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

Glutationa *S*-transferases em macrófitas aquáticas e potencial de biotransformação de biocidas

Rodrigo Nunes dos Santos

Orientador: Juliano Zanette

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Dissertação apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Mestre em Biologia de Ambientes Aquáticos Continentais.

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RESUMO

Biocidas têm sido usados constantemente, seja para proteger a agricultura de pragas ou para controlar organismos marinhos na indústria naval, representando assim risco para o ambiente aquático. Estratégias para remediar esses compostos incluem a fitorremediação por plantas. Enzimas como a glutationa *S*-transferases (GSTs) formam uma superfamília de enzimas de detoxificação e podem ser encontradas em plantas. É possível relacionar a atividade das GSTs contidas em macrófitas com sua capacidade detoxificadora em nível ambiental. Assim, o presente estudo tem como objetivo investigar: (1) a atividade basal de GSTs na raiz, caule e folha de quatorze macrófitas aquáticas utilizando ensaios cinéticos de GST e CDNB como substrato; (2) padrões cinéticos de cinco compostos biocidas no extrato citosólico de origem vegetal, utilizando ensaios competitivos *in vitro*. Quatorze espécies de plantas foram coletadas nos seus respectivos habitats (n = 7 por espécie) para realizar uma triagem da atividade da GST em raiz, caule e folha. Em seguida, foram realizados ensaios competitivos onde a atividade da enzima foi avaliada na presença dos biocidas clorotalonil, DCOIT, diclofluanida, diuron e irgarol para estimar seus padrões de inibição bem como a afinidade pelas GSTs. As espécies de plantas *Nothoscordum gracile*, *Spartina alterniflora* e *Sarcocornia ambigua* apresentaram os maiores níveis de atividade da GST ($p < 0,05$ em relação às demais plantas), sugerindo uma melhor capacidade de fitorremediação via GST. No ensaio competitivo cinético; o clorotalonil e o DCOIT apresentaram alta afinidade às enzimas GST, uma vez que apresentaram baixos níveis de IC_{20} (concentração necessária para causar 20% de inibição) e alta porcentagem de inibição da atividade enzimática. A diclofluanida, por outro lado, apresentou uma alta porcentagem de inibição, porém seu valor de IC_{20} foi três vezes maior do que o observado nos compostos que mostraram maior competição. Além disso, há compostos que apresentam baixa afinidade com as GSTs, assim como o diuron (com alto nível de IC_{20}) e o irgarol, que não competiu com o CDNB, impossibilitando a estimativa dos resultados cinéticos. Todas as espécies demonstraram atividade da GST, logo potencial para uso na fitorremediação; nos ensaios *in vitro* quatro compostos biocidas inibiram a atividade das GSTs, indicando esta como a possível via de detoxificação das substâncias.

Palavras-chave: fitorremediação, xenobiótico, poluição.

ABSTRACT

Biocides have been used constantly, whether to protect agriculture from pests or to control marine organisms on shipping industry, representing thus risk for the aquatic environment. Strategies to remediate those compounds include the phytoremediation by plants. Enzymes such as glutathione *S*-transferases (GSTs) form a superfamily of detoxication enzymes and can be found in plants. It is possible to relate the activity of the GSTs contained in macrophytes with their detoxifying capacity at environmental level. Thus, the present study aim to investigate: (1) basal activity of GSTs in the root, steam and leaf of fifteen aquatic macrophytes using kinetic assays of GST and CDNB as substrate; (2) kinetic patterns of five biocides compounds on plant GST cytosolic extract, using *in vitro* competitive assays. Fourteen species of plants were collected in their natural habitat (n=7 per specie) in order to do a screening of GST activity in roots, steam and leaves. In sequence, competitive assays were performed where activity of the enzyme was evaluated in the presence of biocides chlorothalonil, DCOIT, dichlofluanid, diuron, and irgarol to estimate inhibition patterns as well as their affinity with GSTs. Aquatic macrophytes *Nothoscordum gracile*, *Spartina alterniflora* and *Sarcocornia ambigua* showed the highest levels of GST activity ($p<0.05$ compared to other plants), suggesting a better GST-dependent phytoremediation capability. In the kinetic competitive assay; chlorothalonil and DCOIT presented high affinity to GST enzymes, since they have presented low IC_{20} levels (concentration needed to cause 20% of inhibition) and high inhibition percentage in a given concentration. dichlofluanid, in the other hand, has either presented a high inhibition percentage, however its IC_{20} value was three times greater than that shown in compounds with higher affinity. Besides that, there were compounds which presented low affinity with GST molecules, such as diuron (with high IC_{20} level) and irgarol which did not competed with CDNB making unable to estimate kinetic results. All species showed GST activity, thus potential for use in phytoremediation; in the *in vitro* tests, four biocides inhibited the activity of GSTs, signaling the possible the detoxification pathway of these substances.

