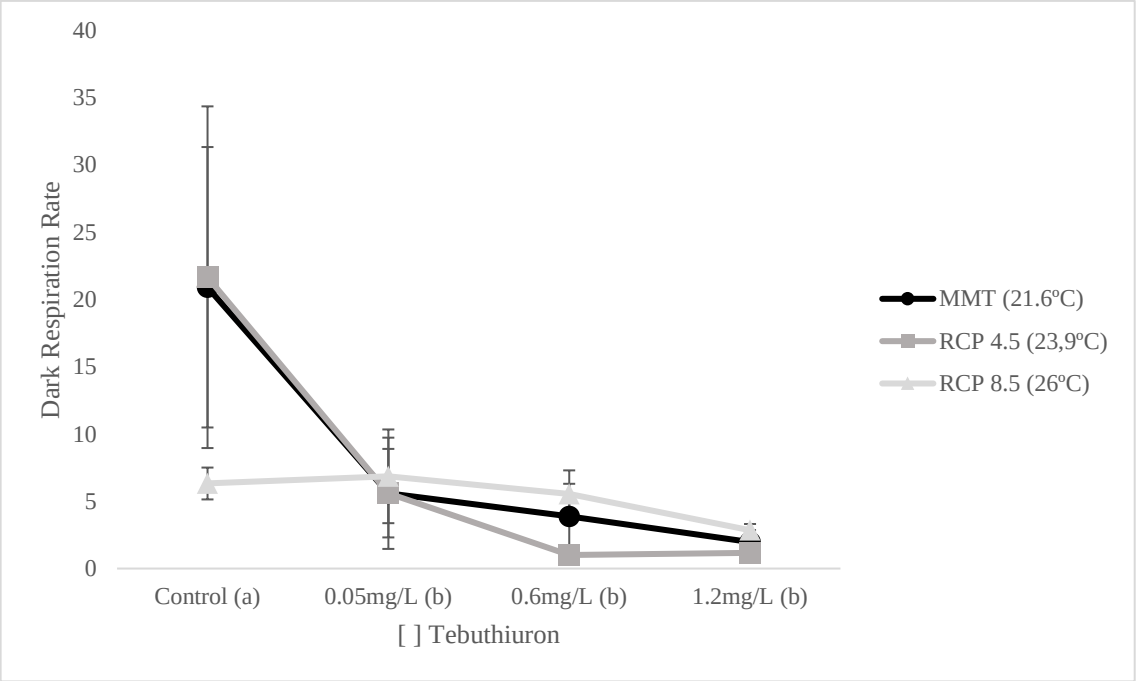

Figure 5 – DRR (dark respiration rate – $mgO_2.g^{-1}DW.h^{-1}$) mean values (n = 5) and standard deviations of *Batrachospermum helminthosum* Skuja after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Different letters mean significant differences ($p < 0.05$) in the two-way Analysis of Variance test.

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CONCLUSÃO GERAL

De maneira geral, os efeitos negativos do herbicida tebuthiuron na fotossíntese das algas testadas foram expressivos, independente da espécie. As espécies de algas verdes testadas apresentaram respostas similares, mostrando efeitos negativos em concentrações acima de 0,6 mg/L. Por sua vez, *Batrachospermum helminthosum*, a espécie de Rhodophyta testada no presente estudo, se mostrou mais sensível ao herbicida, reforçando seu status de potencial bioindicador de boa qualidade de água. Considerando os cenários que envolveram as projeções de aumento da temperatura, a resposta negativa observada para *Oedogonium* sp. não era inicialmente esperada, devido à larga bibliografia que comumente se refere a esta alga como sendo capaz de crescer em um grande gama de temperaturas, razão pela qual, inclusive, seja frequentemente apontada como um organismo com alto potencial biotecnológico. Quanto ao ambiente multi-estressor, um efeito de interação entre o aumento de temperatura e a exposição ao tebuthiuron foi eventualmente observado, porém, um sinergismo negativo foi mais evidente apenas nas concentrações e temperaturas mais elevadas e para as espécies *Nitella microcarpa* var. *wrightii* e *B. helminthosum*.

Tais resultados alertam sobre a potencial toxicidade ambiental desse herbicida, capaz de agir em organismos não-alvo, justamente pelo seu modo de ação específico na cadeia de elétrons do fotossistema II, compartilhado por todos os organismos que apresentam processos fotossintéticos com características tipicamente observadas nos vegetais. Além de seu efeito negativo quando avaliado individualmente, foi possível observar que, em um ambiente multi-estressor causado pelo aquecimento previsto para as próximas décadas, o efeito prejudicial do herbicida pode se tornar ainda mais severo. Dessa forma, o uso desenfreado de tais substâncias em um cenário sem políticas globais de mitigação de aquecimento global constitui um ambiente desafiador para organismos fotossintetizantes de água doce.

Considerando a vasta quantidade de novos pesticidas registrados para uso, é importante que substâncias de origem antrópica sejam avaliadas em estudos com perspectivas ecofisiológicas, uma vez que, juntamente com as diversas variáveis ambientais, tais substâncias podem influenciar processos fisiológicos, o comportamento, o ciclo de vida, os padrões fenológicos, entre outras tantas características funcionais dos organismos. Além disso, apesar de trabalhos analisando as respostas em níveis de espécie serem importantes, futuros estudos podem levar em conta o efeito de ambientes multi-estressores em comunidades de um mesmo nível trófico ou de níveis tróficos diferentes. Seja em estudos "*in vitro*" ou "*in situ*", o conhecimento dos tipos e dos níveis dos impactos da contaminação provocada pela expansão humana no atual cenário de aquecimento global, certamente, contribuirá imensamente para possíveis tomadas de decisão relacionadas ao manejo e conservação da natureza.