



Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais



**A evolução dos estudos ecotoxicológicos sobre a
atrazina e uma análise dos possíveis preditores na
tolerância de microalgas verdes expostas a esse
herbicida**

Muryllo Santos Castro

Orientador: Juliano Zanette
Coorientadora: Camila Martinez de Gaspar Martins

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Aluno: MSc. Muryllo Santos Castro

Orientador: Prof. Dr. Juliano Zanette

Coorientadora: Prof^a. Dr^a. Camila Martins

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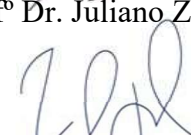


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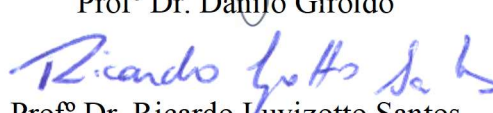
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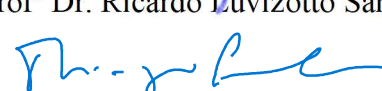
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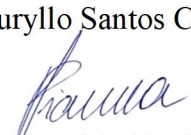
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Muryllo Santos Castro



Prof. Dr. Rogério Tubino Vianna
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Dedico este trabalho às mulheres da minha vida:
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RESUMO

A atrazina (ATZ) é um herbicida utilizado no controle de ervas daninhas na agricultura. Entretanto, têm sido encontrada frequentemente em ecossistemas aquáticos, afetando a saúde de organismos não-alvo, como as microalgas, que desempenham funções cruciais para a manutenção desses ecossistemas. O objetivo geral dessa tese foi avaliar a evolução dos estudos ecotoxicológicos com a ATZ e como as características das microalgas poderiam prever a sua toxicidade para diferentes espécies da classe Chlorophyceae. No primeiro capítulo foram respondidas perguntas como: (i) Qual a tendência global de estudos desenvolvidos avaliando o efeito do herbicida ATZ? (ii) Quais os efeitos biológicos avaliados? (iii) Em que grupos de organismos? Para responder a essas perguntas foi realizado um estudo cienciométrico avaliando a evolução da pesquisa sobre ATZ durante o período de 1959 a 2019, na categoria de Toxicologia da *Web of Science*. Analisamos também a utilização de microalgas e cianobactérias como modelos biológicos para testes de toxicidade com ATZ. Mostramos um aumento no número de publicações científicas durante o período estudado. Embora as microalgas sejam organismos bastante afetados, apenas 7,5% dos 988 estudos compilados avaliavam efeitos da ATZ nesse grupo. Esses achados aponta aos pesquisadores e agências de fomento, as lacunas no conhecimento sobre os efeitos tóxicos da ATZ, e nortear o desenvolvimento de novos projetos de pesquisa e de políticas ambientais. No segundo capítulo foram respondidas as perguntas: (i) Microalgas da classe Chlorophyceae apresentam diferentes níveis de tolerância à ATZ? (ii) Que características intrínsecas dessas espécies poderiam ser preditoras na diferença dessa tolerância? (iii) A inibição da fotossíntese é um possível mecanismo de toxicidade da ATZ em microalgas? A resposta sobre a diferença na tolerância foi obtida através da análise do efeito da ATZ na inibição do crescimento de sete espécies de microalgas verdes. Para descobrir possíveis preditoras, as características morfológicas, fisiológicas, fotossintéticas e bioquímicas dessas espécies foram correlacionadas com os níveis de tolerância demonstrados. Em relação ao mecanismo de toxicidade, as algas foram expostas à ATZ e o rendimento fotossintético foi avaliado. Foi observado que as algas menos tolerantes apresentaram maiores taxas fotossintéticas e menores níveis de atividade da enzima glutathione *S*-transferase (GST) e teor de clorofila-*a*. Já as mais tolerantes apresentaram maior biovolume, ou seja, células maiores apresentam uma maior tolerância à ATZ. Os resultados indicam que a área celular, teor de clorofila-*a*, os níveis fotossintéticos e a biotransformação de GST em combinação, podem ser potenciais preditores para a tolerância de espécies de Chlorophyceae para ATZ. Também foi constatado que a fotossíntese é inibida de forma semelhante em todas as espécies de microalgas quando expostas

às concentrações tóxicas equivalentes (EC30) de ATZ. Isto corrobora a ideia de que a inibição da fotossíntese seja o principal mecanismo de toxicidade deste herbicida em microalgas.

Palavras-chave: algas verdes, bibliometria, ecotoxicologia, pesticida, sensibilidade.

ABSTRACT

Atrazine (ATZ) is a herbicide used to control weeds in agriculture. However, they have been frequently found in aquatic ecosystems, affecting the health of non-target organisms, such as microalgae, which play crucial roles in the maintenance of these ecosystems. The general objective of this thesis was to evaluate the evolution of ecotoxicological studies with ATZ and how the characteristics of microalgae could predict their toxicity to different species of the Chlorophyceae class. In the first chapter, questions such as: (i) What is the global trend of studies developed evaluating the effect of the ATZ herbicide? (ii) What are the biological effects evaluated? (iii) In which groups of organisms? To answer these questions, a scientometric study was carried out evaluating the evolution of research on atrazine during the period from 1959 to 2019, in the Toxicology category of the Web of Science. We also analyzed the use of microalgae and cyanobacteria as biological models for ATZ toxicity tests. We show an increase in the number of scientific publications during the period studied. Although microalgae are highly affected organisms, only 7.5% of the 988 compiled studies evaluated the effects of ATZ in this group. These findings may point out to researchers and funding agencies the gaps in knowledge about the toxic effects of ATZ and guide the development of new research projects and environmental policies. In the second chapter, the questions were answered: (i) Do microalgae of the Chlorophyceae class present different levels of tolerance to ATZ? (ii) What intrinsic characteristics of these species could be predictors of the difference in this tolerance? (iii) Is the photosynthesis inhibition a possible mechanism of ATZ toxicity in microalgae? The answer on the difference in tolerance was obtained by analyzing the effect of ATZ on the growth inhibition of seven species of green microalgae. To discover possible predictors, the morphological, physiological, photosynthetic, and biochemical characteristics of these species were correlated with the presented tolerance levels. Regarding the mechanism of toxicity, the algae were exposed to ATZ, and the photosynthetic yield was evaluated. It was observed that the less tolerant algae showed higher photosynthetic rates and lower levels of activity of the enzyme glutathione S-transferase (GST) and chlorophyll-*a* content. On the other hand, the more tolerant ones presented greater biovolume, that is, larger cells present greater tolerance to ATZ. The results indicate that cell area, chlorophyll-*a* content, photosynthetic levels and GST biotransformation in combination may be potential predictors for the tolerance of Chlorophyceae species to ATZ. It was also found that photosynthesis is similarly inhibited in all microalgae species when exposed to equivalent toxic concentration (EC₃₀) of ATZ. This corroborates the idea that the inhibition of photosynthesis is the main mechanism of toxicity of this herbicide in microalgae.

Key-words: green algae, bibliometry, ecotoxicology, pesticide, sensitivity.

APRESENTAÇÃO

A presente tese traz os resultados obtidos durante meu período no curso de Doutorado em Biologia de Ambientes Aquáticos Continentais – PPGBAC, na Universidade Federal do Rio Grande – FURG. O foco central da tese foi o herbicida atrazina (ATZ), a evolução dos seus estudos ecotoxicológicos, bem como seu efeito no crescimento de microalgas verdes e avaliação de possíveis características intrínsecas dessas algas que pudessem influenciar nas suas respostas a esse herbicida. Ela está estruturada da seguinte forma:

- (i) **Introdução Geral**, formatada segundo as normas da Associação Brasileira de Normas Técnicas (ABNT), que contém as principais informações conceituais a respeito do tema do estudo, a justificativa e os objetivos.
- (ii) **O primeiro capítulo** da tese intitulado “*A scientometric analysis of ecotoxicological studies with the herbicide atrazine and microalgae and cyanobacteria as test organisms*” que traz um panorama sobre o uso da ATZ do período de 1956 a 2019. Esse capítulo já está publicado na revista *Environmental Science and Pollution Research*;
- (iii) **O segundo capítulo** intitulado “*Differential effects of atrazine to chlorophyceae species and association to morphology, photosynthesis, and glutathione S-transferase activity*” abordando sobre o efeito da ATZ no crescimento de espécies de microalgas verdes e possíveis preditores da tolerância dessas espécies à ATZ. Esse artigo está em revisão na revista *Environmental Toxicology and Chemistry*.
- (iv) **As principais conclusões e perspectivas** obtidas com o desenvolvimento da tese e,
- (v) **Anexos**.

As citações e referências bibliográficas de ambos os capítulos seguem as normas de cada revista científica escolhida.

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INTRODUÇÃO GERAL

1. O uso de pesticidas

Todos os químicos usados para matar ou controlar pragas são chamados de pesticidas (Shrivastava and Bajpai 2015). No Brasil, esses produtos são chamados de “agrotóxicos”, entretanto está em discussão um projeto de lei (Projeto de Lei 6.299 2002) que visa alterar essa nomenclatura para “pesticidas”, padronizando o termo adotado entre todos os países membros da Organização para Cooperação e Desenvolvimento Econômico (OECD) (CRISTALDO, 2022). Dentro dos pesticidas podemos classificar ainda esses químicos em herbicidas (controle de ervas daninhas), inseticidas (controle de insetos), fungicidas (controle de fungos), nematocidas (controle de nematóides) e raticidas (venenos de vertebrados) (Shrivastava and Bajpai 2015). O uso de pesticidas aumentou mundialmente nos últimos anos (LUSHCHAK *et al.*, 2018; EUROPEAN PARLIAMENT, 2021). A figura 1 mostra o uso total anual de pesticidas medido em toneladas no mundo. No Brasil, apenas no ano de 2019, cerca de 475 novos “defensivos agrícolas” foram aprovados. Estas substâncias são principalmente químicas e cerca de um terço contém substâncias ativas que foram listadas pela *European Chemicals Association* como proibidas ou severamente restringidas na União Europeia (European Parliament 2021). O uso de pesticidas em grande quantidade se tornou um perigo ambiental pois esses compostos acabam chegando aos ecossistemas aquáticos, onde são frequentemente encontrados (DeLorenzo et al. 2001; Marin-Morales et al. 2013). Na água, esses compostos podem afetar diversos organismos não-alvo, provocando efeitos subletais e letais (DENG *et al.*, 2015; DELLA VECHIA *et al.*, 2016; SANCHEZ *et al.*, 2017; BAUTISTA *et al.*, 2018; RELIGIA *et al.*, 2019). As microalgas são organismos fotossintéticos bastante afetados. Levando em consideração a importância e contribuição desses organismos para a manutenção dos ecossistemas, os riscos devem ser acessados e avaliados e a sua conservação deve ser garantida (Bianchini et al. 2006).

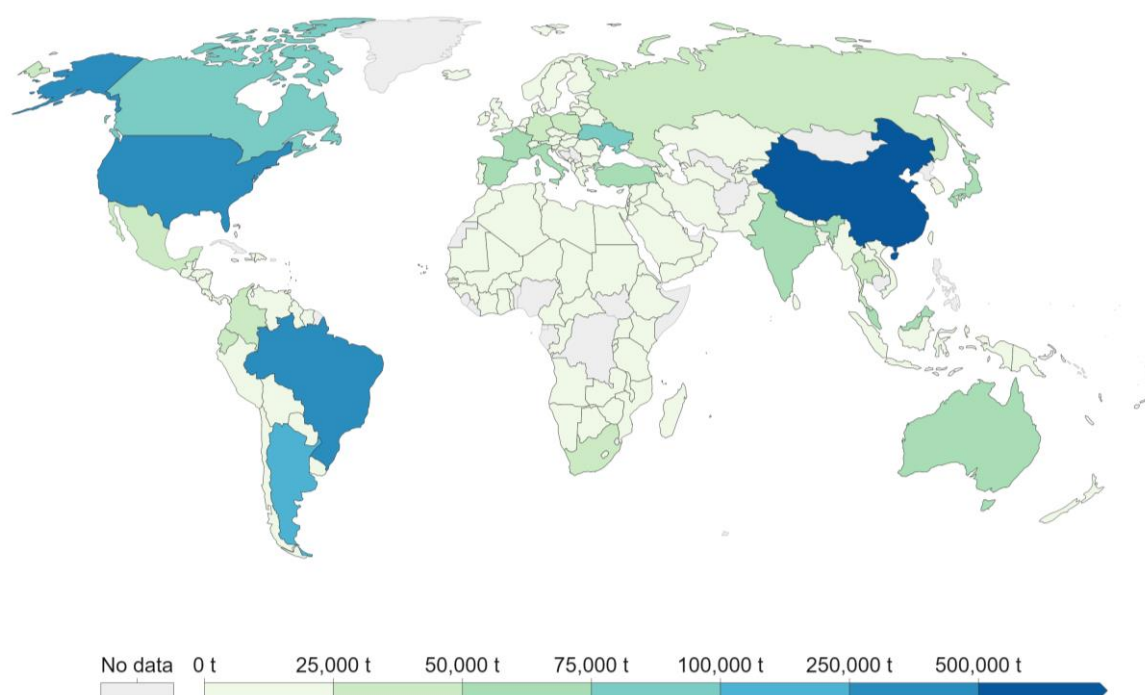
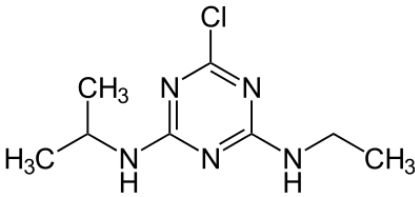


Figura 1. Uso total de pesticidas medido em toneladas de consumo por ano no mundo. Fonte: Adaptado de ROSER, 2019

2. Atrazina

Os herbicidas são substâncias ou agentes biológicos aplicados principalmente na agricultura para proteger as plantações de ervas daninhas durante o crescimento (Lushchak et al. 2018). Entre os herbicidas amplamente utilizados no mundo, podemos citar a atrazina (ATZ) (Tabela 1). A ATZ foi formulada e patenteada no ano de 1952 na Suíça e começou a ser comercializada em 1959 nos Estados Unidos (EUA) (DE ALBUQUERQUE *et al.*, 2020; USEPA, 2016). Este herbicida apresenta alta eficiência, menor toxicidade em comparação com os herbicidas mais antigos e possui baixo custo (Rostami et al. 2021). Tais características a fizeram ocupar o lugar de segundo herbicida mais utilizado no mundo (De Albuquerque et al. 2020). Estudos mostram um alto consumo de ATZ, por exemplo, a China consumia cerca de 2 milhões toneladas de ATZ no ano de 2017, os EUA cerca de 408 milhões de toneladas (ROSER, 2019). No Brasil, em 2017, cerca de 25 mil toneladas foram comercializadas (BRASIL, 2021).

Tabela 1. Fórmulas e características químicas e físicas da atrazina.

Fórmula estrutural	
	
Fórmula química	C ₈ H ₁₄ ClN ₅
Nome geral	Atrazina
Nome IUPAC	2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine
Solubilidade em água (mg/ L)	33 mg/L a 22 °C
Coefficiente de adsorção no solo (K_{oc})	100 cm ³ /g
Coefficiente de partição octanol água (Log K_{ow})	2,75

Fonte: CETESB (2021)

A ATZ é utilizada no controle pré e pós emergente de ervas daninhas e gramíneas de folha larga em plantações como as de milho, sorgo, cana-de-açúcar, abacaxi, grama e pomares (Hansen et al. 2013). É muito utilizada por apresentar menor toxicidade em relação a outros herbicidas. Ainda assim, estudos mostram que a ATZ provoca efeitos adversos em vários grupos de organismos não-alvo (Tabela 2). Em humanos, está associada com desregulação endócrina, citotoxicidade nas células e indução de estresse oxidativo, além de estar na lista da Agência Internacional de Pesquisa sobre o Câncer (IARC) classificada como um herbicida carcinogênico (Gammon et al. 2005; Sass and Colangelo 2006; Singh et al. 2018). Associados a esses efeitos, outras características como alta solubilidade em água, em relação a outros herbicidas, baixa adsorção no solo e alta persistência contribuíram com que a ATZ fosse banida de alguns países como Itália, Finlândia, Alemanha e a União Europeia (Graymore et al. 2001; Nödler et al. 2013). Todavia, atualmente a ATZ está entre os herbicidas mais consumidos nos EUA, Canadá, Argentina, Austrália, Brasil, Ásia, África e Oceania (Hansen et al. 2013; Sharma et al. 2019).

Tabela 2. Efeitos da atrazina em organismos não-alvo.

Grupo	Espécie	Efeitos observados	Referência
Plantas	<i>Elodea canadensis</i>	Inibição da fotossíntese e redução na biomassa	BRAIN <i>et al.</i> , (2012a)
	<i>Zostera marina</i> L.	Inibição de fotossíntese	GAO <i>et al.</i> , (2019)
Algas	<i>Rhodomonas salina</i>	Redução da capacidade fotossintética e inibição do crescimento	THOMAS <i>et al.</i> , (2020)
	<i>Desmodesmus communis</i>	Efeito na funcionalidade mitocondrial	GUIDONY <i>et al.</i> , (2020)
	<i>Chlorella</i> sp.	Inibição fotossintética	SUN, C <i>et al.</i> , (2020)
Crustáceos	<i>Daphnia magna</i>	Anormalidades morfológicas	RELIGIA <i>et al.</i> , (2019)
	<i>Faxonius virilis</i>	Danos ao DNA nas células dos neurônios sensoriais olfatórios.	ABDULELAH <i>et al.</i> , (2020)
	<i>Tigriopus japonicus</i>	Indução de estresse oxidativo	YOON <i>et al.</i> , (2019)
Peixes	<i>Danio rerio</i>	Afetam a qualidade do esperma em peixes, possivelmente resultando em taxas de fertilidade reduzidas	BAUTISTA <i>et al.</i> , (2018)
	<i>Danio rerio</i>	Alteração do ciclo circadiano	(Zhao <i>et al.</i> 2020)
	<i>Cyprinus carpio</i> L.	Estresse oxidativo e dano mitocondrial	WANG, S. <i>et al.</i> , (2019)
Anfíbios	<i>Dendropsophus minutus</i>	Efeitos genotóxico e mutagênicos	GONÇALVES <i>et al.</i> , (2017)
	<i>Rhinella schneideri</i>	Anormalidades morfológicas e alterações na natação e no crescimento	PÉREZ-IGLESIAS <i>et al.</i> , (2019)
	<i>Xenopus laevis</i>	Mortalidade	RIMAYI <i>et al.</i> , (2018)

A ATZ é um herbicida bastante suscetível à lixiviação e escoamento, o que a faz chegar facilmente a corpos d'água, onde tem sido frequentemente encontrada (Cerejeira *et al.* 2003; Lorente *et al.* 2014) tornando-se um problema ambiental. Sua solubilidade e baixa taxa de degradação na coluna de água (meia-vida de 168 dias) faz com que permaneça nesse ambiente provocando efeitos negativos na saúde ambiental, animal e humana (De Souza Barros *et al.* 2021). Alguns países apresentam regulamentações em relação ao limite máximo permitido desse herbicida em águas superficiais, como o Brasil que através da Resolução CONAMA 357/05 define o limite de 2 µg/L (BRASIL 2005), a Agência de Proteção

Ambiental (EPA) dos EUA que fixou 3 µg/L como limite permitido em águas superficiais (USEPA 2001). No entanto, a ATZ já foi encontrada no ambiente aquático em concentrações acima dos níveis autorizado pelas legislações vigentes. Estudos reportam concentrações de 300 µg/L nos ambientes agrícolas nos EUA (Wirbisky-hershberger et al. 2017), de 15 µg /L no Brasil (Sousa et al. 2016), concentrações superiores a 30 µg/L em Portugal (Cerejeira et al. 2003) e concentrações acima de 1000 µg/L em outras partes do mundo (Graymore et al. 2001).

No ambiente aquático, a diminuição do oxigênio dissolvido pode ser um dos principais indícios de exposição à ATZ (Graymore et al. 2001). Isso acontece devido a efeitos adversos na comunidade fitoplanctônica e consequentemente da diminuição da produção primária (Graymore et al. 2001). As microalgas são um grupo bastante afetado pela ATZ, por causa do mecanismo de ação característico desse herbicida, sendo consideradas o grupo mais sensível a esse contaminante, seguido por macrófitas, invertebrados bentônicos, zooplâncton e peixes (Solomon et al. 1996). Como mostra a tabela 3, já se conhecem alguns efeitos que a ATZ pode provocar em diferentes espécies de microalgas. Entretanto, levando em consideração a importância desses organismos para os ecossistemas, realizar mais estudos que avaliem a toxicidade de herbicidas como a ATZ a esses organismos é alternativa importante para a sua conservação.

3. Cienciometria

A cienciometria surgiu na antiga União Soviética, porém tornou-se internacionalmente conhecida no final da década de 1970, com o aparecimento do periódico “*Sciencimetrics*”, na Hungria (VANTI, 2002). Ela faz parte de um grupo denominado de “metrias”, que são estudos denominados como: bibliometria, informetria, cienciometria e webometria. São estudos que avaliam aspectos quantitativos da ciência e da tecnologia, voltados para avaliar a produção científica e tecnológica produzida pela comunidade científica no interior das áreas de conhecimento, representada por artigos, livros, capítulos de livros, trabalhos publicados em anais de eventos, e patentes. A parte que cabe à cienciometria é a avaliação da produção científica através de indicadores numéricos estando relacionada com a produtividade e utilidade científica (HAYASHI, 2013).

O uso da cienciometria, bem como sua importância se elevaram bastante com os avanços tecnológicos que permitiram acesso à informação de artigos e mesmo aos próprios documentos (ALBERGUINI, 1999). Os resultados obtidos a partir dessa avaliação são

relevantes para pesquisadores pois mostram os interesses, carências, tendências, saturações e indicam direcionamentos a partir das lacunas apontadas (SPINAK, 1998; BARBOSA; SCNECK; MELO, 2012). Outro ponto importante é o papel do estudo cienciométrico na elaboração de políticas públicas, pois auxilia na tomada de decisão no que tange à gestão de recursos de qualquer ordem, como no incentivo à geração de informações técnicas que permitam um desenvolvimento de fato sustentável (SPINAK, 1998).