Key-words: bioremediation, xenobiotic, pollution.

APRESENTAÇÃO

Seguindo o modelo sugerido pelo Programa, esta dissertação está dividida em duas partes. A primeira parte é referente à introdução geral abordando a problemática estudada juntamente com as referências utilizadas. Já a segunda, refere-se a um capítulo único, que se trata de um manuscrito a ser submetido à revista Ecotoxicology and Environmental Safety, formatado de acordo com as normas da revista. O manuscrito conta com Introdução, Material e Métodos, Resultados, Discussão, Conclusão, Agradecimentos, Referências, Tabelas e Figuras.

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1 INTRODUÇÃO GERAL

1.1 AS GLUTATIONA *S*-TRANSFERASES

A contaminação de ambientes aquáticos continentais é um tema bastante abordado em linhas de pesquisa recentes. Diante disso, técnicas para compreender melhor a detoxificação de compostos considerados contaminantes vêm sendo enfatizadas e exploradas em ecotoxicologia (Schnoor et al., 1995). Neste cenário, os ensaios enzimáticos *in vitro* mostram-se como importantes ferramentas de estudo para compreender vias metabólicas de detoxificação.

Com um papel fundamental no metabolismo de xenobióticos, as glutationas *S*-transferases (GSTs) são enzimas de detoxificação e podem ser induzidas ou inibidas frente à exposição a certos contaminantes (Haynes et al., 2005). Enzimas de diferentes espécies vegetais são utilizadas como biotransformadoras de vários poluentes, diminuindo as concentrações destes no meio ambiente, permitindo que as plantas possam ser utilizadas como biorremediadoras (Yamada, 2007).

Para explorar plantas visando à remoção e detoxificação de xenobióticos do solo e da água, as enzimas e vias metabólicas dos vegetais locais devem ser consideradas. A existência de várias enzimas de detoxificação tem sido descrita em muitas espécies de angiospermas, gimnospermas e as chamadas "plantas inferiores", como samambaias, musgos e algas marinhas (Pflugmacher, 1998; Schroder et al., 2002). Estudar os caminhos de detoxificação desses vegetais é importante dentre tudo por sua posição na cadeia alimentar como produtores primários, bem como as variadas funções ecológicas nos ecossistemas aquáticos que eles fazem parte (Coleman, 1997).

1.2 A BIOTRANSFORMAÇÃO E A BIORREMEDIAÇÃO

É definido como biotransformação reações químicas que, geralmente mediadas por enzimas, convertem determinada substância em um composto diferente do original (Parkinson e Ogilvie, 2001). Apesar do produto da reação nem sempre ser menos tóxico, para fins de detoxificação no organismo, a biotransformação deve evitar efeitos deletérios na medida em que indisponibiliza o composto tóxico (Cummins et al., 2011). No contexto da biorremediação, a biotransformação tem por objetivo eliminar o composto do ambiente. Tradicionalmente a palavra biorremediação vem sendo utilizada em processos envolvendo microorganismos, entretanto termos como fitorremediação vêm à tona para propriamente descrever a remediação ambiental mediada por plantas (Velázquez-Fernández et al., 2012).

Em um estudo feito com macrófitas e algas marinhas, Pflugmacher (1995) contribui para o conceito de "fígado verde". Resumidamente, o conceito é baseado no fato que plantas e animais possuem mecanismos de metabolização de xenobióticos semelhantes, por exemplo, a biotransformação. Entretanto, diferentemente dos animais, em plantas, o produto da biotransformação em geral é estocado em seus tecidos em um processo denominado fitoestabilização, ficando assim indisponível ao ambiente e logo caracterizando uma modalidade de fitorremediação (Schnoor et al., 1995).

Em animais e plantas, o metabolismo de compostos xenobióticos geralmente envolve dois estágios distintos, comumente referidos como fase I e fase II. A biotransformação de fase I envolve uma oxidação inicial do xenobiótico pelo citocromo P450 (CYP) enquanto a fase II frequentemente envolve reações de conjugação catalizadas pelas GSTs (Keen et al., 1976). Diferentemente de como ocorre nos animais, nas plantas o último estágio do processo (fase III) é quando ocorre a fitoestabilização, onde o xenobiótico é depositado nos vacúolos (Coleman et al., 1997). Conforme a figura abaixo esquematiza.

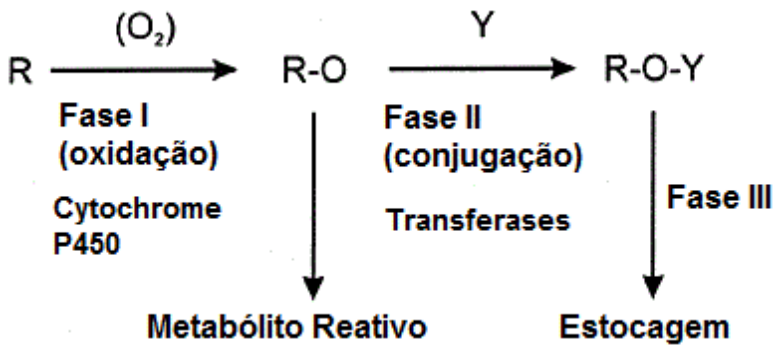

Figura 1 - Metabolismo de compostos xenobióticos: esquema da fase I, II e III

1.3 MACRÓFITAS AQUÁTICAS DO EXTREMO SUL DO PAÍS

São consideradas plantas aquáticas aquelas cujas partes fotossintetizantes estão, ao menos por alguns meses do ano, submersas ou flutuantes na água (Cook, 1996). As regiões alagadas do extremo sul do país possuem alta biodiversidade de macrófitas aquáticas, portanto podem abrigar espécies ainda pouco estudadas que tenham alto potencial de biorremediação via GSTs. A cidade do Rio Grande estando inserida na Planície Costeira da América Latina e contém uma flora diversificada (Irgang e Gastal, 1996), sendo um campo de coleta ideal.

Em um estudo de Pflugmacher e colaboradores (1999), raiz, caule e folha tiveram a atividade enzimática aferida, concluiu-se que nessas estruturas, em detrimento da semente, foi onde a atividade das GSTs demonstrou a maior atividade de biotransformação. O modelo utilizado pelos autores para avaliar a atividade enzimática na fase II é o ensaio enzimático da GST utilizando o 1-cloro-2,4-dinitrobenzeno (CDNB) como substrato. Dessa forma, ao analisar a amostra vegetal, pode-se estimar a atividade GST nela contida.

As plantas estão distribuídas em nove ordens filogenéticas classificadas segundo Angiosperm Phylogeny Group IV (2016) como se observa na Tabela 1. Como é possível que o hábito ecológico da planta tenha influência na sua capacidade de biotransformação, as plantas foram aqui agrupadas em quatro biótipos: flutuantes, emergentes, submersas e anfíbias de acordo com Pedralli (1990).