De acordo com IVANCHEVA, (2008) um estudo cienciométrico aborda sobre:

- a) Estudos quantitativos de cientistas, como projetos, financiamento de pesquisas e infraestrutura de pesquisa;
- b) Estudos quantitativos de publicações, patentes e citações por instituições, países, idiomas, coautorias, campos temáticos;
- c) Identificação das relações entre diferentes disciplinas de pesquisa;
- d) Estudos de internacionalização da ciência;
- e) Revelação de problemas de pesquisa emergentes;
- f) Avaliação da produção de pesquisa e contribuições científicas (de cientistas, instituições, regiões, países) e avaliar o fator de impacto das revistas científicas;
- g) Modelagem de processos científicos e fenômenos, baseados em métodos matemáticos.

Alguns índices são usados para expressar quantitativamente as características bibliográficas de um documento ou conjunto de documentos. Esses indicadores são dados numéricos que representam diferentes características da atividade científica ligadas tanto à produção quanto ao uso da informação (García-Villar and García-Santos 2021).

De acordo com VINKLER (1988) os indicadores podem ser organizados como:

- a) indicadores de publicação (IP), que medem a quantidade e o impacto das publicações científicas e;
- b) indicadores de citação (IC), que avaliam a quantidade e o impacto das ligações e relações entre as publicações científicas, como indicados.

Esses índices permitem que a análise identifique as tendências de crescimento do conhecimento em uma determinada área; as revistas do núcleo de uma disciplina; Permite também prever tendências de publicações, estudar a dispersão e a obsolescência da literatura científica, estudar a produtividade de autores e medir o grau de colaboração entre eles; Além de analisar os processos de citação e co-citação, avaliar aspectos estatísticos da linguagem, das palavras e das frases, medir o crescimento de determinadas áreas e o surgimento de novos temas, desenvolver processos de indexação e classificação de resumos; e obter o número de

citações nos artigos (SPINAK, 1998; HAYASHI, 2013; CABRAL NETTO; LAURINDO, 2015; MOREIRA; SALERNO; TSUNODA, 2020).

Alguns estudos cienciométricos dentro da área de toxicologia já foram desenvolvidos como análises de tendências globais e lacunas para estudos sobre toxicidade de herbicidas como o 2,4-D (Zuanazzi et al. 2020) e o glifosato (Ghisi et al. 2020). Além disso, sobre o uso de metais pesados e sua presença em ecossistemas aquáticos (Ouyang et al. 2018). Todos esses estudos entregam um panorama importante sobre esses compostos e auxiliam nas tomadas de decisões sobre pesquisas futuras. A ATZ é um herbicida muito conhecido e estudado, entretanto há escassez de estudos cienciométricos desenvolvidos sobre esse composto.

4. Microalgas

As microalgas são organismos microscópicos fotossintéticos que podem ser encontradas em ambientes aquáticos e terrestres (Rizwan et al. 2018). São organismos unicelulares que podem viver individualmente ou ainda em colônias com o tamanho que pode variar, dependendo da espécie, alguns micrômetros a algumas centenas de micrômetros (Richmond 2004). Nos ecossistemas aquáticos, esses organismos fazem parte do fitoplâncton, ou seja, algas que estão suspensas na coluna de água (Basset et al. 2018) e colonizam diferentes tipos de habitats como nascentes de água doce, riachos, lagos, lagoas, áreas marinhas costeiras e oceano aberto (Basset et al. 2018).

As microalgas têm grande importância ecológica pois desempenham funções cruciais para o funcionamento dos ecossistemas (Garrido-Cardenas et al. 2018). Elas são os principais produtores primários do ecossistema aquático constituindo o primeiro nível das cadeias tróficas aquáticas (Cid et al. 2012). As microalgas são responsáveis pela produção de pelo menos 60% do oxigênio terrestre (Chisti 2004). Junto a isso elas auxiliam no equilíbrio ecológico, circulação de material, fluxo de energia, fixação de nitrogênio e degradação da poluição (Ramakrishnan et al. 2010). Além dessas funções, também são comercialmente importantes em vários setores industriais e biotecnológicos (Rizwan et al. 2018).

Tabela 3. Efeitos da ATZ a diferentes espécies de microalgas.

Espécie	Classe	Concentração	Efeitos	Referência
<i>Chlorella vulgaris</i>	Chlorophyceae	500 µg/L	Inibição do crescimento	VEBER <i>et al.</i> , (1981)
<i>Anabaena flos-aquae</i>	Cyanophyceae	1050 µg/L	Redução da clorofila	ABOU-WALY <i>et al.</i> , (1991)
<i>Craticula cuspidata</i>	Bacillariophyceae	83 µg/L	Redução na taxa de crescimento	ELSON; OAGLAND; IEGFRIED, (1999)
<i>Oscillatoria limnetica</i>	Cyanophyceae	52 µg/L	Inibição do crescimento	LEBOULANGER <i>et al.</i> , (2001)
<i>Pseudokirchneriella subcapitata</i>	Chlorophyceae	49 µg/L	Inibição do crescimento	WEINER; DELORENZO; FULTON (2004)
<i>Isochrysis galbana</i>	Prymnesiophyceae	91 µg/L	Inibição do crescimento	WEINER; DELORENZO; FULTON (2004)
<i>Raphidocelis subcapitata</i>	Chlorophyceae	110 µg/L	Inibição da fotossíntese	MA <i>et al.</i> , (2006)
<i>Dunaliella tertiolecta</i>	Chlorophyceae	136 µg/L	Alterações na composição macromolecular (alocação de carbono em ácido nucleico, aminoácidos, proteínas e lipídios)	WEINER; DELORENZO; FULTON (2007)
<i>Isochrysis galbana</i>	Prymnesiophyceae	30 µg/L	Inibição do crescimento	DEBELIUS <i>et al.</i> , (2008)
<i>Chaetoceros sp</i>	Bacillariophyceae	43 µg/L	Inibição do crescimento	DEBELIUS <i>et al.</i> , (2008)
<i>Anabaena flos-aquae</i>	Cyanophyceae	96 µg/L	Inibição do crescimento e fotossíntese	BRAIN <i>et al.</i> , (2012b)
<i>Phaeodactylum tricornutum</i>	Bacillariophyceae	100 µg/L	Aumento de espécies reativas de oxigênio (ROS) e alteração na expressão de genes relacionados à fotossíntese	(Bai <i>et al.</i> 2015)
<i>Chlamydomonas reinhardtii</i>	Chlorophyceae	29 µg/L	Aumento nos níveis de ROS e alterações na morfologia nuclear	ESPERANZA <i>et al.</i> (2017)
<i>Desmodesmus communis</i>	Chlorophyceae	20 µg/L	Alteração na integridade da membrana mitocondrial	GUIDONY <i>et al.</i> (2020)

As microalgas são utilizadas em estudos ecotoxicológicos, como organismos teste, para avaliar efeitos de xenobióticos nos ecossistemas aquáticos (LU, *et al.*, 2021). Um xenobiótico é uma substância química estranha a um determinado sistema ecológico ou organismo e com atividade biológica (Godheja *et al.* 2016). Há estudos de microalgas na avaliação da toxicidade de compostos orgânicos, como pesticidas (DU *et al.*, 2019; FELLINE *et al.*, 2019; SUN, Chen *et al.*, 2019; GUIDONY *et al.*, 2020), produtos farmacêuticos e de cuidados pessoais (PPCPs) (ZHANG *et al.*, 2018; XIONG, Q. *et al.*, 2019), poluentes orgânicos persistentes, com o bifenilos policlorados (PCBs) (Halm-lemelle *et al.* 2014; Wang *et al.* 2018) e também compostos inorgânicos, como diferentes tipos de metais (KAPLAN, 2013; WEI; ZHU; LAVOIE, 2014; TORRES *et al.*, 2017; HAZLINA; DEVANTHIRAN; FATIMAH, 2019).

Esses organismos possuem algumas características vantajosas que facilitam seu uso em testes de toxicidade, como: o tamanho pequeno, que facilita obter informações em nível de população e comunidade; a facilidade de manutenção em laboratórios, podendo ser cultivadas também em fotobiorreatores sob condições controladas; o curto tempo de geração e a sensibilidade, sendo capazes de responder rapidamente às mudanças ambientais. Além disso, testes com microalgas são rápidos, eficazes e de baixo custo (Cid *et al.* 2012). Microalgas podem apresentar diferentes níveis de tolerância à um mesmo composto. Estudos mostram a diferença na sensibilidade entre clorofíceas, diatomáceas e cianobactérias expostas ao herbicida fenitrothion (Kent and Currie 1995) e também entre dinoflageladas, diatomáceas e clorofíceas quando expostas à ATZ (Weiner *et al.* 2004). Esses autores atribuem essas diferenças às características intrínsecas dessas algas como diferença na morfologia, membrana celular, composição lipídica, entre outros.

O potencial de biorremediação das microalgas tem sido bastante explorado (Hernández-García *et al.* 2019). O processo de biorremediação é reconhecido como um dos métodos de restauração ambiental seguro, ecologicamente correto, baixo custo-benefício e risco reduzido na geração de poluentes secundários (Nie *et al.* 2019). A utilização desses organismos tem sido frequentemente relatada em estudos para remover nutrientes (RUGNINI *et al.*, 2018; XING-LU *et al.*, 2019; HUBADILLAH *et al.*, 2020), pesticidas (SALMAN; ABDUL-ADEL, 2015; SINGH *et al.*, 2018; NIE *et al.*, 2019), metais (KAPLAN, 2013; RUGNINI *et al.*, 2018; LEONG; CHANG, 2020) e produtos químicos farmacêuticos (XIONG, J. Q.; KURADE; JEON, 2018; ANAMIKA, 2020; RODRIGUES *et al.*, 2020) de ambientes aquáticos. Tendo em vista a capacidade desse grupo, é importante encontrar

espécies que sejam tolerantes aos contaminantes, uma vez que essa é uma característica indispensável para ser um biorremediador.

4.1. Classe Chlorophyceae “Microalgas verdes”

As microalgas pertencentes à classe das clorofíceas são o grupo de interesse no presente trabalho. Elas fazem parte do filo Chlorophyta, um filo que engloba as “algas verdes” (Richmond 2004). Essa classe abrange organismos fitoplanctônicos mais relevantes em águas continentais (Vieira et al. 2016). É uma classe grande e morfológicamente diversa, constituindo um dos três maiores grupos de algas verdes, com aproximadamente 8.000 espécies conhecidas sendo que 90% são de ambientes lacustres (ESTEVES, 2011)

Possuem características como presença de clorofila *a* e *b*, amido como material de reserva e parede celular constituída por celulose (Richmond 2004). Podem ser unicelulares ou multicelulares, não móveis e móveis, podem formar colônias, filamentos ramificados e não ramificados e apresentam talos em forma de lâmina (Leliaert et al. 2012). Essa classe inclui algumas das espécies mais comuns, bem como muitos membros que são importantes ecologicamente, como as espécies usadas nesse estudo.

4.2. Espécies de microalgas utilizadas nesse estudo.

As espécies de microalgas utilizadas nesse estudo foram coletadas nos lagos da Universidade Federal do Rio Grande – FURG, Campus Carreiros com exceção da espécie *P. boryanum* que foi coletada na Lagoa Mirim localizada na porção meridional do estado do Rio Grande do Sul. Todas fazem parte da coleção de microalgas continentais do Instituto de Ciências Biológicas (ICB - FURG). A *Pseudopediastrum boryanum* (CH007) (Figura 3A), pertence à família Hydrodictyaceae, ou Sphaeropleales, é uma espécie cosmopolita encontrada especialmente em águas doces (Lenarczyk and Saługa 2018). Esta espécie caracteriza-se por apresentar colônias em forma de disco e são caracterizadas por projeções periféricas semelhantes a chifres. O número de células por colônia pode variar (BRITÂNICA. 2017). O uso dessa espécie tem crescido em estudos de várias áreas. Ela tem sido utilizada como organismo teste na avaliação da toxicidade de xenobióticos (Puerta et al. 2019), como biorremediadoras no tratamento de águas residuais (Park et al. 2014), na geração de bioprodutos (PARK; CRAGGS; SHILTON, 2014; MEHRABADI; FARID; CRAGGS, 2017) e produtos farmacêuticos (Corrêa da Silva et al. 2020). A *Desmodesmus communis* (CH002) (Figura 3B) é uma microalga verde pertencente à família Scenedesmaceae (Bica et al. 2012).

Organizada estruturalmente em cenóbios que possuem linearmente 4 ou 8 células. Possui uma ampla distribuição em águas doces, sobretudo nas levemente eutrofizadas (Hegewald and Braband 2017). Esse gênero e também a espécie têm sido utilizados em estudos ecotoxicológicos (PUERTA *et al.*, 2019; GUIDONY *et al.*, 2020) e biotecnológicos (Hernández-García *et al.* 2019).

As espécies *Ankistrodesmus densus* (CH001) (Figura 3C) e *Ankistrodesmus falcatus* (CH003) (Figura 3D), pertencem à família Selenastraceae. Ambas são algas representativas no fitoplâncton. Possuem modo de vida colonial encontradas em águas doces de países da América do Sul, Europa e Ásia (GUIRY E GUIRY 2021). Apresentam potencial para organismo teste, como a *A. densus* que têm sido utilizada para avaliar efeitos de metais como o cobre e cádmium (Rocha *et al.* 2021; Rocha and Espíndola 2021) ou a *A. falcatus* usada na avaliação da toxicidade do níquel (Martínez-ruiz and Martínez-jerónimo 2015) além de ter potencial para a produção de biocombustíveis (Wang *et al.* 2020). A *Chlamydomonas puliminiorfes* (CH005) (Figura 3E) é uma espécie unicelular, comumente encontrada em solos e água doce. Embora não haja estudos com a espécie utilizada em nosso trabalho, o gênero é bem relevante. O gênero *Chlamydomonas* é amplamente utilizado como modelo para análises toxicológicas ambientais, pois apresenta sensibilidade a diversos poluentes (Míguez *et al.* 2021). Além de ser usado em biologia molecular e estudos genéticos, uma vez que espécies como a *C. reinhardtii* possui seu genoma completo sequenciado (Merchant *et al.* 2007). A espécie *Raphidocelis subcapitata* (CH012) (Figura 3F), também conhecida por outros nomes, como: *Pseudokirchneriella subcapitata* e *Selenastrum capricornutum* pertence à família Selenastraceae (GUIRY E GUIRY, 2021). Possui uma ampla distribuição em ambientes de água doce e é uma das espécies de algas mais usadas em testes ecotoxicológicos devido à sua sensibilidade a contaminantes orgânicos e metais (Viegas 2021). A *Kirchneriella lunaris* (CH011) (Figura 3G), pertence ao gênero de microalgas dulcícolas da família Selenastraceae. São microalgas muito pequenas, simples e coloniais apresentando 2-4-8 (até 64 células) (Krienitz *et al.* 2011) .

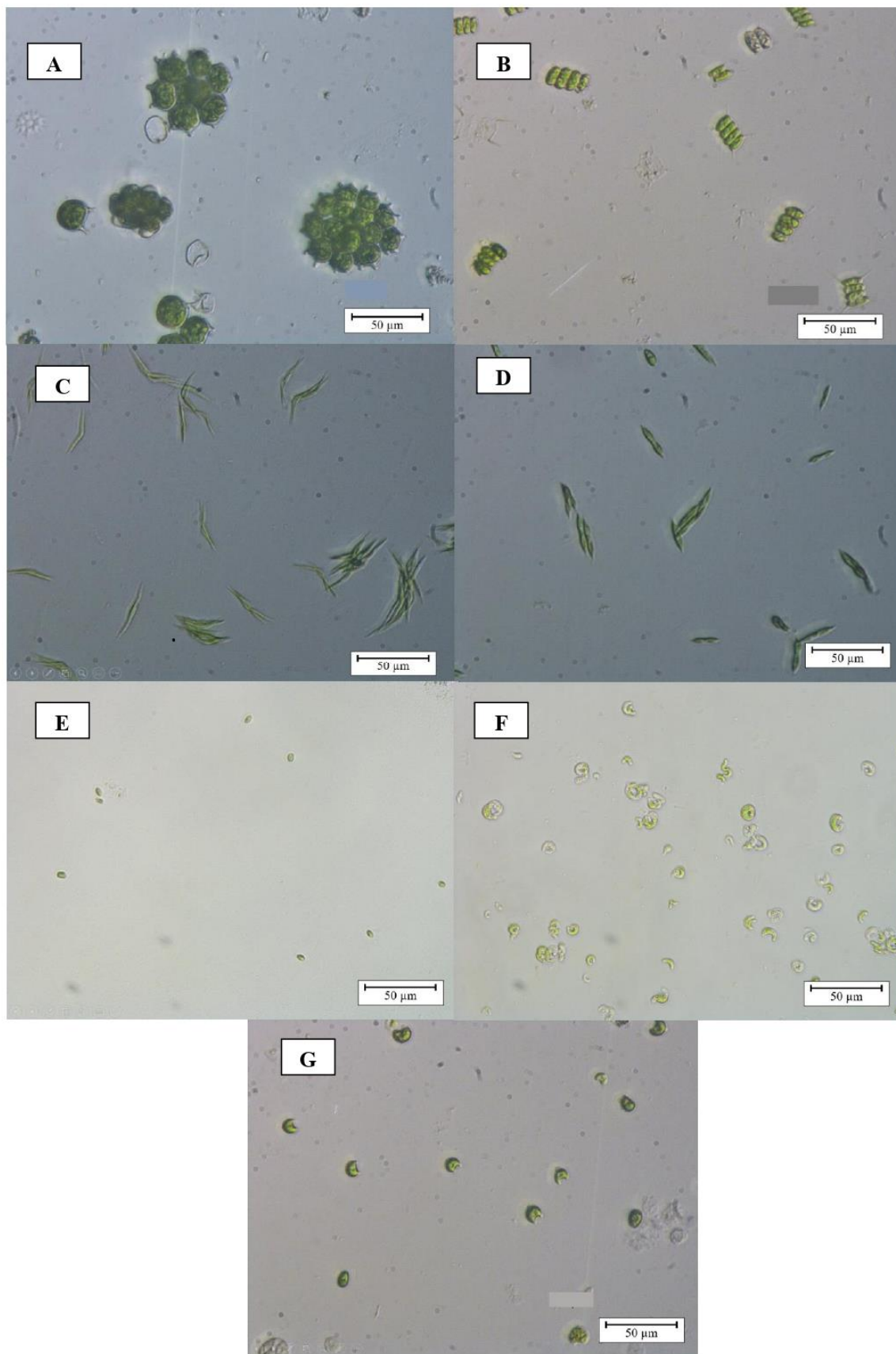


Figura 2. Imagens de microscópio de luz de microalgas verdes (classe chlorophyceae) espécies: (A) *Pseudopediastrum boryanum* (Turpin), (B) *Desmodesmus communis* (E. Hegewald) E. Hegewald, (C) *Ankistrodesmus densus* Korshikov, (D) *Ankistrodesmus falcatus* (Corda) Ralfs, (E) *Chlamydomonas pumilioniformis* Péterfi, (F) *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen & O.M. Skulberg e (G) *Kirchneriella lunaris* (Kirchner) Möbius. As fotos foram tiradas com ampliação de 400x.

5. Ensaio ecotoxicológicos

Nas últimas décadas, a preocupação com a segurança ambiental aumentou devido a quantidade de compostos químicos perigosos que estão sendo gerados, sobretudo pelo fato de que grande parte deles acaba chegando aos ecossistemas aquáticos contaminando esses locais (SOUSA *et al.*, 2016; VAN BRUGGEN *et al.*, 2018; SHARMA *et al.*, 2019). Assim, o desenvolvimento de estudos ecotoxicológicos visando analisar a toxicidade desses compostos também aumentou. Os ensaios ecotoxicológicos são recomendados ou exigidos para avaliação de risco e perigo ambiental ou ecológico de produtos químicos, e partir desses testes pode-se observar os efeitos de xenobióticos em nível individual ou populacional, sendo geralmente baseados na sobrevivência, crescimento, desenvolvimento e reprodução (Rudén et al. 2016). Em relação à ATZ, os testes também aumentaram bastante desde o início de sua comercialização, e vários grupos de organismos têm sido utilizados na avaliação da toxicidade desse composto, como: animais, plantas, fungo e protistas (Castro et al. 2021).

Atualmente, as políticas governamentais visam estimar e monitorar o impacto de produtos químicos prejudiciais à saúde ambiental e humana através do uso de testes dessa natureza (Ceschin et al. 2020) e dessa forma, conseguem regulamentar seu uso. Como já comentado, alguns países como o Brasil e EUA apresentam regulamentação, inclusive para a ATZ, determinando a concentração máxima permitida em alguns ambientes, como os aquáticos (Sass and Colangelo 2006). Um cuidado importante é que os testes são desenvolvidos principalmente para promover confiabilidade, reprodutibilidade e comparabilidade entre os testes e as substâncias de teste, o que permite sua aceitação e que este seja gerado em qualquer país (Rudén et al. 2016). Testes padronizados sob a forma de diretrizes foram formulados por diferentes organizações, como Organização para Cooperação e Desenvolvimento Econômico (OCDE), Agência de Proteção Ambiental dos Estados Unidos (EPA), Organização Internacional de Padronização (ISO), Governo do Canadá (GC) ou América Sociedade de Testes e Materiais (ASTM) (Boros and Ostafe 2020) e, no Brasil pela Associação Brasileira de Normas Técnicas (ABNT).