Gênero	**Espécie**	**Biótipo de ocorrência**
Nothoscordum	*gracile*	Anfíbia
Spartina	*alterniflora*	Anfíbia
Sarcocornia	*ambigua*	Anfíbia
Hydrocotyle	*bonariensis*	Anfíbia
Appalanthe	*granatensis*	Submersa
Eggeria	*densa*	Submersa
Hydrocotyle	*ranunculoides*	Emergente
Typha	*dominguensis*	Emergente
Ludwigia	*repens*	Emergente
Sagitaria	*montevidensis*	Emergente
Nymphoides	*indica*	Flutuante
Eichhornia	*crassipes*	Flutuante
Pistia	*stratiotes*	Flutuante
Salvinia	*auriculata*	Flutuante

Tabela 1 - Distribuição das macrófitas em famílias e espécies de acordo com APG IV e seus biótipos de ocorrência

Algumas destas plantas têm tolerância a altas variações de salinidade, como *Spartina alterniflora* (Rout, 2001), característica que as permite ocupar ambientes estuarinos onde a poluição por biocidas anti-incrustantes, por exemplo, é mais frequente. Já a *Sarcocornia ambigua* é considerada uma planta psamófila (relacionada a solos arenosos), esta planta é

comum em dunas litorâneas abundantes na nossa região, ambiente o qual os contaminantes também podem ser encontrados (Irgang e Gastal, 1996).

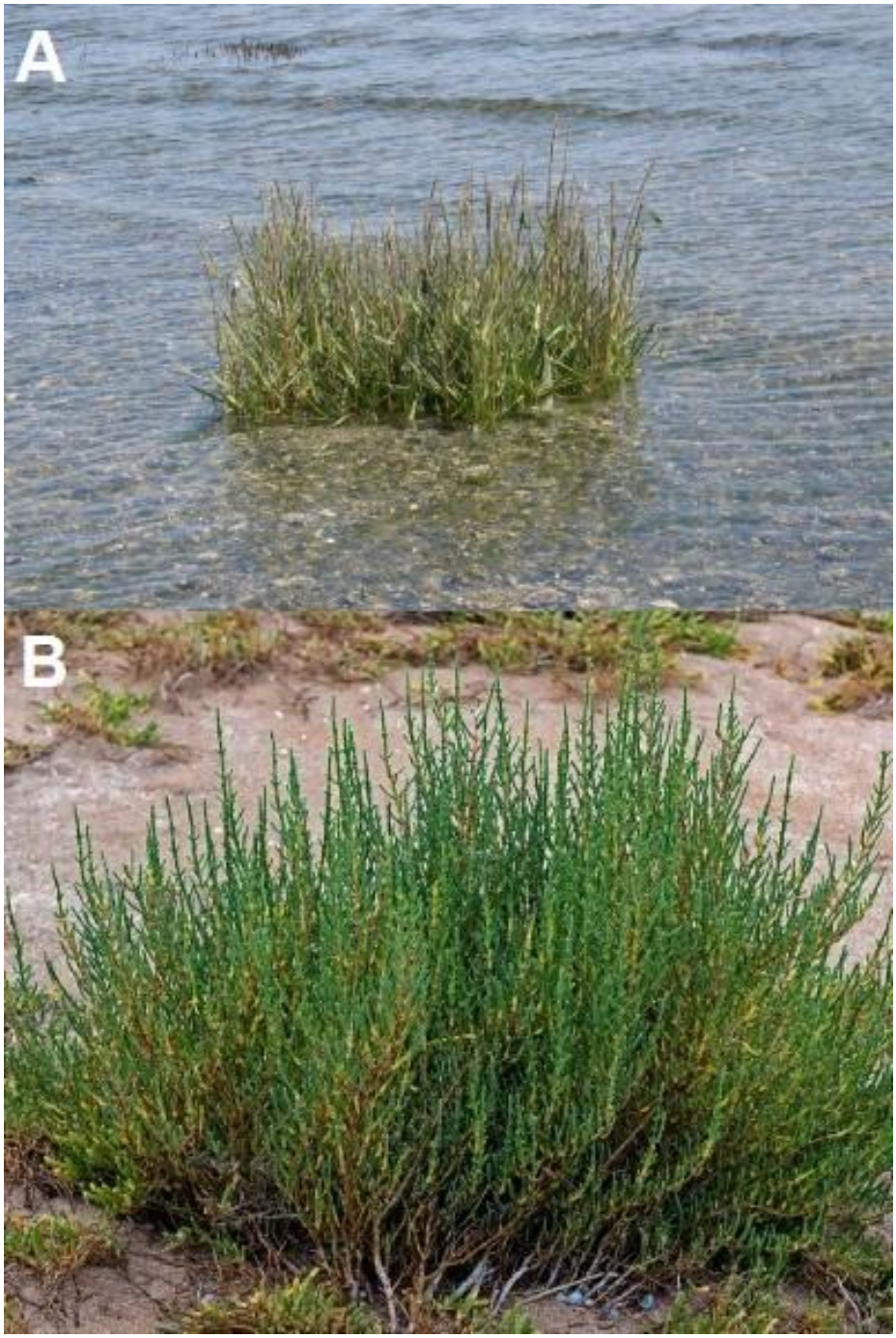

Figura 2 - *Spartina alterniflora* (A), *Sarcocornia ambigua* (B) em seus respectivos ambientes naturais

Outras macrófitas apresentam uma ampla distribuição territorial, como a *Thypha dominguensis*, que ocupa praticamente toda a América Latina, e a *Salvinia auriculata* que ocupa o território do Brasil (Pott e Pott, 2000). Da mesma forma, a ordem *Alismatales* possui

distribuição cosmopolita, sendo representada pela *Pistia* stratiotes e *Sagittaria* montevidensis (Irgang e Gastal, 1996).