5.1. Ensaios toxicológicos com microalgas

5.1.1. Inibição de crescimento

Existem várias formas de se avaliar a toxicidade de um composto. No caso das microalgas, um dos métodos mais utilizados é a análise da biomassa da cultura, por densidade celular (obtida através de medidas de absorbância a 750 nm) ou por taxa de crescimento (ABNT NBR 12648:2011). O efeito de inibição sobre uma população de algas, após um intervalo de tempo, é determinado comparando-se o crescimento observado na presença de agente tóxico com o crescimento normal observado em um sistema livre de agente tóxico, o qual é chamado de controle, sendo que nestes testes, parâmetros a luminosidade e temperatura são rigorosamente controlados para não afetar significativamente o crescimento das algas (COSTA et al, 2003).

5.1.2. Rendimento fotossintético

A ATZ atua no organismo-alvo inibindo a fotossíntese, com bloqueio do transporte de elétrons no fotossistema II (Jones et al. 1986) (Figura 3). O bloqueio ocorre porque a ATZ se liga ao sítio de ligação da plastoquinona QB (aceptor de elétrons), interrompendo o fluxo de elétrons entre os fotossistemas.

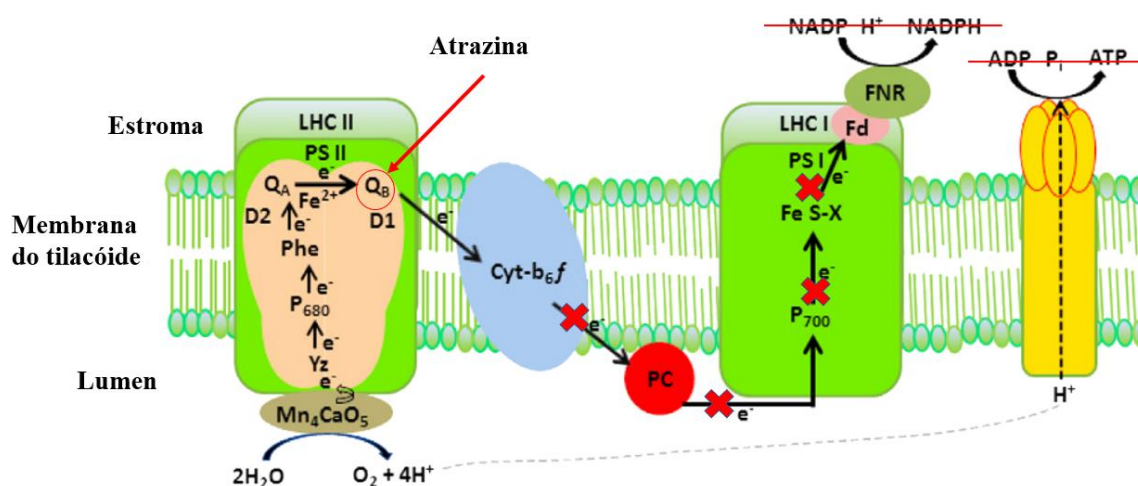


Figura 3. Representação esquemática das reações luminosas da fotossíntese em algas. As setas pretas lineares indicam o transporte de elétrons. O x e as listras em vermelho representam os impactos negativos da atrazina. Fonte: Adaptado de KUMAR et al., (2014)

A fotossíntese é o principal processo que leva à produção primária na biosfera, através dela ocorre a conversão de energia solar, onde compostos inorgânicos (água e CO_2) e energia luminosa são convertidos em matéria orgânica (carboidratos) e O_2 (Masojídek et al. 2013). Este processo é dividido em duas etapas, as chamadas reações de luz (fotoquímica) e reações de escuridão (fase química) (Figura 4) (Johnson 2016). As reações de luz estão ligadas às membranas fotossintéticas, onde a energia da luz é convertida em energia química, fornecendo um redutor bioquímico NADPH_2 e um composto de alta energia - ATP. Já as reações escuras, ocorrem no estroma, e o NADPH_2 e o ATP formados na reação anterior são utilizados na redução bioquímica sequencial do dióxido de carbono em carboidratos. (RENGER, 2007).

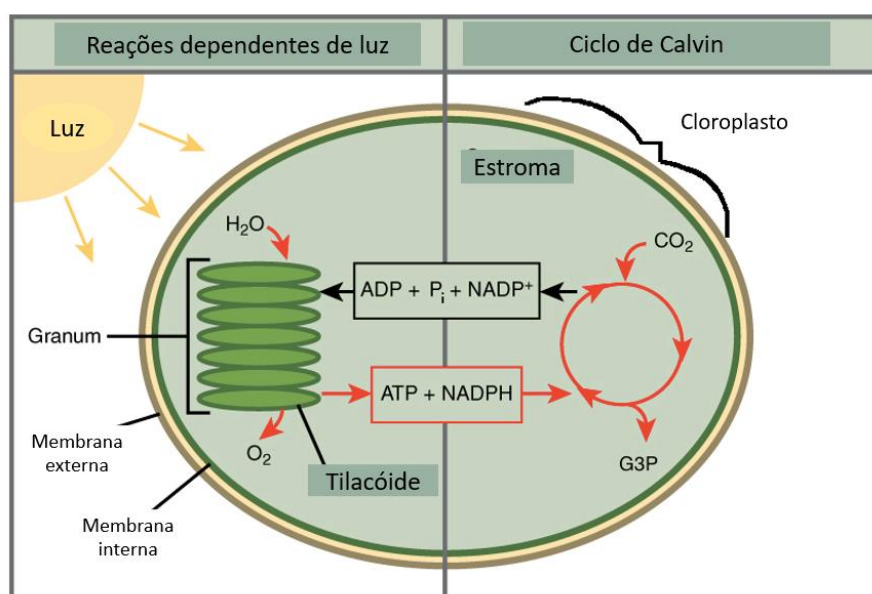


Figura 4. Estágios principais da fotossíntese: as reações dependentes de luz (fase clara) e o ciclo de Calvin (fase escura). O processo de fotossíntese oxigenada é dividido em duas etapas, as chamadas reações de fase clara e reações de fase escura. Essas fases incluem, respectivamente, absorção de luz, transferência de elétrons e translocação de elétrons e prótons, resultando na produção de NADPH_2 , ATP e O_2 e a redução do dióxido de carbono e síntese de carboidratos a partir do NADPH_2 e do ATP produzidos na fase clara. Fonte: Modificado de "Overview of photosynthesis OpenStax College, Biology 2014.

A fotossíntese é um processo dependente da absorção de luz e para isso, os organismos fotossintetizantes contêm pigmentos orgânicos utilizados para coletar essa energia luminosa. Existem três classes principais de pigmentos: clorofilas, carotenóides e ficobilinas (Masojídek et al. 2013). Desses, o principal pigmento é a clorofila *a*, que absorve intensamente as luzes vermelha e azul, sendo a luz verde predominantemente refletida (MADIGAN, 2016). Os pigmentos são encontrados ligados a proteínas embutidas na

membrana, conhecidas como complexos coletores de luz (LHCs) que por sua vez, são denominados fotossistemas (Johnson 2016)

Existem dois tipos de fotossistemas: fotossistema I (PSI), e fotossistema II (PSII), onde o PSI está localizado na lamela do estroma do tilacóide, enquanto o PSII está no domínio grana empilhado. (GAO *et al.*, 2018). Os fotossistemas possuem duas partes distintas: um complexo de captura de luz (complexo antena) onde se encontram um aglomerado de pigmentos (principalmente clorofila) captando a energia luminosa de excitação e a transferindo por ressonância ao centro de reação, que seria a segunda parte do fotossistema. (Figura 4) (GAO *et al.*, 2018). As reações que ocorrem na fotossíntese são representadas pelo “esquema Z” (Figura 5) que descreve o processo de fotofosforilação, pois a produção de energia, na forma de ATP, está vinculada a uma cadeia não-cíclica de elétrons, iniciada com a fotólise da água e encerrada na produção de NADPH.

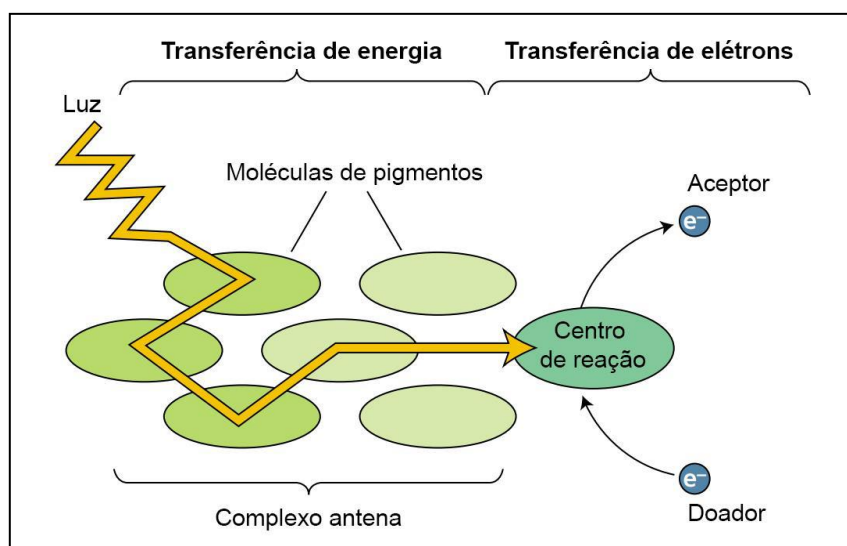


Figura 5. Esquema da transferência de energia em um fotossistema. A luz é coletada através dos pigmentos (complexo antena) e sua energia é transferida para o centro de reação, logo após elétrons (e^-) da clorofila são transportados para uma molécula aceptora. Fonte: Adaptado de TAIZ e ZEIGER (2002).

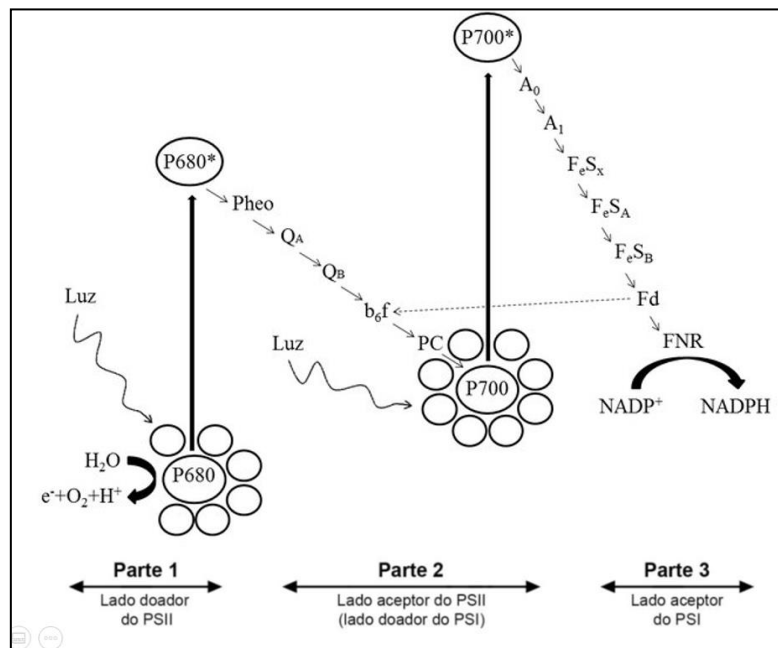


Figura 6. Representação básica do esquema Z da fotossíntese. (1) o lado doador do PSII inclui as reações responsáveis pela entrada de elétrons no fotossistema a partir da água; (2) a cadeia de transporte de elétrons intersistêmica, composta por uma série de carreadores; e (3) o lado aceptor do PSI, onde ocorre a redução direta de NADP⁺ em NADPH, o qual será exportado para o Ciclo de Calvin. O asterisco (*) representa o estado reduzido de ambos os pares. Adaptado de TAIZ e ZEIGER (2002).

A fluorescência da clorofila é uma das técnicas mais populares em fisiologia vegetal devido à facilidade com que o usuário pode obter informações detalhadas sobre o estado do PSII e tem um papel importante na compreensão dos mecanismos fundamentais da fotossíntese (Endo and Omasa 2004; Kalaji et al. 2017). A possibilidade de estudo da fisiologia vegetal a partir da fluorescência da clorofila decorre do princípio básico de que a energia luminosa incidente sobre o pigmento não é utilizada em sua totalidade (Murchie and Lawson 2013). Três processos podem ocorrer com a energia luminosa absorvida pelas moléculas de clorofila, que são: (i) conduzir a fotossíntese (fotoquímica); (ii) ser reemitido como calor; ou (iii) ser reemitida como luz (fluorescência) e esses três processos não existem isoladamente, mas sim em competição uns com os outros, ou seja, a alteração na eficiência de um desses processos resultará em consequente mudança nos demais (Murchie and Lawson 2013). Assim, o rendimento da emissão de fluorescência da clorofila nos dá informações valiosas sobre a eficiência quântica da fotoquímica e dissipação de calor (Kalaji et al. 2014).

Outro ponto importante é que o sinal emitido pela fluorescência é rico em informações sobre organismos fotossintetizantes pois através dessa técnica pode-se obter respostas às mudanças ambientais (Kalaji et al. 2014). O sinal de fluorescência da clorofila é, em sua maior parte, causada pela atividade do PSII. Devido a sua sensibilidade a fatores externos, bióticos ou abióticos, realizar a análise da fluorescência ajuda na compreensão dos

mecanismos fotossintéticos desses organismos e como eles respondem ao ambiente (MURCHIE; LAWSON, 2013). A medição da fluorescência da clorofila é um método sensível, versátil, robusto, não-invasivo e eficiente na obtenção de dados qualitativos e quantitativos a respeito da fotossíntese, sendo eficaz para quantificar e analisar o fitoplâncton de água doce e do mar *in situ* (Zeng and Li 2015).

A possibilidade de se analisar e avaliar as reações fotossintéticas primárias e mecanismos de dissipação energética em estudos fisiológicos com fitoplâncton se tornou possível devido a um método bastante utilizado que é a fluorometria de pulso de amplitude modulada (PAM) (Figuerola et al. 2013). A técnica PAM combina diferentes tipos de luz para analisar a cinética de indução da fluorescência (SCHREIBER, 2004) e são obtidos através da “curva Kautsky” que é uma representação das principais mudanças ocorridas no rendimento de fluorescência da clorofila *a* de um organismo fotossintético quando este é adaptado a uma condição de escuro é logo após transferido para uma condição de exposição à luz (MAUZERALL; GREENBAUM, 1989). Através dela vários parâmetros podem ser estimados, como os sinais básicos de fluorescência da clorofila que são: fluorescência máxima em adaptação ao escuro (F_m), fluorescência mínima em adaptação ao escuro (F_o), fluorescência máxima de células adaptadas à luz ($F'm$) e fluorescência mínima de células adaptadas à luz ($F'o$). Através desses parâmetros é possível calcular uma série de variáveis importantes para estudos fisiológicos como os rendimentos quânticos máximo e efetivo do PSII, os coeficientes de decaimento da fluorescência, a taxa de transporte de elétrons e as três medidas fundamentais extraídas das chamadas Curvas Rápidas de Luz (MASOJIDEK; VONSHAK; TORZILLO, 2010).

5.1.3. Morfologia

O tamanho da célula do fitoplâncton varia muito entre os diferentes gêneros ou mesmo entre diferentes indivíduos variando de alguns micrômetros (ou até menos de 1 mm) a alguns milímetros (Fonseca et al. 2014). O estudo da morfologia, mais especificamente, o biovolume de microalgas é importante no desenvolvimento de diversos estudos (SUN; DONGYAN, 2003). O tamanho individual de uma célula pode nos dar respostas a algumas perguntas pois estão relacionadas a processos como a absorção de nutrientes, afinidade à luz, fotossíntese e respiração, taxas de acomodação, transporte físico e interações planta-herbívoro (Vadrucci et al. 2013). O biovolume também é usado para a conversão de contagens de células em unidades de carbono em estudos de fluxos de matéria orgânica em comunidades aquáticas (Sun and Dongyan 2003). Estudos também têm utilizado esse parâmetro pra explicar a

diferença na tolerância de microalgas a um mesmo composto (por exemplo, pesticidas) já que o tamanho da célula pode estar relacionado com a quantidade de composto absorvido (Kent and Currie 1995; Weiner et al. 2004). Inclusive alguns, estudos atribuem a esse parâmetro a diferença de sensibilidade entre microalgas clorofíceas e cianobactérias (KENT; CURRIE, 1995; WEINER; DELORENZO; FULTON, 2004)

Existem alguns métodos automatizados ou semiautomatizados para a estimativa do biovolume, como o uso de sistema de análise de imagem micrográfica, citometria de fluxo e tecnologia de varredura holográfica (SUN; DONGYAN, 2003). Entretanto o método geral para calcular o biovolume das células do fitoplâncton é baseado na atribuição geométrica (HILLEBRAND *et al.*, 1999; SUN; DONGYAN, 2003; VADRUCCI; MAZZIOTTI; FIOCCA, 2013). O cálculo de biovolume baseado em modelos geométricos de células fitoplanctônicas e sua biomassa de conversão relacionada são populares em estudos de ecologia do fitoplâncton. O método aplica o princípio de modelos ou formas geométricas que são mais semelhantes à forma real do organismo (Figura 6) (Vadrucci et al. 2013).

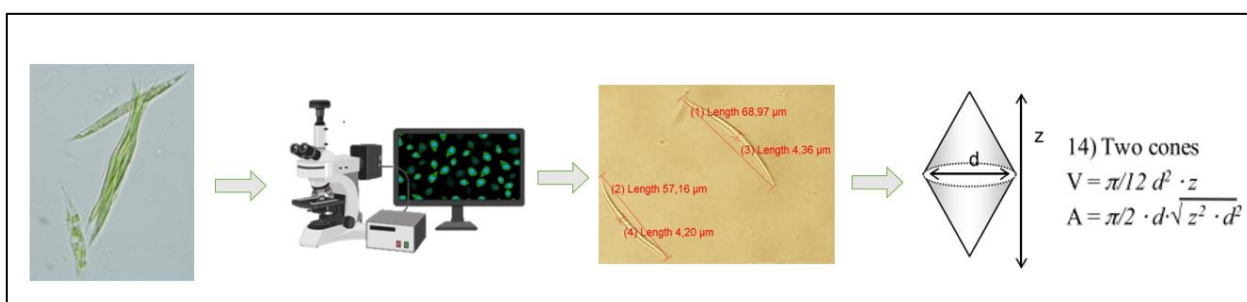


Figura 7. Esquema da metodologia de obtenção dos parâmetros morfológicos (biovolume e área). A microalga é analisada através de um microscópio óptico e as medidas mensuradas e usadas posteriormente para calcular biovolume e área de acordo com a forma geométrica e cálculos previstos por protocolos já estabelecidos. Fonte: O autor.

5.1.4. Glutathione S-transferase

As glutathione S-transferases (GSTs) compõem uma família de proteínas multifuncionais que atuam como enzimas e também como proteínas de ligação em vários processos de desintoxicação e metabolismo celular (Tsuchida and Yamada 2014). As GSTs geralmente se encontram no meio biológico principalmente na forma de homô ou heterodímeros, apresentando dois sítios ativos por dímero cujas atividades são independentes uma da outra (Huber et al. 2008). Cada sítio ativo consiste no mínimo de duas regiões de ligação, um para a

co-enzima glutathione reduzida (GSH) que é muito específico para este tripeptídeo, e outro sítio de ligação com menor especificidade para substratos eletrófilos (Huber et al. 2008).

As GSTs estão presentes em todos os organismos vivos e podem ser classificadas em três superfamílias quanto a localização celular: GST citossólica, GST mitocondrial e GST microsomal (HUBER; ALMEIDA; FÁTIMA, 2008; TSUCHIDA; YAMADA, 2014; CHATZIKONSTANTINO *et al.*, 2017). A família GST citossólica é a mais abundante no grupo das plantas (CHATZIKONSTANTINO *et al.*, 2017). A nomenclatura para as GSTs dessa família foi dada baseada nas semelhanças das suas estruturas primárias e na divisão em classes com base na identidade das sequências de aminoácidos. Essas classes foram designadas por letras gregas, incluindo Alpha, Mu e Pi. Isoformas de GST que pertencem a mesma classe são distinguidos por algarismos arábicos, como por exemplo, GST-Alpha1 e GST-Alpha2. As proteínas diméricas nativas ou formas moleculares, por sua vez, foram nomeadas de acordo com sua composição de subunidades.

Nos vegetais as classes de GSTs encontradas são phi, tau, teta, zeta, lambda, desidroascorbato redutase (DHAR), tetraclorohidroquinona desalogenase (TCHQD) e subunidade γ do fator de alongamento da tradução eucariótica 1B (EF1Bg), hemeritrina GST e iota GST (Edwards et al. 2000; Lan et al. 2009; Chatzikonstantinou et al. 2017).

As GSTs têm um papel importante na tolerância a herbicidas em plantas e microalgas. Estas enzimas formam um complexo de conjugação xenobiótico/GSH que está envolvido na desintoxicação de várias classes de herbicidas, inclusive a ATZ (Huber et al. 2008). A detoxificação enzimática de xenobióticos, como os herbicidas se dá em três fases distintas (Figura 7), porém estritamente relacionadas, onde nas fases I e II, geralmente se observa a transformação do xenobiótico em uma espécie que apresenta maior solubilidade em água, além de uma menor toxicidade, e na fase III, estes metabólitos são transportados para o exterior da célula e então excretados (Huber et al. 2008). As enzimas da fase II catalisam a conjugação dos xenobióticos ou de seus metabólitos oriundos da fase I com substratos endógenos, tornando-os mais solúveis em água. Quantitativamente, a formação de conjugados com a glutathione é a principal reação observada para muitos xenobióticos durante a fase II. Assim, de fato, o mecanismo de detoxificação via glutathione pode representar uma adaptação biológica fundamental para a sobrevivência e garantia da perpetuação de muitas espécies (Iyanagi 2007; Huber et al. 2008). Certos herbicidas também podem ser inativados diretamente pela conjugação com GSH catalisada por GST. No caso de microalgas, os herbicidas conjugados são subsequentemente transportados para vacúolos para armazenamento e / ou metabolismo adicional (Yu and Powles 2014).