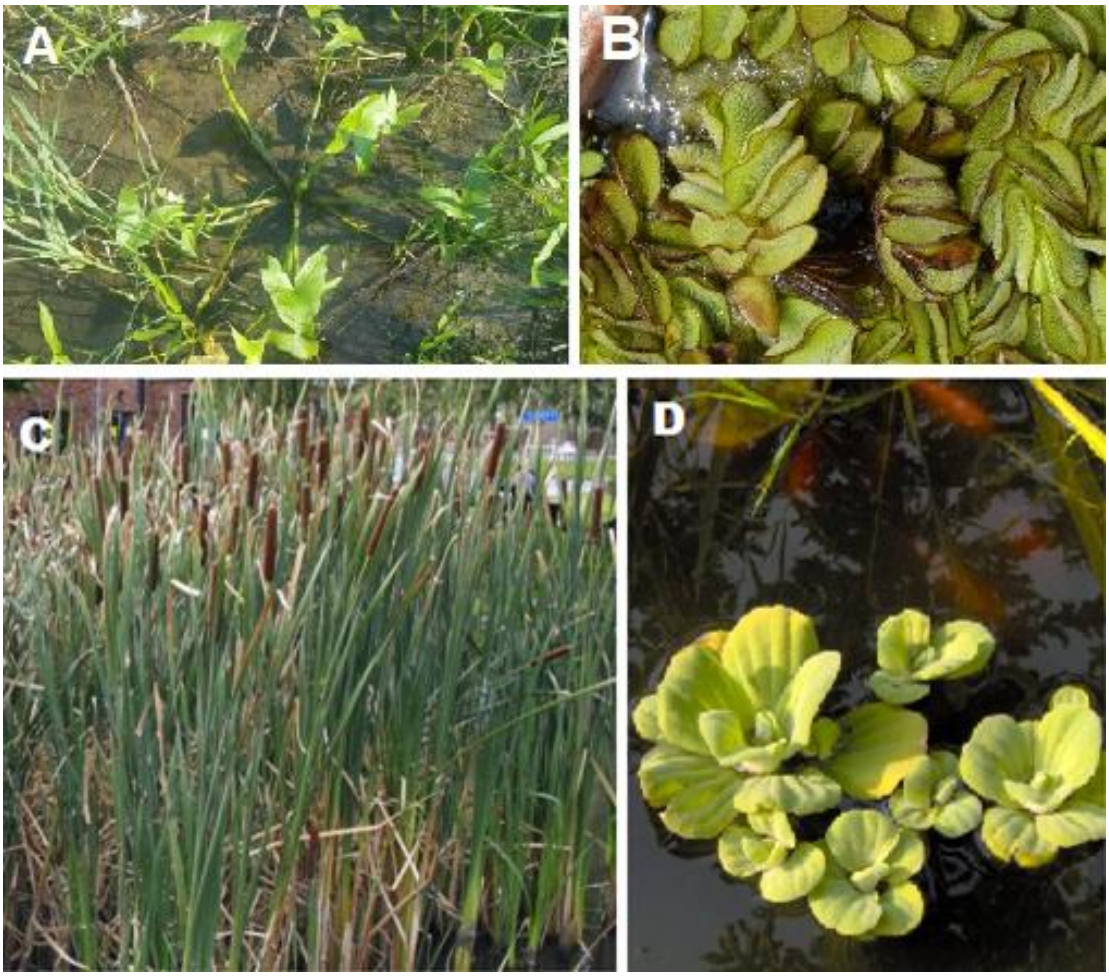

Figura 3 - *Sagittaria montevidensis* (A), *Salvinia auriculata* (B), *Typha dominguensis* (C) e *Pistia stratiotes* (D)

Macrófitas do gênero *Hydrocotyle* possuem diversas características morfológicas em comum, entretanto diferem quanto ao biótipo de ocorrência. A *Hydrocotyle ranunculoides* é uma planta emergente que é encontrada com mais frequência em regiões com lâmina d'água, enquanto a *Hydrocotyle bonariensis* ocupa geralmente ambientes terrestres (Amaral et al., 2009).

Figura 4 - *Hydrocotyle ranunculoides* (A) e *Hydrocotyle bonariensis* (B) ocupando seus habitats

1.4 OS BIOCIDAS E O ENSAIO ENZIMÁTICO COMPETITIVO

Uma questão bem conhecida que afeta intensamente o ambiente aquático é o impacto ambiental e a toxicidade dos biocidas que contaminam águas continentais (Konstantinou e Albanis, 2004). Estes podem ser utilizados na agricultura para controlar pragas e/ou para evitar a bioincrustação em navios (Castro et al, 2011), fazendo com que estas diferentes aplicações afetem os ecossistemas aquáticos com frequência. Uma propriedade conhecida como biomagnificação é passível de ocorrer podendo atingir diferentes sistemas além do seu local de descarga (Sarkar et al., 2006). O fenômeno aconteceria quando certos contaminantes são expostos à água; ainda segundo este autor, as substâncias podem inclusive persistir através dos diferentes níveis tróficos da cadeia alimentar.

Os biocidas irgarol (irgarol 1051), clorotalonil (2,4,5,6-tetrachloroisophthalonitrile), diclofluanida (N-dichlorofluoromethylthio-N′,N′-dimethyl-N-phenylsulfamide), DCOIT (dichloroctylisothiazolinon) e diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) são utilizados como ingredientes ativos em produtos destinados ao controle de organismos (tais como pragas em lavouras, comunidades bioincrustantes em embarcações), entretanto foi demonstrado que são potencialmente perigosos a nível ambiental (Qian et al., 2013). O irgarol é um composto altamente nocivo a algas marinhas e de água doce, sendo considerado fitotóxico (Dyer et al., 2016). O diuron atua de forma semelhante ao irgarol, inibindo a fotossíntese nos organismos (Giacomazzi e Cochet, 2004), ambos os compostos são altamente perigosos para as plantas em geral (Castro et al, 2011). Clorotalonil e diclofluanida são fungicidas protetores usados na agricultura com uma ampla gama de ação contra um grande número de organismos, e desde os anos 80 têm sido usados princípios ativos anti-incrustantes (Castro et al, 2011; Qian et al., 2013). O clorotalonil é tóxico para peixes e invertebrados aquáticos (Kumar et al., 2016). O

DCOIT é um princípio ativo anti-incrustante incipiente, com impacto ambiental de longo prazo pouco conhecido (Cima et al., 2013), demandando ter a toxicidade estudada.