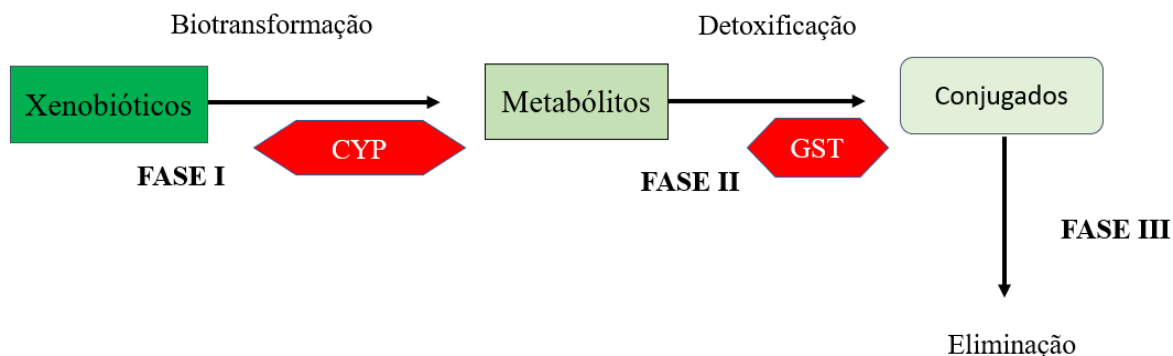


Figura 8. Esquema das fases de detoxificação enzimática de xenobióticos. Fonte: Adaptado de LUSHCHAK (2011)

5.1.5. Teor de clorofila – *a*

A clorofila é um componente bioquímico importante no aparato molecular responsável pela fotossíntese (Mandal and Dutta 2020). Ela é o principal pigmento coletor de luz em plantas, algas e cianobactérias. A energia solar é absorvida pela clorofila situada nos fotossistemas intrínsecos da membrana e usada para separação de carga, que impulsiona a produção de ATP e NADPH (Büchel 2020). Esse pigmento está presente na organela celular especializada chamada cloroplasto e, portanto, é nele que a fotossíntese ocorre dentro da célula (Mandal and Dutta 2020). A clorofila ocorre em várias formas distintas, que variam de acordo com as espécies, entretanto as clorofilas *a* e *b* são os principais tipos encontrados em plantas superiores e algas verdes (Figura 9) (TAIZ; ZIEGER, 2002).

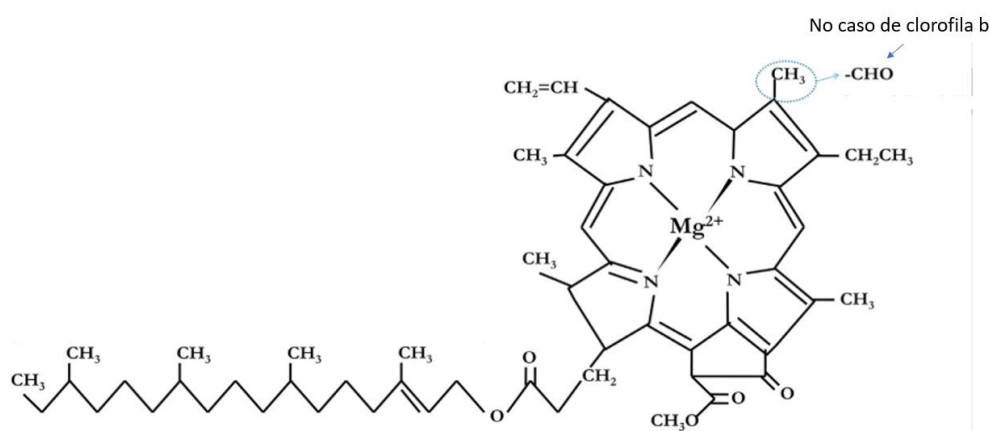


Figura 9. A estrutura química da molécula de clorofila típica. A clorofila *a* contém o grupo metil ($-\text{CH}^3$) e a clorofila *b* contém o grupo aldeído ($-\text{CHO}$). Fonte: Adaptado de MANDAL; DUTTA, 2020.

As clorofilas são formadas por complexos derivados da porfirina, tendo como átomo central o magnésio (Mg). Esse composto é formado por uma estrutura macrocíclica assimétrica totalmente insaturada constituída por quatro anéis de pirrol (SCHOEFS, 2002). As clorofilas *a* e *b* encontram-se na natureza numa proporção de 3:1, respectivamente, e diferem nos substituintes de carbono C-3. Na clorofila *a*, o anel de porfirina contém um grupo metil (-CH₃) no C-3 e a clorofila *b* contém um grupo aldeído (-CHO), que substitui o CH₃ (Streit et al. 2005). Além das moléculas de clorofilas, outros pigmentos acessórios participam do processo de fotossíntese, são eles as ficobilinas e os carotenóides. A energia absorvida pelos pigmentos é transferida para sítios bem definidos chamados centros de reação. Há dois centros de reação, um deles absorvendo em 680 nm e outro em 700 nm, os quais interagem entre si através de transportadores de elétrons (Streit et al. 2005). É a partir da molécula de clorofila, a qual absorve em 680 nm no espectro visível, que os elétrons oriundos da água são transferidos para a cadeia transportadora de elétrons da fotossíntese (KLUGE, 2004).

A clorofila – *a* também é um indicador de saúde nas plantas e algas. O teor de clorofila é usado para avaliar a capacidade fotossintética e estado de crescimento das plantas. Nessa tese, o teor de clorofila foi avaliado como preditor de tolerância de microalgas à ATZ, uma vez que, a clorofila está diretamente relacionada à quantidade de fotossistema II na célula, que é o principal alvo bioquímico deste herbicida (Delorenzo et al. 2004).

JUSTIFICATIVA

O aumento do uso de pesticidas, sobretudo no Brasil, traz um cenário preocupante pois esses químicos acabam chegando a ecossistemas importantes e causando efeitos adversos em organismos não alvo. Como já exposto, a ATZ é um herbicida que está presente em ambientes aquáticos provocando efeitos negativos nas espécies que habitam o local, principalmente organismos fotossintetizantes, como as microalgas. Tendo em vista a importância desses dois elementos, um como uma ameaça ambiental e outro como organismo de relevância ambiental ameaçado, ambos serão o foco da tese: A atrazina e as microalgas.

Devido ao extensivo uso da ATZ é necessário ter conhecimento sobre esse herbicida. O conhecimento da evolução do uso da ATZ bem como o que se produziu de estudos na área de toxicologia sobre ela é importante pois pode trazer lacunas que ajudem em tomadas de decisões sobre futuros estudos a serem desenvolvidos. Nesse contexto, desenvolver estudos cienciométricos e avaliar a pesquisa científica dentro de áreas ambientais e mais especificamente em áreas como a ecotoxicologia é importante. E estudos dessa natureza sobre a ATZ são escassos. Assim realizamos um estudo cienciométrico para obter informações a partir da literatura, mas especificamente na literatura dentro da toxicologia, o que se conhece e o que se pesquisa sobre o herbicida ATZ e seus efeitos em microalgas.

Outro ponto importante é conhecer os efeitos da ATZ nas microalgas, uma vez que esses organismos são importantes para a manutenção do ecossistema desempenhando funções cruciais. Sabe-se que as microalgas podem apresentar diferentes níveis de tolerância a um mesmo composto, inclusive a ATZ (Kent and Currie 1995). Estudos mostram que a ATZ é capaz de inibir o crescimento de microalgas e também inibir o rendimento fotossintético, uma vez que a inibição da fotossíntese é o principal alvo da ATZ (Marchello et al. 2018). Desse modo, investigar os possíveis preditores que influenciam na diferença da tolerância de microalgas verdes à ATZ bem como avaliar se a inibição do rendimento estaria relacionada à inibição do crescimento são importantes.

OBJETIVOS

Objetivo geral

Construir um panorama da evolução dos estudos com ATZ na área de toxicologia e avaliar a diferença na tolerância de espécies de microalgas da classe Chlorophyceae à ATZ relacionando a tolerância às características morfológicas, fotossintéticas e bioquímicas destas espécies.

Objetivos específicos

- O primeiro capítulo teve como objetivo: (1) Quantificar os trabalhos na área de toxicologia que utilizaram ATZ, (2) averiguar quais países que mais publicaram, (3) analisar o fator de impacto dos periódicos, (4) analisar quais os grupos de organismos mais usados na análise de toxicidade da ATZ, (5) quantificar os trabalhos que utilizaram microalgas e cianobactérias, (6) averiguar que tipo de testes foram utilizados.
- O segundo capítulo teve como objetivos: (1) Avaliar a tolerância de sete espécies de microalgas clorofíceas com base na avaliação da inibição do crescimento, (2) mensurar o biovolume, área e relação superfície volume das microalgas, parâmetros fotossintéticos, teor de clorofila – *a* e atividade basal da enzima GST das sete espécies, (3) relacionar as características avaliadas para as espécies com a tolerância diferencial das mesmas e (4) avaliar se a fotossíntese é inibida de forma semelhante em todas as espécies de microalgas quando expostas a concentrações tóxicas equivalentes (EC30) de ATZ

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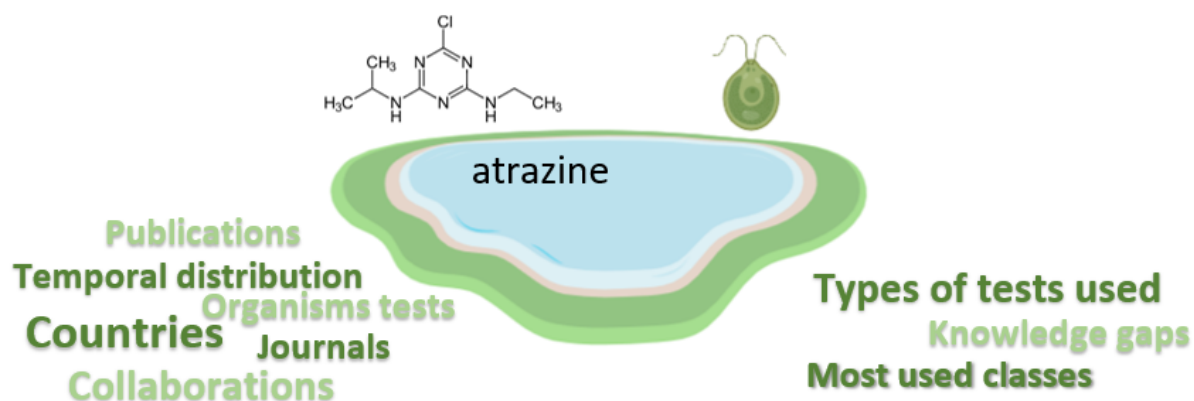
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CAPÍTULO 1

A scientometric analysis of ecotoxicological studies with the herbicide atrazine and microalgae and cyanobacteria as test organisms



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A scientometric analysis of ecotoxicological studies with the herbicide atrazine and microalgae and cyanobacteria as test organisms

Muryllo Santos Castro ^{1,2}, Fabiana Gonçalves Barbosa², Pablo Santos Guimarães ², Camila Martinez De Gaspar Martins ^{1,2}, Juliano Zanette ^{1,2 *}

¹ Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, 96203-900, Brazil.

² Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, 96203-900, Brazil.

* Corresponding author:

Juliano Zanette, Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande (FURG), Av. Itália Km 8, Rio Grande, RS, 96203-900, Brazil

Tel: +55-53-32975196 Fax.: +55-53-32336633

E-mail: julianozanette@furg.br; juliano.zanette@pq.cnpq.br

ABSTRACT

Atrazine (ATZ) is one of the most widely used herbicides in the world. A scientometric study was conducted to analyze the evolution of research on ATZ. The study also looked at the use of microalgae and cyanobacteria as biological models for toxicity tests during the period from 1959 to 2019, in the category of Toxicology of Web of Science. The results show an increase in the number of scientific publications mainly in the United States of America, Canada, and China. Most papers were published in journals with high impact factors, demonstrating the relevance of such studies. About 83% of the studies aimed to evaluate the effect of ATZ on non-target organisms. From those, 7.5% included microalgae and cyanobacteria. Most of them worked with chlorophyceae to perform toxicity bioassays of ATZ and analyze its sublethal effects. The gaps identified by this analysis included: a small number of collaborations between research groups from different countries; the number of studies with terrestrial organisms, which are larger in comparison to aquatic organisms; and the fact that none of the studies with ATZ and microalgae were performed in the field. These findings can point out to researchers and funding agencies the gaps in knowledge on the toxic effects of ATZ and guide the development of new research projects as well as environmental policies.

Keywords: chlorophyceae, pesticide, pollution, research productivity, web of science, toxicity

Introduction

Atrazine (ATZ) is a herbicide used for pre and post-emergence weed control in agricultural and forestry systems (Graymore et al. 2001). This herbicide was formulated in 1952 in Switzerland and since 1959 it has been commercialized (USEPA 2002). The extensive and common use of ATZ makes this pesticide the second most widely used in the world (Singh et al. 2018). However, ATZ has shown to cause toxic effects in non-target species (Van Der Kraak et al. 2014), which can vary from organisms living in soil and aquatic environments (Jablonowski et al. 2011) to humans living in agricultural areas (Pathak and Dikshit 2011). This compound has been often found in aquatic environments due to its characteristics such as low adsorption in soils, moderate aqueous solubility, and high leaching (Singh et al. 2018; De Souza et al. 2019). Studies report the presence of the compound in concentrations that can vary from 0.001 to 88 $\mu\text{g/L}$ in rivers and water reservoirs in the United States of America (USA), Canada, Europe, Africa, and Australia, with a half-life of up to 6 months (Graymore et al. 2001).

Various studies have summarized the toxic effects of ATZ on an individual and community level in aquatic environments (Graymore et al. 2001; De Souza et al. 2019; de Albuquerque et al. 2020). ATZ is a potential threat to photosynthetic organisms, such as microalgae and cyanobacteria, as it inhibits photosynthesis by blocking the transport of electrons during the Hill reaction of photosystem II (PSII) (Jones et al. 1986). It is important to mention that photosynthetic organisms perform functions that are important for the maintenance of the aquatic ecosystem and its biodiversity (McCormick and Cairns 1994).

Photosynthetic organisms are the main primary producers of the aquatic ecosystem, responsible for about 60% of the Earth's primary production (Chisti, 2004). Thus, any disturbance in these populations can impact other organisms (Cid et al., 2012) and potentially affect the aquatic ecosystems (McCormick and Cairns 1994). Therefore, microalgae and cyanobacteria have ecological importance. Also, due to their sensibility to various pollutants, ease of laboratory cultivation, rapid growth rate, and short life cycle (DeLorenzo et al. 2001), some species have been used as model organisms in the field, or in laboratory experiments, to assess the toxicity and negative effects of different pesticides (DeLorenzo et al. 2001), including ATZ (El-Sheekh et al. 1994; Didur et al. 2012; Kabra et al. 2014).

Scientometric analysis can identify patterns, gaps, or trends that may exist in publications on a given topic and/or field study (Barbosa et al. 2012). This information helps to systematically evaluate and quantify advances in knowledge as well as providing a more

integrated view of the research trends regarding a specific topic. According to The Organization for Economic Co-operation and Development (OECD), scientific research is the base for economic growth, thus “the scientific publication is considered a component of knowledge-based economy construction” (Nguyen and Pham 2011). Data obtained through this kind of study can help decision-makers agree on which research activities are necessary to achieve economical and political goals (Mingers and Leydesdorff 2015). In parallel, scientometric studies are useful for promoting scientific partnerships (Mascarenhas et al. 2018), as well as to identify specific gaps in knowledge to guide future research (Blettler et al. 2018).

In ecotoxicology, scientometric studies have included studies on several pollutants, including pesticides (Barboza and Gimenez 2015; Blettler et al. 2018; Vanzetto and Thom 2019). There are scientometric analyses on paraquat and glyphosate that show an increase in the number of scientific publications from 1962 to 2015 for paraquat, and from 1978 to 2015 for glyphosate (Zyoud et al. 2017; Zyoud 2018). Moreover, studies on herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Zuanazzi et al. 2020) and glyphosate (Ghisi et al. 2020) showed a higher rate of publication from developed countries than from low-income countries.

In this context, the present study performed for the first time a scientometric analysis on ATZ focusing on toxicological studies, and considering papers published from 1959 onwards, the year of its commercialization. The aim is to provide an overview of the type of research that has been carried out addressing this herbicide. We also focus in studies using microalgae and cyanobacteria as model organisms for testing, considering the importance of this group. The following questions were asked: (i) Did the number of studies grow over the years? (ii) Which countries have published the most? (iii) Are there international collaborations between groups? (iv) What is the proportion between individual research and international collaborations? (v) What are the main scientific journals publishing on ATZ? And (vi) Which organisms are used to investigate the toxicity and sublethal effects of ATZ? For the studies involving microalgae and cyanobacteria, the following questions were asked: (i) Did the number of studies grow over the years? (ii) Were the tests carried out in the laboratory or field? (iii) What type of analysis are most used (toxicity bioassays and/or biomarker)? (iv) Were the tests acute or chronic? And (v) Which taxonomic classification of microalgae and cyanobacteria are most used in the tests with ATZ? The answers should support researchers with important information for future studies on ATZ, and environmental policy discussion.

Methodology

The Clarivate Analytics Web of Science database (WoS) was used to search for scientific studies on atrazine (updated in August 2020). We used the keyword “atrazine” for the topic search. In our search, we set the following filters: (i) only articles published in English, (ii) only journals classified in the Toxicology Web of Science category, to select studies focused on assessing the effects of atrazine on the environment, and on non-target organisms and (iii) period from 1959 to 2019. The WoS has been widely recognized as an important database by the international scientific community (Li et al. 2018) and covers 93 journals in the Toxicology Web of Science category. We selected the year 1959 since it was the year ATZ was introduced on the market.

Initially, our search resulted in a total of 1471 articles. Next, we manually checked all articles to exclude works that were not related to ATZ and this approach reduced the number of articles to 1009 (Fig. 1) (see a list of all articles used in the present study in the Supporting Information Table S1). For each article, we retrieved the following information: (i) year of publication, (ii) country of publication, (iii) journal of publication, and (iv) organisms used to evaluate the effects of ATZ. The same workflow was used for our scientometric analysis on the use of microalgae as a test organism in studies on ATZ (Fig. 1). For each article, we retrieved the following information: (i) year of publication, (ii) use of microalgae in the study, (iii) field or laboratory experiment, (iv) method of analysis (toxicity bioassay and/or biomarkers), (v) whether it was a chronic or acute test, and (iv) taxonomic classification of microalgae and cyanobacteria.

Fig. 1 Research methodological framework.

For scientometric analysis we downloaded the articles in the BibTeX file format and, then we analyzed the information using the bibliometrix package (Aria and Cuccurullo 2017) in the R software (R Core Team, 2019). We used a simple linear regression to identify trends over time in the number of articles per year. The type of scientific collaboration between countries was determined by the country of the corresponding author of each article: (i) the term “Single Country Publications” was assigned for articles where the corresponding author and co-authors were from the same country and (ii) the term “Multiple Country Publications” was assigned for articles where the corresponding author and co-authors were from different countries (Li et al. 2009). The VOSviewer software was used to build a map of the

international collaboration network (Van Eck and Waltman 2010). We obtained the impact factor (IF) and the h-index of each journal from the 2019 Journal Citation Reports (JCR).

Results and discussion

A total of 1009 articles on ATZ were published between 1959 and 2019 in journals classified in the Toxicology WoS category. The first record of scientific publication on ATZ indexed in the WoS occurred in 1973, despite the formulation being released back in 1952 by the Geigy Company, a herbicide company in Switzerland (USEPA, 2002). In this first publication, the effect of ATZ was evaluated on the reproduction of rats (Peters and Cook 1973). From this first study, the number of published articles increased every decade (Fig. 2), showing a strong correlation with the amount of time ATZ was released for use (R squared: 0.9357). In the 60s, no articles concerning ATZ were published, and in the 70s and 80s, an average of 1 to 4 articles was published by year, respectively. In the following decades, publications on ATZ increased, wherein the 90s, 00s, and 2010-2019 the average annual production reached 20.5, 37.5, and 40 studies, respectively. This result is consistent with those from Van Der Kraak et al. (2014), who mentioned that the potential effects of ATZ on organisms have been a subject of interest since the 90s. The increase in the number of articles on ATZ may be associated with the increase in herbicide use in agricultural systems, mainly after the 00s (Gianessi 2013). With that, the need to understand the compound increased, and that consequently increased the number of scientific researches. Most studies have assessed the potential effects of ATZ on human health, non-target species, and the environment. Similarly, scientometric studies with the herbicides glyphosate (Zyoud et al. 2017b; Ghisi et al. 2020), paraquat (Zyoud 2018), and 2,4-dichlorophenoxyacetic acid (Zuanazzi et al. 2020), found a growing scientific interest in recent decades. These trends follow the general pattern described by Köhler and Triebkorn (2013) who reported that research on the effects of pesticides has continuously increased since the 90s, especially those belonging to the triazine group, such as ATZ. This may be associated with the concern around the use of these compounds, and their relationship with contamination and environmental degradation.

Fig. 2 Number of articles on atrazine (ATZ) published between 1959 and 2019 in the toxicology category from WoS.