Se à medida que a concentração *in vitro* do biocida aumenta, a atividade das GSTs decai, o composto é considerado inibidor da atividade enzimática. O ensaio competitivo baseia-se nesta premissa; demonstrando a competição entre o CDNB (substrato de amplo espectro) e os biocidas testados pelas GSTs. O ensaio enzimático competitivo é a técnica aqui escolhida que permite estimar o tipo de interação que cada biocida exerce nas enzimas. Em enzimologia, convencionou-se o valor de IC_{20}: a concentração da substância inibidora necessária para diminuir 20% da atividade enzimática de uma dada amostra biológica (Sebaugh, 2011). O IC_{20} é um dado de referência importante e permite estimar o efeito inibitório de diferentes substâncias na atividade enzimática, e compará-las entre si.

Uma vez com os resultados da cinética enzimática, pode-se traçar o gráfico de Lineweaver-Burker, conhecido como duplo recíproco, que proporciona um método gráfico útil para analisar a relação da velocidade da reação em função da concentração de substrato. Contendo duas ou mais retas, apresenta a primeira representando a reação na ausência do inibidor e a outra com uma concentração deste (Nelson e Cox, 2000).

Ao analisar os gráficos gerados, se conclui se a inibição é não competitiva quando as retas lineares do modelo interceptam o eixo y em pontos diferentes e são paralelas entre si (C). Da mesma forma, a inibição poderá ser competitiva caso as retas se interceptem no eixo y no ponto que corresponde a $V_{máx}^{-1}$ (A), ou mista se as retas se interceptarem em um ponto diferente (B) (Nelson e Cox, 2000). Na Figura 7 estão ilustrados os possíveis modelos que podem ser obtidos a partir dos resultados.

Figura 5 - Representação do padrão de inibição competitiva (A), mista (B) e não-competitiva (C) em gráficos Lineweaver-Burker

Pode-se definir se a diminuição na atividade enzimática pelos biocidas é competitiva, não-competitiva ou mista em ensaios utilizando GST de origem vegetal (Segel, 1975). Esta

classificação leva em conta a interação do inibidor com a enzima. No caso da inibição competitiva, o inibidor se liga ao sítio ativo, impedindo assim que o substrato o ocupe, enquanto que na não-competitiva o inibidor altera o formato do sítio ativo impossibilitando que o substrato ali se ligue (Figura 8). Na inibição mista, ambos os padrões podem ser observados.

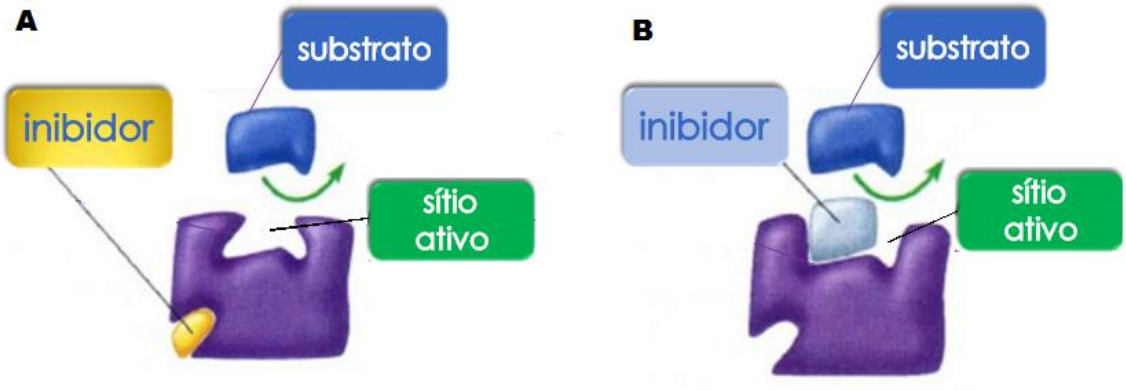

Figura 6 - Representação do padrão de inibição não-competitiva (A) e competitiva (B)

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo deste trabalho é estimar os níveis de atividade enzimática das GSTs de macrófitas presentes em ambientes aquáticos e avaliar o potencial de biotransformação via GSTs de cinco contaminantes biocidas utilizando um ensaio competitivo *in vitro*.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a atividade da GST em raiz, caule e folha de quatorze espécies de macrófitas aquáticas distribuídas em diferentes biótipos usando ensaios cinéticos de GST e CDNB como substrato;
- Estimar a biotransformação *in vitro* via GSTs de clorotalonil, diclofluanida, irgarol, diuron e DCOIT através de ensaios competitivos com CDNB utilizando o extrato citosólico de macrófitas aquáticas.