According to the present study, researchers from 54 countries published articles on ATZ between 1959 and 2019 in the Toxicology category from the WoS. Researchers from the

United States of America (USA) were responsible for 41 % the of articles, followed by China (8 %) and Canada (7%) (Table 1). This result has been the standard in scientometric studies on pesticides: American, Chinese, and Canadian researchers tend to publish more than researchers from other countries (Zyoud et al. 2017; Zyoud 2018; Ghisi et al. 2020; Zuanazzi et al. 2020). The United States has programs and laws developed to regulate the use of pesticides in the country, such as the Office of Pesticide Programs of the Environmental Protection Agency (EPA), as well as the Federal Law on Insecticides, Fungicides, and Rodenticides that governs the registration, distribution, sale, and use of pesticides in the USA. This is one of the reasons that many studies on pesticides are developed in the country. In China, policies have improved to control pesticides, as well as, in technological applications and implementing scientific knowledge to guide pesticide use (Xing-Lu et al. 2019). This could also be associated with the budget increase from the National Science Foundation of China since 2000 (Yang 2016). Another factor that may influence the high number of published studies is the need for regulation on ATZ in these countries. Some countries have included ATZ in the list of controlled herbicides, and the European Union has banned its use since 2004 (De Albuquerque et al. 2020). However, in the United States (USA) and China, its use continues without effective regulatory control (Sun et al. 2017).

According to Abt (2007), rapid globalization contributed to international collaboration in science. The present study, however, shows that 82% of the articles were single country publications (SCP) while 18% of the articles were multiple country publications (MCP) (Table 1). This result indicates that research on ATZ lacks international collaboration, and there is a need to increase international academic partnerships. About 24 countries collaborated in research on the herbicide ATZ (Fig. 3). The USA played an important role in this scientific network and had close cooperation with the majority of countries analyzed, especially with China and Canada. According to Witze (2016), the collaboration between these three countries has been growing since 2013. The collaborative relationship between the USA and Canada is probably higher due to easier geographical proximity (Katz 1994). On the other hand, aligning science and technology knowledge has been one of the main factors for the scientific relation between the USA and China (Wagner et al. 2015). We note that the top 10 countries that have carried out most of the international collaborations are in Europe (Italy, France, Spain, and Serbia). Two factors can explain the higher percentage of international collaborations: the population size of the country and, consequently, the size of the scientific community. Our results show that the USA, China, Brazil and India (the countries with the largest population in the ranking) are the ones that least performed international

collaborations, probably due to the number of researchers in these countries. According to OECD (2020) in 2017 the USA had 325.1 million inhabitants where approximately 0.92% were researchers, and in China, which in 2019 had 1.393 billion inhabitants, approximately 0.24% were researchers. On the other hand, countries with a smaller population number tend to have fewer researchers and thus carry out more international partnerships seeking collaborators and new techniques to enrich their studies (Glanzel et al. 1999). That was seen in Italy, Spain, and Australia (the countries with the lowest population in the ranking), which carried out more international collaborations. For example, Spain in 2019 had 46 million inhabitants where approximately 0.7% were researchers, and in Italy, with 60 million inhabitants in 2019, approximately 0.6% were researchers (OECD, 2020). In this context, this second hypothesis justifies the largest collaborations in European countries, as well as explaining the fact that the USA, Germany, and Brazil are the countries that present the least amount of articles on ATZ involving international collaborations.

Fig. 3 Map of the international collaboration network between countries on atrazine from 1959 to 2019 in the toxicology category from WoS. On the map, the circles and their sizes represent the countries and the number of articles, respectively, and the lines and their thickness represent their cooperation and the degree of collaboration, respectively.

Articles on ATZ were published in 92 journals in the Web of Science in the toxicology category. The top ten journals in the ranking account for 70% of total publications on ATZ (Table 2). The journal *Environmental Toxicology and Chemistry* was ranked first with 181 articles, followed by *Bulletin of Environmental Contamination and Toxicology* (105 articles), *Ecotoxicology and Environmental Safety* (104 articles), and *Archives of Environmental Contamination and Toxicology* (93 articles). Additionally, journals with a higher impact factor were the *Environmental Health Perspectives* (IF = 8.3, h-index = 269), *Ecotoxicology and Environmental Safety* (IF = 4.9, h-index = 121), and *Aquatic Toxicology* (IF = 3.9, h-index = 133). According to JCR (2019), 70% of the top ten journals on publication with ATZ have an impact factor (IF) above the average (2.754). The impact factor and h-index are the main metrics used to evaluate the scientific journals, commonly associated with the journal's prestige. This demonstrates that research on ATZ is extremely relevant to science, as shown by other scientometric studies (Zyoud et al. 2017; Zyoud 2018; Zuanazzi et al. 2020).

Of the 1009 studies on the herbicide ATZ, 841 of them (83% of the studies) aimed to evaluate the effect of ATZ on non-target organisms through bioassays, and 168 (17%)

addressed other chemical analyses such as identification and quantification of the herbicide in aquatic and terrestrial environments. In general, information on the hazards of pesticides is based on the knowledge of toxicity features and their effects on living organisms (Köhler and Triebkorn 2013) (EPA 1994). In 76% of the studies, animals were used to address toxicity (Fig. 4). Since most of the articles on ecotoxicology research use animals such as fish, insect, and to a lower extent, birds, amphibians, and arachnids in tests, this was expected (Köhler and Triebkorn 2013). Animal experimentation dates back many years ago and is extremely important to evaluate the impact of pesticides on human health via oral exposure, application to the skin, and the respiratory system (Jang et al. 2014). This result follows the publication by Humane Society International (HSI), which reports that the majority of tests to evaluate the toxicity of herbicides before commercial release is done in animals (HSI 2015).

Fig. 4 Number of articles published on atrazine herbicide in the toxicology category of the WoS, filtered by the organism used in the study.

Lytle and Lytle (2001) reviewed the use of plants for toxicity assessment in estuarine environments in compound bioaccumulation tests, biomonitoring, biotransformation of toxic substances, and phytoremediation. The review also indicated that plants tend to be more sensitive to environmental toxins such as pesticides, than animals. However, it was observed that only 15% of the tests with atrazine are performed with plants (Fig. 4). Given the sensitivity of this group to a variety of compounds, including ATZ, there is a need for further studies using them as a test organism. Another approach found in the studies analyzed here was the phytoremediation potential of microalgae and cyanobacteria species to the herbicide ATZ (Tang et al. 1998; Weiner et al. 2004; González-Barreiro et al. 2006; Chelinho et al. 2012).

Microalgae are distributed in three groups: Plantae (green microalgae of the class Chlorophyceae), monera (Cyanophyceae), and protist (other groups of microalgae) (Cavalier-smith 2004; Manoylov 2014). Microalgae are the most commonly used aquatic organism in toxicity studies for testing herbicides (DeLorenzo et al. 2001). Over sixty studies were found (64) where microalgae and cyanobacteria were used as biological models. According to our results, the first article published in the toxicology category from WoS using microalgae was in 1981 and assessed the accumulation and toxic effects of ATZ in the green microalgae *Chlorella vulgaris* (Veber et al. 1981) (Fig. 5). From 1981 to 1999, 18 articles were published using microalgae as a biological model, and 46 articles from 2000 to 2019. The largest

amount of published data occurred in recent years, from 2011 to 2019, where the number of publications doubled (Fig. 5). This boost in knowledge published mainly from the years 2000s explains the lack of information existent previously on the effects of atrazine on aquatic organisms (Van Der Kraak et al. 2014).

Fig. 5 Cumulative number of articles on atrazine herbicide using microalgae and cyanobacteria as test organisms in the toxicology category from WoS.

All of the studies with microalgae and cyanobacteria were performed in laboratory settings, probably since it is possible to simulate what can happen in aquatic environments with reasonable approximation and it is easier to establish a cause and effect relationship (Zagatto and Bertolotti 2008). But it is important to keep in mind that these studies were focused on the environmental impact of the pesticide. Although tests in the field using microalgae and cyanobacteria are important for environmental protection, they are uncommon (Moreira-Santos et al., 2004). Determining environmental impacts caused by contaminants based only on laboratory tests, however, can lead to wrong decisions regarding the legislation on this topic (Moreira-Santos et al., 2004). According to Chappie and Burton (2000), field tests allow evaluation of the effects of contaminants under natural conditions without discarding the biological, chemical, and physical processes of the environment. An assessment under local conditions is useful to reduce uncertainties, complement laboratory data, and to contextualize it ecologically (Castro et al. 2003; Casabé et al. 2007). Among the articles analyzed in our study, 83% performed toxicity bioassays where microalgae were exposed to sub-lethal concentrations of ATZ (Fig. 6). Of these bioassays, 77 % were acute tests aimed to assess the sensitivity of these organisms to ATZ by determining the EC50 (effective concentration capable of immobility on 50% of exposed organisms) based on parameters as growth inhibition (e.g., Baxter et al., 2016) or chlorophyll fluorescence (e.g., Vallotton et al. 2008; Didur et al. 2012). These are the parameters typically measured in tests with microalgae and cyanobacteria (Lewis 1995; Prado et al. 2009). According to Rand and Petrocelli (1985), acute toxicity tests are the most common since these evaluate severe and rapid responses from organisms as to EC50. The others 23% were chronic bioassays analyzing sub-lethal effects of atrazine in the microalgae and cyanobacteria. About 11% of the articles that evaluated the effects of ATZ on microalgae and cyanobacteria used biomarkers (Fig. 6). Biomarkers are responses that organisms manifest when exposed to some xenobiotic compound and incorporate a series of parameters (e.g., biochemical, behavioral,

physiological) at the individual level. They started to be used among toxicological tests in 1997 (Blaise and Gagné 2009), probably the reason why there are not many articles published using biomarkers alone to evaluate the effects of ATZ. In the articles compiled, the effects of the pesticide ATZ on the activities of the enzymes catalase (CAT), glutathione s-transferase (GST), superoxide dismutase (SOD), ascorbate peroxidase (APx), and glutathione peroxidase (GPx) were the biomarkers most used (Lozano et al. 2014; Esperanza et al. 2015; Esteves et al. 2018). ATZ causes oxidative damage in microalgae, and these enzymes are responsible by the antioxidant defense of these organisms. Measuring the antioxidant response and oxidative damage shows the degree of stress of microalgae when exposed to sublethal concentrations of pollutants (Esteves et al. 2018). Of the published articles, 6% used toxicity bioassays on EC50 combined with the use of biomarkers (Fig. 6). Studies of this nature complement each other, bringing more information about the compound. The toxicity bioassays show an irreversible cause-effect assessment, and, through the use of biomarkers, the sublethal effects of contaminants in low doses or concentrations can be determined, therefore having a response of high ecological relevance. (Prado et al. 2009).

Fig. 6 Types of analysis used on microalgae and cyanobacteria to evaluate the toxicity and effects of atrazine.

The choice of the microalgae species is very important for the toxicity test and requires some criteria such as taxonomy, ecological preferences, and physiological requirements (Lewis 1995). In this sense, Chlorophyta and Bacillariophyta are present in 61% and 24% of published studies, respectively (Fig. 7). Generally, chlorophyceae (Chlorophyta) is the most common in ecotoxicological tests, being the first class chosen for such studies, since 1960 (Nalewajko and Olaveson 1998), due to its sensitivity to different pollutants (DeLorenzo et al. 2001). Chlorophyceae and cyanobacteria are recommended as model organisms by the Organization for Economic Cooperation and Development (OECD) test guideline. Regarding diatoms, these have different tolerances to water quality parameters, which have contributed to their use to assess the health of aquatic environments since 1900 (Chintapenta et al. 2018). Two other groups that also appear as test organisms are cyanobacteria and haptophytes, in 13% and 2% of the works, respectively (Fig. 7). Although cyanobacteria are a group widely used in tests of this nature (Hornstrom 1990) they were not the species that stood out in the articles evaluating the toxicity of ATZ, perhaps due to their resistance to this herbicide (Lockert et al. 2006; Chalifour et al. 2016). As already mentioned,

sensitivity to xenobiotics is an important feature in the choice of the organism. The haptophyte phylum is essentially marine and, generally, atrazine has been detected at lower levels in marine or estuarine environments due to dilution and degradation (Graymore et al. 2001). This may explain the lack of interest in the use of marine species, giving greater relevance to algae from continental environments.

Fig. 7 Taxonomic classification of microalgae and cyanobacteria species used to evaluate the toxicity and effects of atrazine.

Conclusion

This scientometric study provides an overview of the increase of studies on atrazine from 1959 to 2019, focusing on microalgae and cyanobacteria as biological models of toxicity testing. The USA, China, and Canada are the countries that published the most on this herbicide. It is a topic of interest to several countries. The articles have been published in journals with a good impact factor and relevant in the area of toxicology. Animals are the most used organisms in ecotoxicological bioassays with this herbicide. Microalgae studies have grown, but they are still scarce.

We have also identified gaps in the field, such as the low exchange between research groups from different countries, a high number of studies with terrestrial organisms in comparison to aquatic, and that none of the studies with ATZ and microalgae were performed in the field. These findings could help prioritize future research efforts related to atrazine toxicity.

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Declarations

Ethics approval and consent to participate.

Not applicable in this section.

Consent for publication.

Not applicable in this section.

Availability of data and materials.

The authors declare that (the/all other) data supporting the findings of this study is available within the article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

MSC and FGB conceived and designed the study. MSC conducted the literature search. MSC and FGB were involved in the analysis and interpretation of data. MSC and FGB drafted the manuscript. The study was supervised by FGB, PSG, CMDGM and JZ. All authors read and approved the final manuscript.

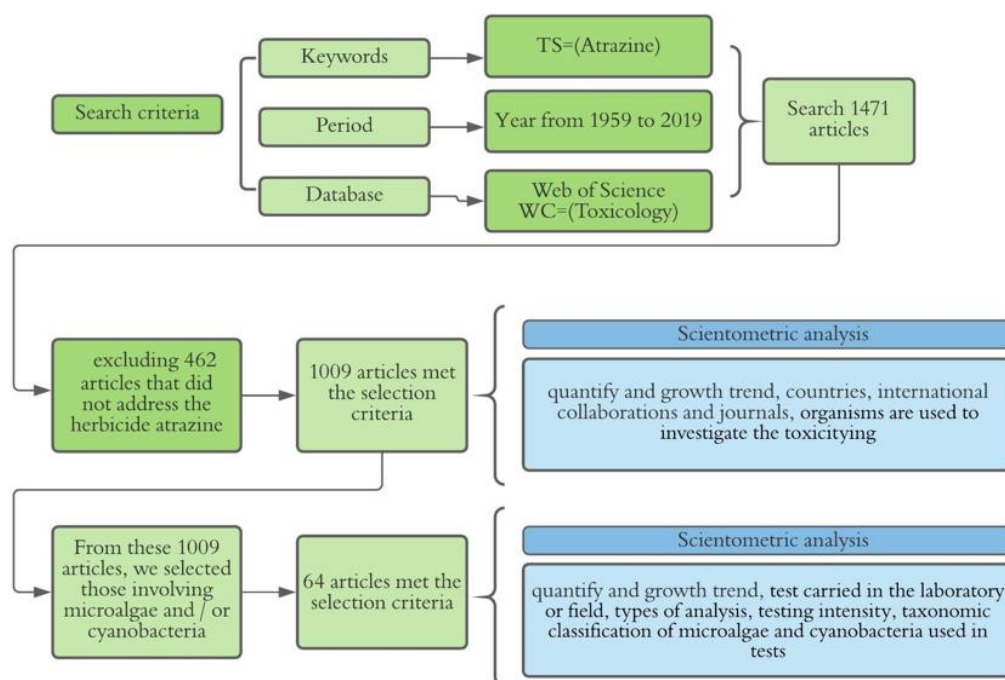


Figure 1. Research methodological framework.

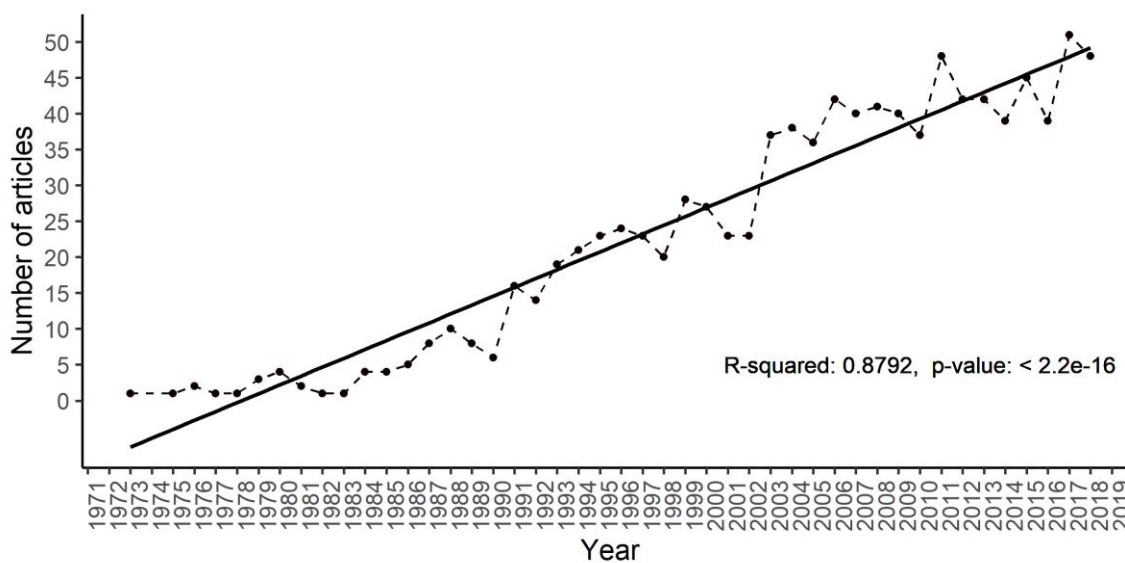


Figure 2. Number of articles on atrazine (ATZ) published between 1959 and 2019 in the toxicology category from WoS.

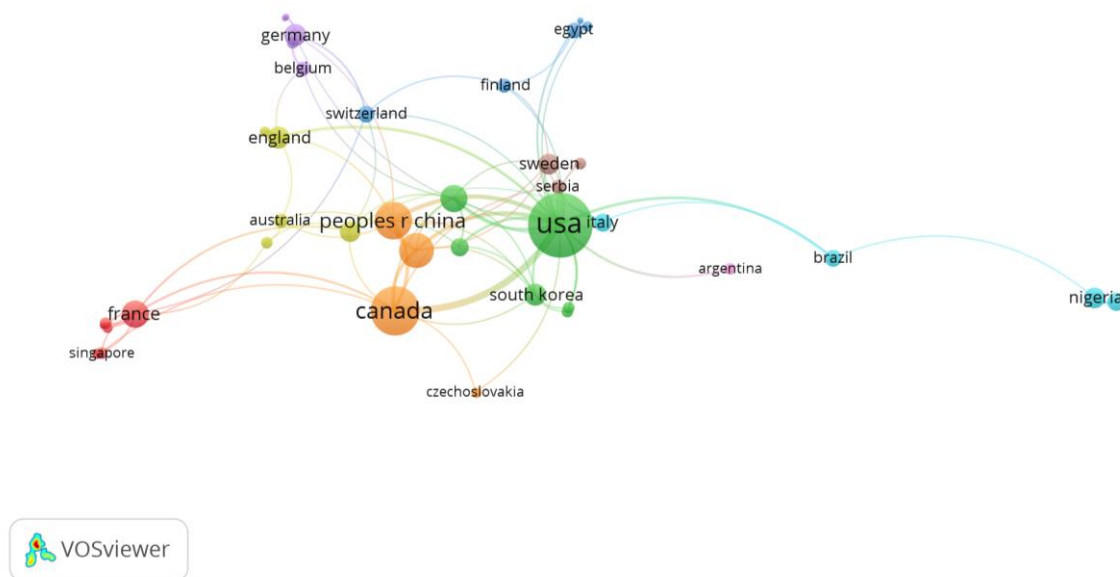


Figure 3. Map of the international collaboration network between countries on atrazine from 1959 to 2019 in the toxicology category from WoS. On the map, the circles and their sizes represent the countries and the number of articles, respectively, and the lines and their thickness represent their cooperation and the degree of collaboration, respectively.

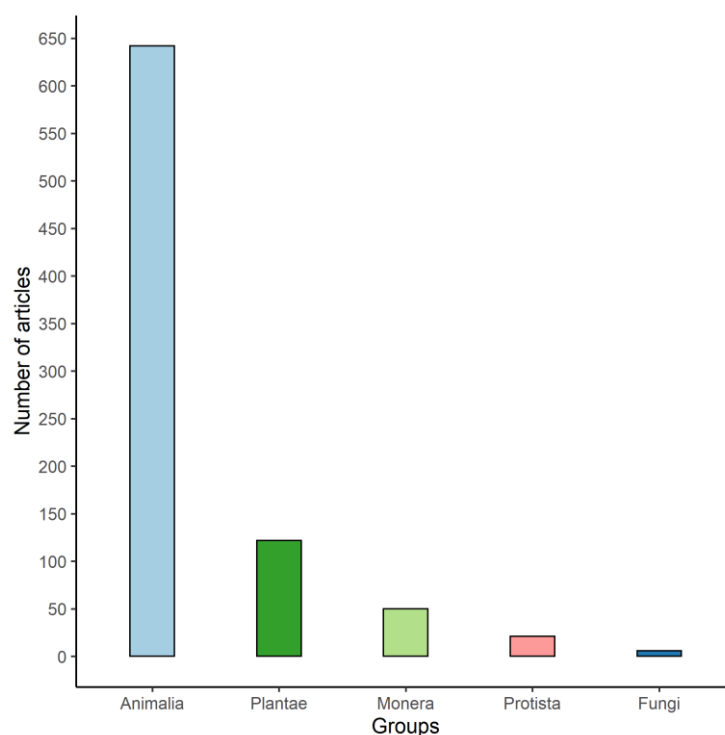


Figure 4. Number of articles published on atrazine herbicide in the toxicology category of the WoS, filtered by the organism used in the study.

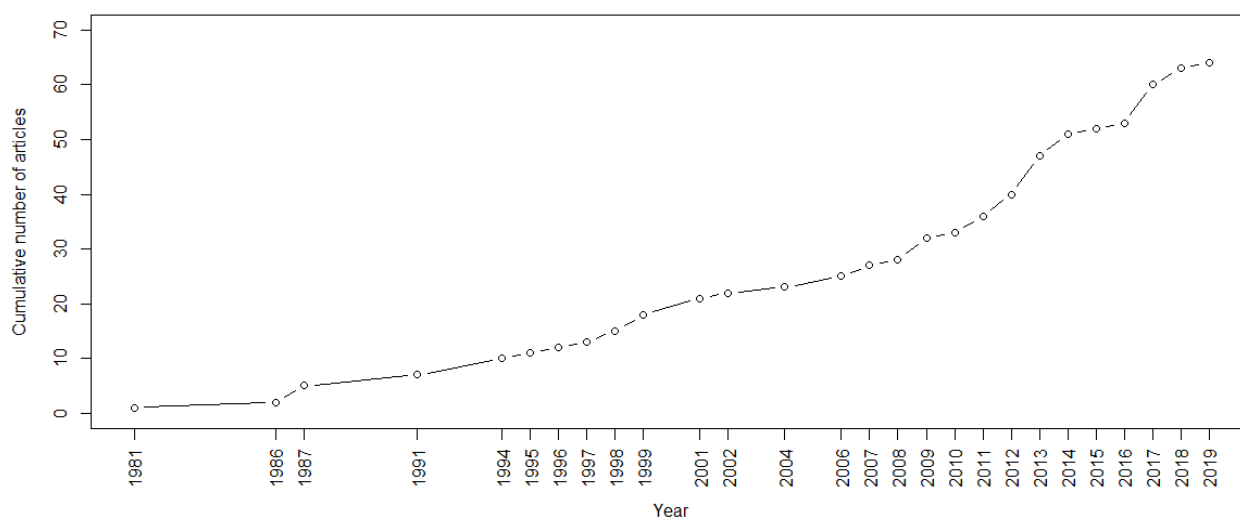


Figure 5. Cumulative number of articles on atrazine herbicide using microalgae and cyanobacteria as test organisms in the toxicology category from WoS.

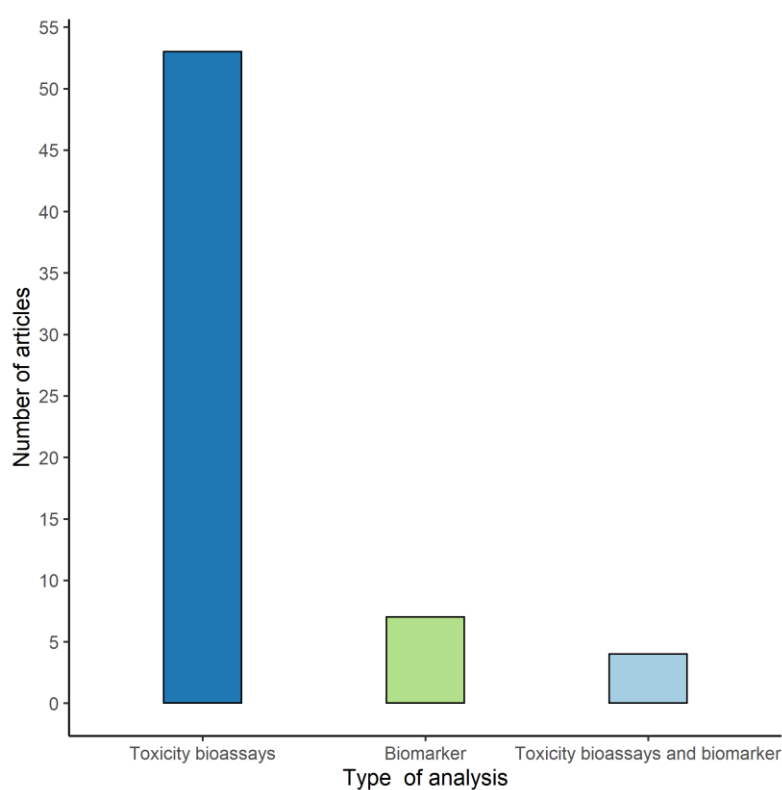


Figure 6. Types of analysis used on microalgae and cyanobacteria to evaluate the toxicity and effects of atrazine.

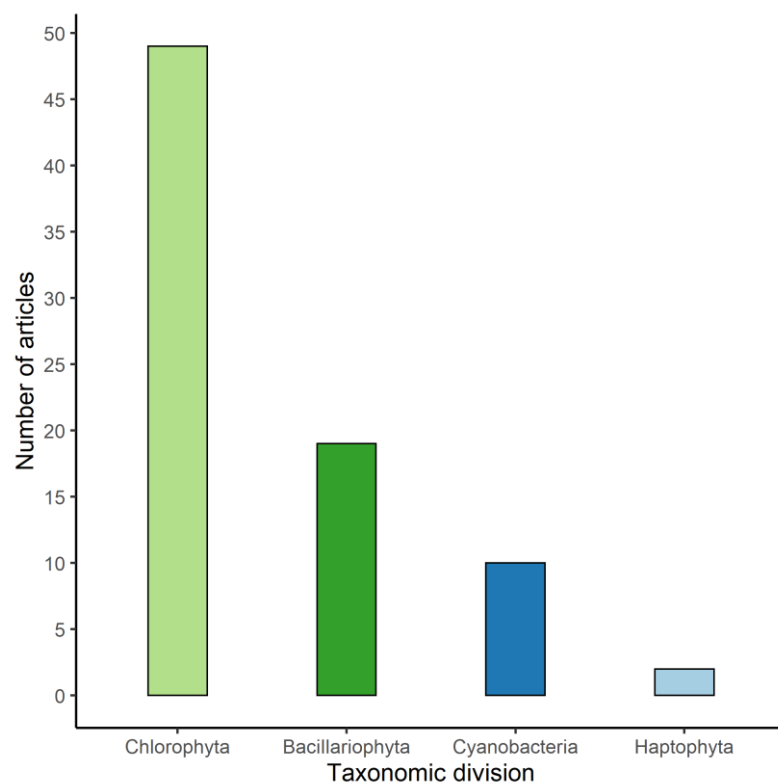


Figure 7. Taxonomic classification of microalgae and cyanobacteria species used to evaluate the toxicity and effects of atrazine

Table 1. Characteristics of the ten most-productive countries that published articles on atrazine between 1959 and 2019 in the toxicology category from Clarivate Analytics Web of Science database.

Rank	Country	Number of articles	SCP	(%) ¹	MCP	(%) ²
1	USA	418	388	93%	30	7%
2	China	83	74	89%	9	11%
3	Canada	75	62	83%	13	17%
4	France	53	44	83%	9	17%
5	Brazil	35	32	91%	3	9%
6	Spain	27	22	81%	5	19%
7	Italy	21	15	71%	6	29%
8	India	19	15	79%	4	21%
9	Serbia	18	14	78%	4	22%
10	Australia	17	12	71%	5	29%
Total³		766	678	-	88	-
Average (%)⁴		-	-	82%	-	18%

USA: United States of America. SCP: number of single country publications. MCP: number of multiple country publications. ¹ Percentages of SCP in respect to total publication in the country, ² Percentages of MCP in respect to total publication in the country, ³ Total number of published articles, ⁴ Average percentage total.

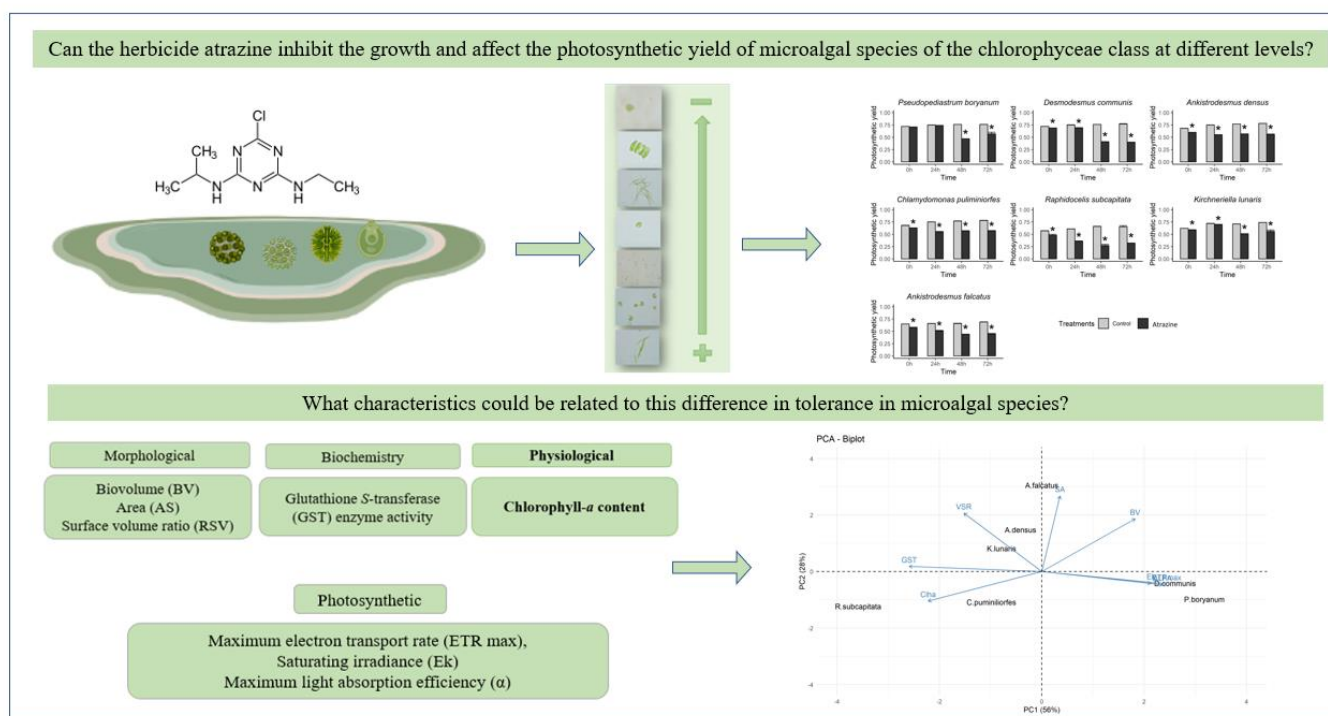
Table 2. Characteristics of the ten journals that most published articles on atrazine between 1959 and 2018 in the toxicology category from Clarivate Analytics Web of Science database

Rank	Journal	Number of articles	H index	IF ¹
1	Environmental Toxicology and Chemistry	181	164	3.2
2	Bulletin of Environmental Contamination and Toxicology	105	68	1.7
3	Ecotoxicology and Environmental Safety	104	121	4.9
4	Archives of Environmental Contamination and Toxicology	93	104	2.4
5	Aquatic Toxicology	69	133	3.8
6	Toxicological Sciences	46	175	3.7
7	Environmental Health Perspectives	34	269	8.3
8	Ecotoxicology	33	86	2.5
9	Reproductive Toxicology	19	106	3.1
10	Toxicology	17	153	4.1

¹ Impact factor of 2019 (JCR)

CAPÍTULO 2

Differential effects of atrazine to chlorophyceae species and association to morphology, photosynthesis, chlorophyll – *a* content and glutathione *S*-transferase activity



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Differential effects of atrazine to Chlorophyceae species and association to morphology, photosynthesis, chlorophyll content and glutathione *S*-transferase activity

ABSTRACT

Atrazine (ATZ) is an herbicide widely used in the control of weeds in crops such as corn, sugar cane, and sorghum. It is often found in aquatic environments, where can endanger non-target organisms such as microalgae. This study evaluated ATZ toxicity to seven different species of Chlorophyceae and the tolerance of the species to the herbicide was related to morphological, photosynthetic, chlorophyll-*a* content and the activity of the glutathione *S*-transferase enzyme (GST). The comparison of EC50 values for growth inhibition indicates higher toxicity of ATZ for *Pseudopediastrum boryanum* and *Desmodesmus communis*, intermediate toxicity for *Ankistrodesmus densus*, *Chlamydomonas puliminiorfes* and *Raphidocelis subcapitata* and lower toxicity for *Kirchneriella lunaris* and *Ankistrodesmus falcatus* (EC50: 38, 42, 66, 103, 248, 1,004 and 1,585 $\mu\text{g L}^{-1}$ ATZ, respectively). Principal component analysis (PCA) with algal characteristics suggested that the ATZ-tolerant algae *P. boryanum* and *D. communis* were positively associated with photosynthetic levels and negatively associated with GST activity and chlorophyll-*a* content. The PCA also suggested that the ATZ-tolerant algae *A. falcatus* and *K. lunaris* were positively associated with morphological parameters, where the larger the cell size, the more tolerant. Although it is difficult to associate a single characteristic of algae as the key factor determining the tolerance to ATZ, the results indicate that the cell area, the photosynthetic parameters (mainly saturating irradiance), chlorophyll-*a* content, and the biotransformation by GST in combination may be potential predictors for the differential tolerance of Chlorophyceae species to the herbicide.

Keywords: algae, pesticide, ecotoxicology

INTRODUCTION

Atrazine (ATZ) is a herbicide in the group of s-triazines, used in the pre and post-emergence control of weeds in crops such as corn, sugar cane, and sorghum (De Albuquerque et al. 2020). It has been commercialized for more than 50 years and due to its efficiency and low cost ATZ has been a widely used herbicide (Singh et al. 2018), being the second most-consumed pesticide in the world (Singh et al. 2018) with records showing a global annual consumption of about 70,000–90,000 tons (Singh et al. 2018). Characteristics of ATZ such as susceptibility to leaching and runoff to water bodies, high solubility, and low degradation rates in the water column (half-life of 168 days) make it frequently found in aquatic ecosystems (Graymore et al. 2001; Singh et al. 2018) where it can be harmful non-target organisms (Graymore et al. 2001).

The mode of action of ATZ in plants is the inhibition of the photosynthesis by competing with plastoquinone B (QB) for the QB-binding site, blocking photosynthetic electron transport (Jensen et al. 1977; Jursinic and Stemler 1983). Therefore, as photosynthetic organisms microalgae are sensitive to ATZ (Castro et al. 2021). Studies show that ATZ reduces the chlorophyll-*a* content of cells, inhibit population growth, and affect the cell viability (Hu et al. 2021; Míguez et al. 2021). As primary producers, microalgae perform important ecological functions in aquatic ecosystems. They are responsible for 60% of the oxygen production (Chisti 2004), assist in ecological balance, material circulation, energy flow and the degradation of some environmental contaminants (Ramakrishnan et al. 2010). Considering that ATZ is widely present in surface waters, it is important to develop studies concerning its toxicological effects on primary producers and understand how this may differ between species. This information may provide ways to better manage the use of this compound to protect aquatic ecosystems (Tang et al. 1997; Dupraz et al. 2016).

Results from literature show differences in the sensitivity of different classes of microalgae when exposed to the same compound (Weiner et al. 2004; Martínez-Ruiz and Martínez-Jerónimo 2018), including ATZ (Tang et al. 1997; Kabra et al. 2014). This may be related to cell size and consequently to the capacity for bioconcentration, where smaller cells with higher surface area to volume ratios will incorporate more compound and, in general, will be less tolerant to the exposure (Kent and Currie 1995; Weiner et al. 2004). According to Weiner et al. 2004, the use of a greater diversity of classes of microalgae can prevent the correlation of species with cellular parameters. Therefore, for this study we used organisms belonging to one class, Chlorophyceae, to avoid influence of genetic characteristics in the algae responses due to the phylogenetic distance. In this sense, we investigated the relationship of some intrinsic characteristics of seven microalgae species in relation to differences in tolerance to ATZ. Chlorophyceae class constitutes one of the most relevant phytoplankton groups in continental waters (Vieira et al. 2016). The choice of the species was based on different morphological characteristics such as shapes, sizes, and structure of thallus (colonial, unicellular), on the relevance of species to ecotoxicological and biotechnological studies in addition to commercial importance (Martínez-Ruiz and Martínez-Jerónimo 2018; Puerta et al. 2019; Rocha et al. 2021).

We analyzed morphological aspects (biovolume [BV], surface area [SA], and surface volume ratio [RSV]), related with area of adsorption or absorption (Kent and Currie 1995; DeLorenzo et al. 2004), and parameters related to photosynthesis, such as fast light curves (maximum electron transport rate [ETR_{max}], saturating irradiance [E_k] and maximum light absorption efficiency [α]), as well as chlorophyll-*a* (Chl-*a*) content since this pigment is the main target of the ATZ (Antonacci et al. 2018). The activity of the glutathione *S*-transferase enzyme was also analyzed as a biochemical parameter related to

the detoxification process of ATZ (Rea et al. 2009), which may be related to microalgae tolerance to this herbicide.

MATERIALS AND METHODS

Cultivation and pre-cultures of microalgae

The Chlorophyceae species used in the present study were *Ankistrodesmus densus* Korshikov, *Ankistrodesmus falcatus* (Corda) Ralfs, *Chlamydomonas pumilioniformis* Péterfi, *Pseudopediastrum boryanum* (Turpin) E. Hegewald, *Desmodesmus communis* (E. Hegewald) E. Hegewald, *Kirchneriella lunaris* (Kirchner) Möbius and *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen & O.M. Skulberg. They were obtained from cultures belonging to the Freshwater Microalgae Cultures Collection of the Biological Sciences Institute at the Federal University of Rio Grande, FURG, Brazil, seen **Figure 1**. Algae images were obtained using a light microscope (Nikon Eclipse Ni DS-Fi 2, Japan) with 400x magnification, digital camera (Sight DS-U3, Japan), and NIS-Element F 4.00.00 software.

Each microalgae species was cultivated separately under axenic conditions in WC culture medium (Guillard and Lorenzen 1972) made in distilled water (autoclaved at 121 °C, 20 min, pH 7.0) in 500 mL Erlenmeyer flasks (termed as mother culture). The microalgae were maintained at a light intensity of 130 $\mu\text{mol}/\text{m}^2/\text{s}$ photons, 12h:12h light/dark cycle, and 23 ± 2 °C. All tests started when the initial cell concentration of algae reached 10^5 to 10^6 cells mL, determined by counting using a Neubauer chamber with 40x magnification. Two separate aliquots of 1 mL obtained from the “mother culture” were added to two Erlenmeyers filled with 49 mL of culture medium (1 mL in each Erlenmeyer). These new cultures, named as precultures, were allowed to grow until the exponential phase (around 3 days) in the same conditions of “mother culture”. The preculture from one Erlenmeyer was used for ATZ toxicity tests based on growth inhibition, and to evaluate ATZ effects on

maximum photosynthetic yield of algae. The second preculture was not submitted to ATZ exposure and were used to evaluate the morphology, photosynthesis, Chl-*a* content, and GST activity of microalgae. The procedure was repeated for each microalgae species. The whole experimental design is shown in [Figure 2](#).

ATZ exposure and growth inhibition

Toxicity tests with ATZ were carried out following OECD guideline 201 (OECD, 2011). The tests were performed in transparent glass Erlenmeyer flask (100 mL) filled with 50 mL of experimental medium, which was the WC culture medium without ATZ (control), or with ATZ diluted in 0.01% of dimethyl sulfoxide (DMSO) solvent. Inoculated with 10⁴ cells mL of exponentially growing cultures, the following treatments were performed: a control group without the DMSO solvent (exposure medium only), a control with the solvent (exposure medium + 0.01% DMSO), and the treatments with ATZ herbicide (Sigma-Aldrich, purity > 98%) with concentrations of 5, 12.5, 32, 80, 200, 500, or 1250 µg L⁻¹ (exposure medium + ATZ herbicide + 0.01% of DMSO). The tests lasted 72 h. All treatments were performed with 5 replicates and were maintained in the same conditions described for cultivations and pre-cultures. Culture flasks were gently shaken (manually) twice a day until the end of the experiment.

The growth inhibition rate was estimated by optical density (OD) at 750 nm using a spectrophotometer (Shimadzu UV-mini, 1240), with measurements at the beginning (0 h) and the end (72 h) of the experiment, according to the Equation 1.

Equation 1:

$$GRI = (\ln df - \ln di) / (tf - ti)$$

Where, *GRI* = Growth rate inhibition; *df* = the final optical density; *di* = the initial optical density; *tf* = final time; and *ti* = initial time.

Microalgal growth curves were adjusted using the package “drc: Analysis of Dose-Response Curves (drc)” in R software. This package fits the data to a three-parameter logistic regression model. The upper limit of the curve was normalized to 1 and the lower limit to 0. From the curves, we estimated the effective concentrations that inhibited the growth of microalgae by 30% (EC30) and 50% (EC50). To compare the EC50 values, the CompParm function of the drc package was used. This function performs a t-test between the EC50 values ($p < 0.05$).

Morphological, photosynthetic, Chl-a content, and enzymatic characteristics of microalgae (non-exposed to ATZ)

Thirty cells of each microalgae species were randomly selected from an aliquot of 1 mL collected from the non-exposed precultures to determine their cell volume and the surface area. The length and width measurements were estimated using a microscope (Olympus BX53) with a camera attached (Olympus DP72). The biovolume of each species was calculated using geometric shapes and equations already described by Hillebrand et al. (1999).

To evaluate the photosynthetic parameters, we use the PhytoPAM fluorometer (Heinz-Walz, Germany). An aliquot of 3 mL of a 50 mL preculture was dark adapted for 20 min and the rapid light saturation curve (RLC) determination initiated. It was performed by increasing pulses of photosynthetically active radiation (PAR) applied to the samples at regular intervals (20 ms). Relative electron transport rate (rETR) values for each light intensity were calculated from the multiplication of PAR ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) by the effective quantum yield. The curves were adjusted according to the mathematical model proposed in Platt et al. 1981 and the parameters of angular coefficient at the very beginning of the RLC (α), maximum electron transport rate (rETR_{max}), and saturation irradiance (E_k) were obtained.

To measure the GST enzyme activity, 0.5 L of microalgae were added in Erlenmeyer flasks filled with 4.5 L of culture media under constant aeration (CO₂) and left over 8–9 days to obtain a high number of cells sufficient for the dosage. The cells (5 L) were centrifuged at 4,000 rpm and the pellet was collected and homogenized. For homogenization, the pellet (4 g of wet biomass) was resuspended with 4 mL of sodium phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 14 mM DTT dithiothreitol (DDT), and 1 mM ethylene diamine tetraacetic acid (EDTA) and was macerated on ice using a mortar and pestle (4 mL buffer per g of tissue) according to Monferran et al. 2007. Alumina (Al₂O₃) (Sigma-Aldrich) was added to the biomass to aid in the steeping process. After that, the macerated fraction was centrifuged at 9,000 ×g (4 °C) for 20 min, and the supernatant was collected and stored at –80 °C for subsequent GST activity and protein content analysis. The GST activity was analyzed according to Keen et al. 1976. It was used 96-well microplates with a final volume of 260 µL, with 100 mM of potassium phosphate buffer (pH 7.0), 1 mM of 1-chloro, 2,4-dinitrobenzene (CDNB), 1 mM of glutathione (GSH), and sample were added. This determination was performed in triplicate and the absorbance was recorded at 340 nm, 30 °C for 3 min. The total protein concentration was determined using a biuret-based method (total protein; Labtest. Kit, MG, Brazil) (Gornall et al. 1949). The GST activity was expressed in mU mg⁻¹ protein where one unit (U) of GST is defined as the amount of enzyme able to form 1 µmol of the product 2.4 dinitrophenyl glutathione per min. The activity of the GST enzyme was calculated as follows (Equation 2).

Equation 2:

$$\text{GST activity (mU mg protein}^{-1}\text{)} = (\Delta A_{340} / \text{min} \times \text{volume of reaction (mL)}) / e_{mM} \times \text{volume of sample (mL)}$$

Where, ΔA_{340} = final A_{340} read – initial; A_{340} read; min = minute; and e_{mM} (extinction coefficient for CDNB conjugate at 340 nm using 96-well microplate) = 5.3 mM⁻¹ cm⁻¹.

For the determination of Chl-*a* concentration, aliquots of 5 mL of preculture microalgae (non-exposed to ATZ) were sampled. They were centrifuged at 4000 rpm for 10 min. Then supernatant was removed, and 3 mL of methanol was added to the pellet and left over 24 h for Chl-*a* extraction. After this period, the samples were centrifuged (4000 rpm for 10 min), the supernatant was transferred to the spectrophotometer cuvette and readings at wavelengths of 663 and 750 nm were conducted. Methanol was used as blank. Concentration of Chl-*a* ($\mu\text{g L}^{-1}$) was calculated according to Equation 3 (Mackinney, 1941).

Equation 3:

$$\text{Chl} - a \ (\mu\text{g } \mu\text{m}^{-3}) = \left[\left(\frac{\text{abs (663} - 750) \times 12,63 \times \text{metanol volume (L)}}{\text{sample voume (L)}} \right) / df \right] / BV(\mu\text{m}^3)$$

Where, A665 = absorbance at 665 nm; A750 = absorbance at 750 nm; 12.63 Chlorophyll extraction correction factor; df = final density of microalgae at 750 nm; and BV= microalgae biovolume (μm^3).

Results were presented as mean $\pm$ SE. Differences between algae species for the morphological parameters and photosynthesis were evaluated using the Kruskal–Wallis tests followed by Dunn’s test ($p > 0.05$). Differences between algae species for the GST activity and Chl-*a* were compared using ANOVA followed by the Tukey test ($p > 0.05$). We used a principal component analysis (PCA) with z-score normalization to assess the relationship between parameters related to morphology, photosynthesis, and GST activity of microalgae species.

Effect of ATZ in the maximum photosynthetic yield of algae

The effect of ATZ in the maximum photosynthetic yield of microalgae was evaluated daily in a 72 h exposure experiment with the following groups: (1) control (exposure medium only); (2) control group with solvent added (exposure medium + 0.01% of DMSO); and (3) ATZ treatment in the concentrations of $209 \mu\text{g L}^{-1}$ for *A. falcatus*, $58 \mu\text{g}$

L⁻¹ for *K. lunaris*, 31 µg L⁻¹ for *R. subcapitata*, 42 µg L⁻¹ for *C. pulimniorfes*, 30 µg L⁻¹ for *A. densus*, 18 µg L⁻¹ for *P. boryanum*, 21 µg L⁻¹ for *D. communis*. The ATZ concentrations chosen for this experiment was the concentrations that caused 30 % inhibition grow (EC30) estimated for each species. Thus, we used equipotent concentrations. The experiment was also performed with five repetitions per group and maintained in the same conditions of light, photoperiod, and temperature described in for the “mother culture”. The experiment was prepared in 100 mL glass Erlenmeyer flasks filled with 50 mL of the experimental medium and gently shaken twice a day manually until the end of the experiment. From each sample, a 3 mL aliquot was pre-adapted to the dark (20 min) to allow complete oxidation of the reaction centers of photosystem II (PSII), before the measurements of maximum quantum yield (ΦM).

The ΦM was determined in a PhytoPAM fluorometer (Heinz-Walz, Germany) at room temperature according to Lombardi and Maldonado (2011), where the maximum quantum yield of PSII represents the difference between the maximum and minimum fluorescence divided by the maximum fluorescence ($F_v / F_m = (F_m - F_o) / F_m$) (Schreiber 2004). The effects of ATZ on the photosynthetic yield of each microalga were compared using the Kruskal–Wallis tests followed by Dunn’s test. Comparisons were made between the different treatments to control, including the solvent control (data not shown) every 24 h. The software R was used for all analyses (R Core Team, 2019).

RESULTS

Growth inhibition effects of ATZ in microalgae

The species *P. boryanum* and *D. communis* showed lower EC50 values than the other species ($p < 0.05$) indicating that these two species are less tolerant to ATZ exposure. In sequence, *A. densus*, *C. pulimniorfes*, and *R. subcapitata* presented intermediate tolerance, whereas *K. lunaris* and *A. falcatus* were the more ATZ tolerant species. The EC50 values of

these last two species were higher than the EC50 values estimated for the others ($p < 0.05$) (Table 1).

Morphological, photosynthetic, chlorophyll-a content and enzymatic characteristics of microalgae (non-exposed to ATZ)

As shown in Table 2, the species *A. falcatus* presented greater BV, SA, and VSR compared to the other microalgae, while the species *R. subcapitata* presented the lowest values of BV and SA and *P. boryanum* the lowest VSR. For the Chl-*a* content, the microalgae *R. subcapitata* had the highest value while the *D. communis* had the lowest. *R. subcapitata* also showed the greatest detoxification capacity via GST activity.

The Figure 3 shows that the two main components of the PCA explained 84% of the overall variation. The first main component (PC1) expressed 56% of the total variance of the data and the second main component (PC2) expressed 28%. The less ATZ-tolerant species *D. communis* and *P. boryanum* were positively associated with PC1. For this axis, the photosynthetic parameters and GST activity were the most representative, where ETR max, α , and E_k were positively associated, and the Chl-*a* content and GST enzyme activity was negatively related (Table 3). Of the parameters related to cell size, SA was the least related to PC1. The microalgae *R. subcapitata*, which was one of the most ATZ-tolerant species (Table 1) presented the highest Chl-*a* content and the highest activity of the GST enzyme among the studied algae. It also presented a negative association with PC1 which, in turn, was negatively related to the Chl-*a* and GST activity and positively associated with the photosynthetic parameters (ETR max, E_k and α) (Table 3). On the other hand, the microalgae *A. falcatus* and *K. lunaris*, which were the more tolerant to ATZ (Table 1), were positively associated with PC2 (Figure 3). This association was particularly evident for *A. falcatus*. For PC2, the parameters related to cell size (BV, SA, and VSR), were the most representative (Table 3).

Effect of ATZ in the maximum photosynthetic yield of algae

All microalgae exposed to their respective EC30 had a significant reduction ($p < 0.05$) in the photosynthetic levels as denoted by a reduction in the maximum quantum yield (ΦM). Except for *P. boryanum*, for all species the effect was observed from the beginning of the exposure, specifically at the first sampling time, which was performed after 20 min in the dark condition (Figure 4). The major reductions in ΦM were observed for *R. subcapitata*, which fell over the 72 h of evaluation, and *D. communis*, which showed a sharp drop after 48 h of exposure. Both species showed inhibitions of 49% and 48% in ΦM at 72 h of exposure to ATZ, respectively (Table 4).

DISCUSSION

The present study evaluated the tolerance of seven Chlorophyceae species to ATZ and associated this with intrinsic morphological, photosynthetic, physiological (Chl-*a*), and enzymatic characteristics of the different algae species. In agreement with other studies (e.g.: Magnusson et al., 2008), we observed a negative effect of ATZ in the growth and photosynthetic rate of microalgae. It is expected given the mode of action of ATZ as an herbicide.

As seen in Table 1, the ATZ EC50-72h based on growth inhibition was between 38 to 1,585 $\mu\text{g L}^{-1}$. As far as we know there are no previous records about ATZ EC50 for the microalgal species tested in our study. However, there are results for other Chlorophyceae species such as *Scenedesmus arcuatus* (EC50 = 48.95 $\mu\text{g L}^{-1}$), *Scenedesmus quadricauda* (EC50 = 86.37 $\mu\text{g L}^{-1}$) (Chin et al. 2019), *Clamydomonas reinhardtii* (EC50 = 77.6 $\mu\text{g L}^{-1}$) (Harshkova et al. 2021) and *Selenastrum capricornutum* (EC50 = 23 $\mu\text{g L}^{-1}$) (Zhao et al. 2018). In these studies microalgae were exposed to ATZ over 72 h or 96 h and the endpoint analyzed was growth inhibition. Comparing the EC50s reported in the Table 1 and in the

studies mentioned above, the species *K. lunaris* and *A. falcatus* are the most ATZ-tolerant Chlorophyceae with EC50 of 1,004 and 1,585 $\mu\text{g L}^{-1}$ ATZ, respectively.

Responses of phytoplankton to ATZ at different concentrations can be particularly important to higher trophic levels since their growth and abundance can determine the potential productivity of the entire ecosystem. The difference in tolerance level to xenobiotics for certain species is a problem that, in the case of microalgae, can harm microalgal communities and aquatic ecosystems by selective pressure (Lopez-Rodas et al. 2001; Marv et al. 2010). The environmental pollution can cause population explosion or community dominance by tolerant microalgae species in certain water bodies and reduce the algal species diversity of the local ecosystem (Lopez-Rodas et al. 2001; Marv et al. 2010; Dupraz et al. 2016).

ATZ has its limit in surface water regulated in some countries, being 2 $\mu\text{g L}^{-1}$ in Brazil (BRASIL, 2005), 1.8 $\mu\text{g L}^{-1}$ in Canada (PMRA, 2017), 13 $\mu\text{g L}^{-1}$ in Australia (APVMA, 2008), 15 $\mu\text{g L}^{-1}$ in the United States (USEPA 2016), and 3 $\mu\text{g L}^{-1}$ in China (He et al. 2019). However, studies conducted in aquatic environments have shown the presence of ATZ at concentrations of 15 $\mu\text{g L}^{-1}$ in Brazil (Sousa et al. 2016), higher than 30 $\mu\text{g L}^{-1}$ in Portugal (Cerejeira et al. 2003), 100 $\mu\text{g L}^{-1}$ in the Midwest of the USA (USEPA 2016) and concentrations above 1,000 $\mu\text{g L}^{-1}$ in other parts of the world (Graymore et al. 2001). Although these extremely high levels are considered rare or anomalous, it is important to note that they exceed the regulated concentrations and are very close to the ATZ EC50 estimated for the microalgae in this study.

Results presented here also show that the degree of effect of ATZ in growth inhibition were significant different between the algae species, suggesting a differential tolerance/sensitivity between them. In this sense, *D. communis* and *P. boryanum* were the more sensitive compared to the other Chlorophyceae. These species are cosmopolitan,

predominant in continental aquatic environments (Shubert et al. 2014) and are used as test organisms in ecotoxicological studies (e.g. Da Luz et al. 2016; Puerta et al. 2019). Given their sensitivity and considering their extensive natural occurrence, it would be interesting to consider the possibility of the inclusion of those two species in future legislation, like the *R. subcapitata* in the US regulation. On the other hand, the use of tolerant microalgae has gained prominence in bioremediation studies as an efficient and low-cost process (Nie et al. 2019; Kalra et al. 2020). In this sense the microalgae *A. falcatus* and *K. lunaris* presented the higher resistance to ATZ. Besides that, they are easy to cultivate, are abundant in the phytoplankton and cosmopolitan. Thus, we suggest that these microalgae to be tested as potential bioremediators of ATZ.

There are other studies comparing the tolerance of different classes of microalgae (Chlorophyceae and Diatoms; Chlorophyceae and Cyanobacteria) to different herbicides, including ATZ (Kent and Currie 1995; DeLorenzo et al. 2004; Moore et al. 2016); however none of them investigated the photosynthetic parameters and the activity of the biotransformation GST enzyme as possible predictors. Through the analysis of PCA, we observed that the high levels found in the photosynthetic parameters and the low Chl-*a* content and low activity of the GST enzyme were associated to the least ATZ-tolerant microalgae, the *P. boryanum*. Thus, we suggest that these characteristics may be correlated of this response (sensitivity).

The increased tolerance of some species of microalgae and plants to the ATZ herbicide has been associated with mutations that may occur in the chloroplast gene protein *psbA*, leading to a decreased binding of atrazine and QB (Lu et al. 2019; Melero-Jiménez et al. 2021). However, this tolerance may be accompanied by pleiotropic effects. The same mutation that causes tolerance to ATZ might also increases sensitivity to other classes of herbicides (Murphy and Tranel 2019) in addition to cause structural and functional problems

in the PSII complex (Lu et al. 2019; Melero-Jiménez et al. 2021). For example, Holt et al. (1981) observed that biotypes more tolerant to ATZ as *Senecio vulgaris* L. show lower levels of light absorption and electron transfer rates when compared to sensitive species. Wilski et al. (2006) tested various mutants of the D1 protein of PSII by transforming the microalgae *Chlamydomonas reinhardtii* and noticed that some mutants had their photosynthesis reduced about 60% compared with the wild type. Melero-Jimenez et al. (2021) showed that herbicide-resistant microalgae showed alterations in photosynthetic performance, with a significant decrease in the herbicide-resistant than in the wild-type cells. We observed the same pattern in the microalgae *D. communis* and *P. boryanum* (less tolerant species), where these showed higher levels of photosynthetic rates, unlike *A. falcatus* and *K. lunaris* (more tolerant species). We did not analysed any genetic aspect; but we hipothetize that the differences in tolerance to ATZ observed in the Chlorophyceae species used in this study can be related to specific genes that codify key components of the photosystem (e.g. *psbA*).

The two less tolerant species (*P. boryanum* and *D. communis*) presented the lower levels of GST activity whereas the more tolerant presented the higher levels. The activity of GST can be an important component of resistance to ATZ. The GST is a phase II biotransformation enzyme that plays an important role in the detoxification of many xenobiotic compounds (Dos Santos et al. 2021) including ATZ (Cummins et al. 2011). Studies show that this enzyme is one of the main enzymes of the defense system of microalgae (Chatzikonstantinou et al. 2017). The microalgae *P. boryanum* also presented the less amount of Chl-*a*. It is known that algae with higher levels of Chl-*a* tend to be more tolerant to ATZ, as this parameter is directly related to the amount of PSII in the cell, which is the main biochemical target of ATZ. Thus, the more photosynthetic targets available in the cell, the more ATZ would be needed to block it (DeLorenzo et al. 2004). It is curious

that *P. boryanum* was the species that showed the greater activity in the parameters related to photosynthesis. A lower amount of Chl-*a* in the antenna complex can increase the efficiency of solar conversion into photosynthesis, thus preventing light from being wasted as heat (Gu et al. 2017). Studies show that both mutant green algae and higher plants show better efficiency in photosynthetic solar energy conversion and productivity when they have lower chlorophyll-*a* content (Pettigrew et al. 1989; Melis 2009; Gu et al. 2017).

Regarding cell morphology, the PCA analysis showed that the highest values of BV, SA, and VSR (morphological characteristics) had greater relationship with the microalgae more tolerant to ATZ. The *A. falcatus*, the species with the highest BV, SA, and VSR values, was the more tolerant species. The second species in ATZ tolerance was *K. lunaris*, which showed high values of SA and VSR but low value of BV. Thus, we suggest that the morphological characteristics may influence the tolerance of these species to ATZ. This result corroborates with others who reported that characteristics such as cell size and morphology influence the tolerance of microalgae to herbicides (Kent and Currie 1995; Tang et al. 1998). For example, cell size can contribute to differences in bioconcentration of ATZ (Kent and Currie 1995; Tang et al. 1997; Tang et al. 1998). Likely, the entry of toxic components into cells and the subsequent response may also be a function of cell size, where smaller cells need fewer internal levels of ATZ to cause inhibition. However, this concept cannot be generalized, the *R. subcapitata* is the smallest algae, with the lowest BV and SA, but was in the group of the most tolerant to ATZ. This may be related to its high GST activity and high Chl-*a* content.

The ATZ EC30 significantly reduced the photosynthetic efficiency in all microalgae. It worth noting that ATZ concentrations that cause 30 % of growth inhibition (EC30) were able to cause similar inhibition (%) of photosynthesis considering the average of the seven species tested (33.4 ± 11.1 %; mean $\pm$ SE). This indicates an important

association between these two parameters. Our results also show that the negative effect of ATZ on photosynthesis manifests itself at the beginning of the exposure to the herbicide and can be worsened over time (72 h). Even considering that algae have different levels of tolerance to ATZ, all the Chlorophyceae species tested in this study presented photosynthesis impairment. Thus, negative effect in growth could be related to the mode of action of ATZ binding to QB-binding niche on the D1 protein and blocking the electron transport in the PSII (Jones et al. 1986; Antonacci et al. 2020). This process results in the consequent inhibition of photosynthesis (Antonacci et al. 2018). Although algae can rapidly recover from exposure to ATZ because of the non-covalent binding of the analyte to the target receptor (Brain et al. 2012), it is possible that long duration exposures cause negative effects in algae community.

CONCLUSION

We showed that Chlorophyceae species have different levels of tolerance to ATZ and that morphological, photosynthetic and Chl-*a* levels as well as activity of the GST enzyme are correlated with this differential tolerance. Less tolerant microalgae have higher values of ETR max, α and E_k and lower values of GST enzyme activity and Chl-*a* content, in addition to being lower in BV, SA and VSR.

Furthermore, even concentrations that can be found in the environment affected growth and photosynthesis in all species tested in this study. Thus, it is urgent to divert resources and efforts to minimize the use of ATZ, since microalgae play important roles for aquatic ecosystems.

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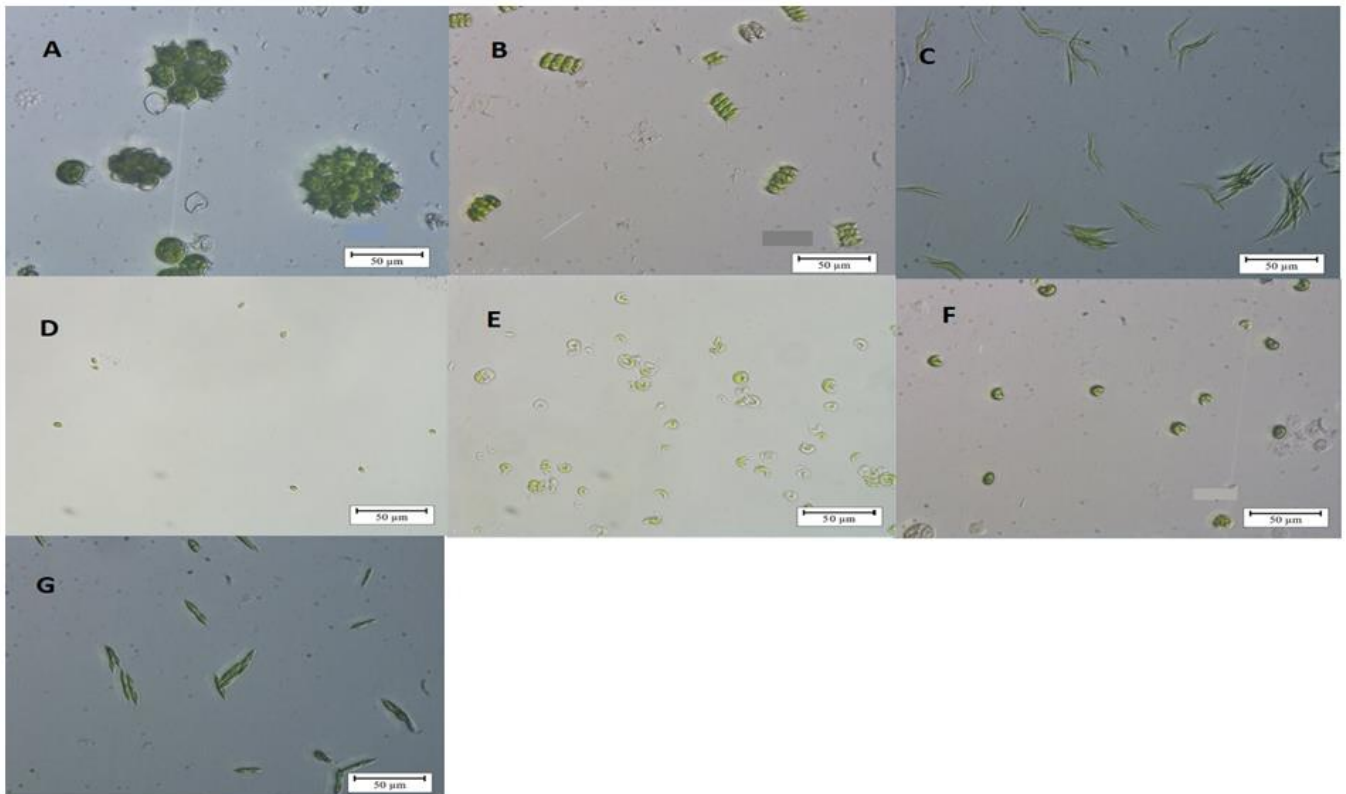


Figure 11. Light microscope images of the Chlorophyceae microalgae species *Pseudopediastrum boryanum* (Turpin) investigated in this study. (A), *Desmodesmus communis* (E. Hegewald) E. Hegewald, (B) *Ankistrodesmus densus* Korshikov, (C) *Chlamydomonas pumilioniformis* Péterfi, (D) *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen & O.M. Skulberg, (E) *Kirchneriella lunaris* (Kirchner) Möbius (F) and *Ankistrodesmus falcatus* (Corda) Ralfs (G). All images with 400x magnification.

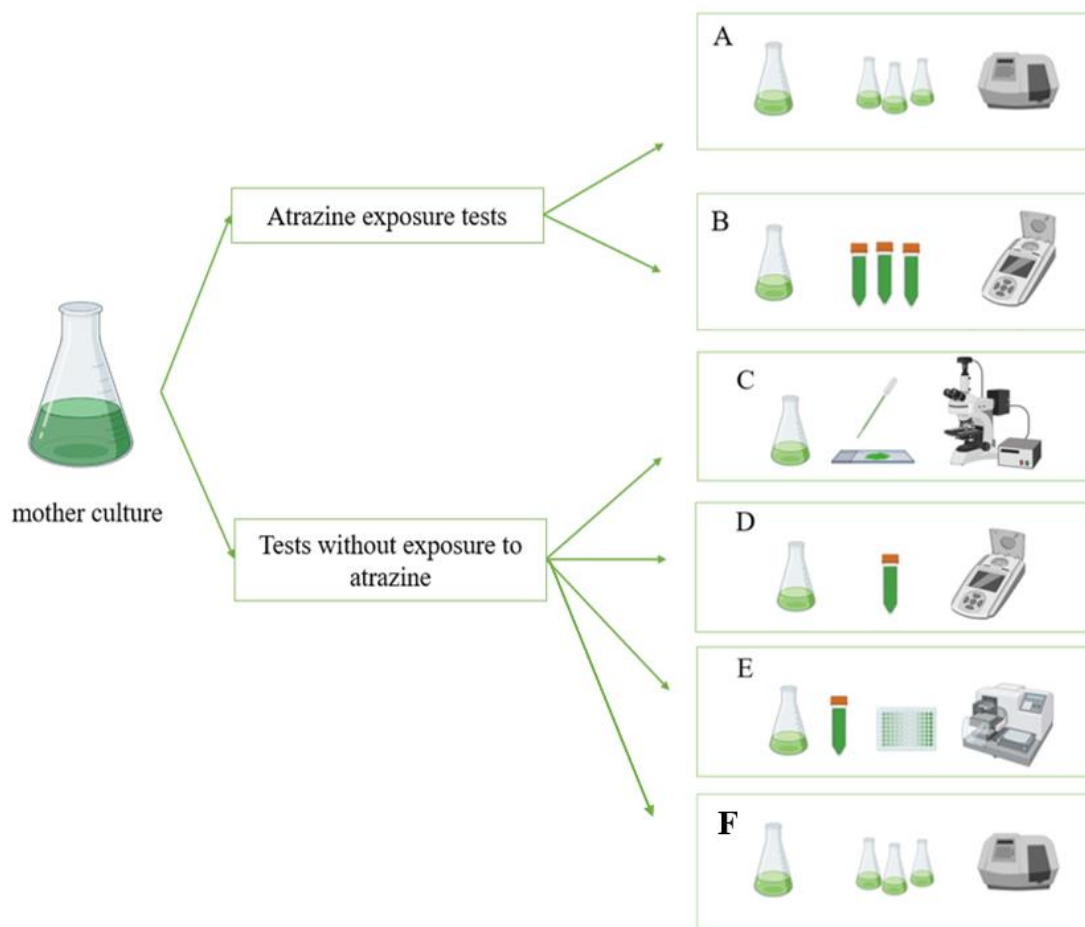


Figure 2. Methodology scheme. Atrazine exposure tests: A - ATZ exposure and growth inhibition (EC50 estimated by optical density at 750 nm using a spectrophotometer according to OECD guideline), B - Effect of ATZ in the maximum photosynthetic yield of algae (The Φ_M was determined in a PhytoPAM fluorometer according to Lombardi and Madonado 2011). Tests without exposure to atrazine: C – Morphological (The length and width measurements were estimated using a microscope with a camera attached, the biovolume was calculated according to Hillebrand et al. 1999), D – Photosynthetic (using a PhytoPAM fluorometer), E – enzymatic (enzyme extraction according to Monferran et al. 2007 and GST activity was analyzed according to Keen et al. 1976) parameters and F - Chlorophyll-*a* content (according to Mackinney, 1941)

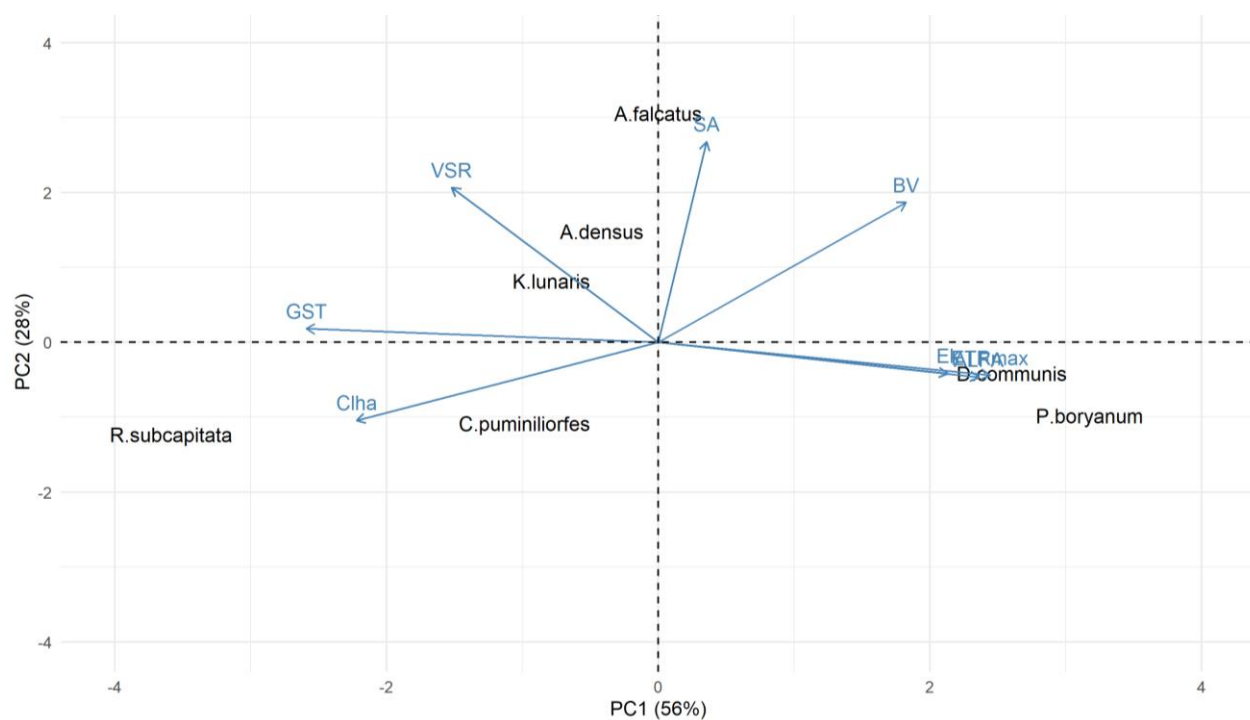


Figure 12 Main component analysis plot showing the relationship among morphological, photosynthetic, chlorophyll content and enzymatic characteristics of Chlorophyceae microalgae species. BV= biovolume (μm^3), SA= surface area (μm^2) e VSR= volume surface ratio, ETR max= Maximum electron transport rate ($\mu\text{mol electrons m}^{-2} \text{s}^{-1}$), α = Light absorption coefficient ($\text{mg C. mg Chl a}^{-1} [\mu\text{mol photons m}^{-2} \text{s}^{-1}]$), E_k = Saturation light index ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$), GST = glutathione *S*-transferase enzyme activity ($\text{mU.mg protein}^{-1}$), Chl-*a* (mg.um^{-3}).

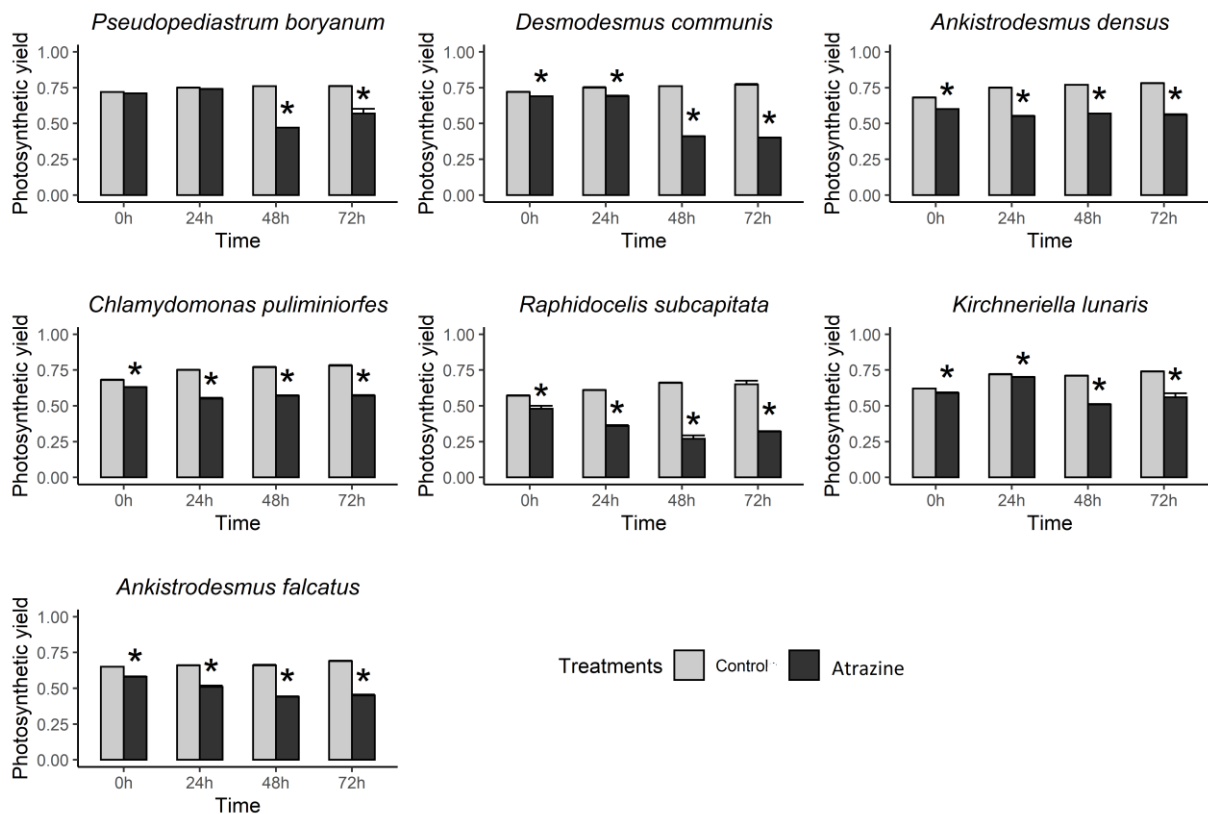


Figure 13. Maximum photosynthetic yield of the microalgae exposed to ATZ (EC30 based on growth inhibition) and control. Values are the average $\pm$ (standard deviation) and different letters represent significant differences (Kruskal Wallis followed by Dunn's test; $p < 0.05$). (*) Represents difference between ATZ and control.

Table 1: Concentration of ATZ ($\mu\text{g L}^{-1}$) necessary to cause 50 % of effect (EC_{50}) in the cellular growth of Chlorophyceae microalgae species during 72 h of exposure (average $\pm$ SE).

Species	72 h EC_{50} ($\mu\text{g L}^{-1}$ ATZ) ^{a *}
<i>Pseudopediastrum boryanum</i>	38 $\pm$ 3 A
<i>Desmodesmus communis</i>	42 $\pm$ 4 A
<i>Ankistrodesmus densus</i>	66 $\pm$ 7 B
<i>Chlamydomonas pulimniorfes</i>	103 $\pm$ 13 C
<i>Raphidocelis subcapitata</i>	248 $\pm$ 55 D
<i>Kirchneriella lunaris</i>	1004 $\pm$ 457 E
<i>Ankistrodesmus falcatus</i>	1585 $\pm$ 635 E

^a Lower EC_{50} values indicates higher inhibitory effect (sensitivity) caused by ATZ.

*Different letters mean significant differences between groups (ANOVA followed by Tukey HSD test; $p > 0.05$)

ATZ = atrazine

Table 2. Summary of cell morphological, photosynthetic and glutathione *S*-transferase activity for Chlorophyceae microalgae species (average $\pm$ SE).

Species	Morphological characteristics			Photosynthetic characteristics			Biochemical characteristics	Physiological characteristics
	BV ^a	SA ^a	VSR ^a	ETR max ^a	α ^a	Ek ^a	GST ^b	Chl- <i>a</i> ^b
<i>P. boryanum</i>	659 $\pm$ 75 A	188 $\pm$ 15 A	0.3 A	292 $\pm$ 1 A	0.75 $\pm$ 0.007	388 $\pm$ 4 A	2 $\pm$ 6 A	1x10 ⁻⁵ $\pm$ 1x10 ⁻⁶ CD
<i>D. communis</i>	256 $\pm$ 23 BC	233 $\pm$ 14 A	1.0 B	284 $\pm$ 1 AB	0.75 $\pm$ 0.003 A	377 $\pm$ 1 AB	3 $\pm$ 1 A	5x10 ⁻⁶ $\pm$ 7x10 ⁻⁷ E
<i>A. densus</i>	336 $\pm$ 27 B	2015 $\pm$ 160 B	6.0 C	179 $\pm$ 4 CD	0.73 $\pm$ 0.005 AB	246 $\pm$ 6 BC	7 $\pm$ 3 A	6x10 ⁻⁶ $\pm$ 9x10 ⁻⁷ DE
<i>C. puliminiorfes</i>	60 $\pm$ 7 D	71 $\pm$ 6 C	1.4 D	168 $\pm$ 2 DE	0.68 $\pm$ 0.012 BC	248 $\pm$ 7 BC	6 $\pm$ 3 A	3x10 ⁻⁵ $\pm$ 9x10 ⁻⁶ BC
<i>R. subcapitata</i>	32 $\pm$ 2 D	58 $\pm$ 3 C	1.8 E	133 $\pm$ 8 E	0.61 $\pm$ 0.017 D	217 $\pm$ 7 C	22 $\pm$ 6 B	2x10 ⁻⁴ $\pm$ 8x10 ⁻⁶ A
<i>K. lunaris</i>	187 $\pm$ 13 C	1124 $\pm$ 79 D	6.0 C	233 $\pm$ 0 BC	0.64 $\pm$ 0.003 CD	365 $\pm$ 2 AB	13 $\pm$ 3 C	5x10 ⁻⁵ $\pm$ 1x10 ⁻⁵ B
<i>A. falcatus</i>	1343 $\pm$ 207 E	8059 $\pm$ 1242 E	6.0 C	168 $\pm$ 2 DE	0.65 $\pm$ 0.007 CD	257 $\pm$ 5 BC	6 $\pm$ 3 A	3x10 ⁻⁵ $\pm$ 2x10 ⁻⁶ BC

^a Same letters represent the absence of significant differences (Kruskal Wallis followed by Dunn Test; $p > 0.05$).

^b Same letters represent the absence of significant differences (ANOVA followed by Tukey HSD test; $p > 0.05$).

BV = Biovolume (μm^3); SA = surface area (μm^2); VSR = volume surface ratio; ETR max = Maximum electron transport rate ($\mu\text{mol electrons m}^{-2} \text{s}^{-1}$); α = maximum light absorption coefficient ($\text{mg C. mg Chl a}^{-1} [\mu\text{mol photons m}^{-2} \text{s}^{-1}]$); Ek = saturation index light ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$); GST =Activity enzymatic of GST ($\text{mU/mg.protein}^{-1}$); Chlorophyll content (mg.um^{-3}).

Table 3. Loadings of the principal component analysis (PCA) for the parameters ETR max, GST, α , Ek, VSR, BV and SA measured in the cultured algae species used in the study.

Parameters	PC 1	PC 2
ETR max	0.89	-0.16
GST	-0.95	-0.06
α	0.86	-0.17
Chl- <i>a</i>	-0.81	-0.38
Ek	0.78	-0.15
VSR	-0.56	0.75
BV	0.67	0.68
SA	0.13	0.98

Table 4. Photosynthetic yield (ϕ M) in control and ATZ-exposed algae and % inhibition of ϕ M caused by ATZ. Each algae species was 72h-exposed to a particular ATZ concentration that caused 30 % growth inhibition (EC30) as determined in a preliminary toxicity assay.

Species	control (ϕ M)	ATZ (ϕ M)	% inhibition
<i>Pseudopediastrum boryanum</i>	0.76	0.57	25
<i>Desmodesmus communis</i>	0.77	0.40	48
<i>Ankistrodesmus densus</i>	0.78	0.56	28
<i>Chlamydomonas puliminiorfes</i>	0.75	0.57	24
<i>Raphidocelis subcapitata</i>	0.65	0.33	49
<i>Kirchneriella lunaris</i>	0.75	0.57	24
<i>Ankistrodesmus falcatus</i>	0.70	0.45	36

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A presente tese trouxe informações sobre a evolução da ATZ como composto estudado na área da toxicologia e o uso de microalgas nesses estudos. Bem como, analisar se alguns parâmetros intrínsecos de microalgas verdes poderiam ser preditores na diferença de tolerância dessas espécies à ATZ. Desse modo, o estudo foi conduzido utilizando abordagens teóricas e práticas e ambos trazem resultados interessantes.

No primeiro capítulo foi realizado um estudo cienciométrico, um panorama dos estudos na área de toxicologia onde o herbicida ATZ é o composto foco foi apresentado. Foi observado que a atrazina demorou alguns anos para se tornar objeto de estudo científico. Ela foi patenteada em 1956 e os primeiros estudos foram realizados no ano de 1973, porém a partir de então, cresceu, sobretudo a partir dos anos 2000. Muitos países estudam sobre a toxicidade da ATZ, dentre eles, EUA, China e Canadá são os países que mais publicaram sobre ela. Inclusive, estudos que foram publicados em periódicos bem avaliados na área de toxicologia. Em relação aos organismos utilizados nesses estudos, o grupo de animais, sejam eles vertebrados ou invertebrados, foram mais utilizados. O grupo de vegetais, principalmente aquáticos, não é um grupo com muitos estudos desenvolvidos. Nesse mesmo trabalho, foi mostrado que estudos onde microalgas e cianobactérias foram organismos teste na avaliação da toxicidade da ATZ, foram pouco realizados. Essas foram algumas lacunas encontradas e que podem ser importantes e de grande ajuda no direcionamento de futuras pesquisas sobre esse herbicida. Os resultados podem incentivar o desenvolvimento de mais trabalhos onde organismos aquáticos sejam foco, devido a tendência de se encontrar ATZ com facilidade nesses ambientes e da pouca produção com esses organismos.

O segundo capítulo aborda sobre características morfológicas, fotossintéticas e bioquímicas que podem estar relacionadas com a tolerância diferenciada de algumas espécies de microalgas clorofíceas para a ATZ. Dentre as sete espécies de microalgas avaliadas através de ensaios de inibição de crescimento, as algas *P. boryanum* e *D. communis* foram as menos tolerantes (2 e 3% menos tolerantes, respectivamente) em relação às algas mais tolerantes. Observamos que os parâmetros morfológicos, fotossintéticos, fisiológicos e bioquímicos avaliados em conjunto podem estar ligados à tolerância a ATZ. Nas microalgas menos tolerantes, as taxas fotossintéticas basais foram mais altas, ao contrário das mais tolerantes que tem taxas mais baixas, talvez por um efeito pleiotrópico provocado por uma mutação, a mesma que confere a resistência à ATZ. Além disso, as algas menos tolerantes apresentaram uma baixa atividade da enzima GST, enzima que é importante para a

detoxificação de xenobióticos nessas algas, inclusive na detoxificação da ATZ. As mais tolerantes, por sua vez, apresentaram maiores valores de parâmetros morfológicos, como biovolume e área, o que pode estar relacionado à capacidade de absorção do composto. Quanto menor o biovolume, menores os níveis internos de atrazina necessários para provocar a inibição. Em relação ao teor de clorofila-*a*, algas menos tolerantes obtiveram menores valores, ao contrário de microalgas mais tolerantes como *R. subcapitata* e *K. lunaris* que obtiveram maior teor de clorofila. A clorofila pode estar relacionada com a resistência, uma vez que, quanto mais clorofila-*a*, mais PSII (alvo da ATZ) o organismo possui e, quanto mais alvos fotossintéticos disponíveis, maior a concentração de ATZ seria necessária para bloquear uma quantidade representativa dos mesmos. Esse trabalho mostrou que concentrações de ATZ que causam 30 % de inibição do crescimento, também causam inibição da fotossíntese. Deixo como sugestão que outros estudos sejam desenvolvidos a partir desse, onde o potencial biorremediador das algas mais tolerantes possa ser testado e as mais sensíveis possam ser usadas como possíveis organismos testes para estudos que visam analisar a toxicidade de compostos e que requerem a utilização de organismos sensíveis.

ANEXOS

1. Artigo publicado em 2021 onde fui coautor do estudo.

FERNANDES, Ubirajara Lima; CASTRO, Muryllo Santos; GIROLDO, Danilo. Morphological features of the *Geminella protogenita* (Kützinger) West (Chlorophyta, Trebouxiophyceae): a rare freshwater green alga found in southern of Brazil. *Biologia*, [s. l.], v. 76, n. 10, p. 2889–2894, 2021. Disponível em: <https://doi.org/10.1007/s11756-021-00861-3>