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CAPÍTULO ÚNICO – MANUSCRITO

Glutathione *S*-transferases in freshwater macrophytes and biotransformation potential for biocides

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Glutathione *S*-transferases in freshwater macrophytes and biotransformation potential for biocides

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Highlights:

- GST-dependent phytoremediation capability was estimated based on GST activity of macrophytes.
- Four of five biocides tested compete for the active site of GST enzymes present in aquatic plants.
- Chlorothalonil and DCOIT are the biocides which have highest affinity to GST enzymes tested.
- Inhibition patterns obtained from Lineweaver-Burk plots can be related with bioremediation via GSTs performance.

ABSTRACT

Biocides have been used constantly, whether to protect agriculture from pests or to control marine organisms on shipping industry, representing thus risk for the aquatic environment. Strategies to remediate those compounds include the phytoremediation by plants. Enzymes such as Glutathione *S*-transferases (GSTs) form a superfamily of detoxication enzymes and can be found in plants. It is possible to relate the activity of the GSTs contained in macrophytes with their detoxifying capacity at environmental level. Thus, the present study aim to investigate: (1) basal activity of GSTs in the root, steam and leaf of fifteen aquatic macrophytes using kinetic assays of GST and CDNB as substrate; (2) kinetic patterns of five biocides compounds on plant GST cytosolic extract, using *in vitro* competitive assays. Fourteen species of plants were collected in their natural habitat (n=7 per specie) in order to do a screening of GST activity in roots, steam and leaves. In sequence, competitive assays were performed where activity of the enzyme was evaluated in the presence of biocides chlorothalonil, DCOIT, dichlofluanid, diuron, and irgarol to estimate inhibition patterns as well as their affinity with GSTs. Aquatic macrophytes *Nothoscordum gracile*, *Spartina alterniflora* and *Sarcocornia ambigua* showed the highest levels of GST activity ($p<0.05$ compared to other plants), suggesting a better GST-dependent phytoremediation capability. In the kinetic competitive assay; chlorothalonil and DCOIT presented high affinity to GST enzymes, since they have presented low IC_{20} levels (concentration needed to cause 20% of inhibition) and high inhibition percentage in a given concentration. dichlofluanid, in the other hand, has either presented a high inhibition percentage, however its IC_{20} value was three times greater than that shown in compounds with higher affinity. Besides that, there were compounds which presented low affinity with GST molecules, such as diuron (with high IC_{20} level) and irgarol which did not competed with CDNB making unable to estimate kinetic results. All species showed GST activity, thus potential for use in phytoremediation; in the *in vitro* tests, four biocides inhibited the activity of GSTs, signaling the possible the detoxification pathway of these substances.

Key-words: bioremediation, xenobiotic, pollution.

1. Introduction

Continental aquatic environments are extensively exposed to anthropogenic contaminants. In view of this situation, several methods of environmental decontamination have been proposed, among them the bioremediation by plants (i.e. phytoremediation) (Schnoor et al., 1995). Phytoremediation is one of the detoxification's modalities, in this technique plants with high potential of biotransformation are used to eliminate toxic substances of the environment (Cummins et al., 2011).

If one wishes to exploit plants for the removal and detoxification of xenobiotics from soil and water, the enzymes and metabolic pathways of the local vegetables have to be considered. The existence of various detoxification enzymes has been described in many species of Angiosperms, Gymnosperms and the so-called "lower plants" such as ferns, mosses and seaweeds (Pflugmacher and Steinberg, 1998; Schröder and Collins, 2002). However, macrophytes native from South America remain poorly studied. Studying detoxication paths of this kind of vegetable is important as their position in the food chain as primary producers as well as the varied ecological functions in aquatic ecosystems they do.

Biotransformation is defined as chemical reactions that, generally mediated by enzymes, convert a substance into a different compound from the original (Parkinson and Ogilvie, 2001). For purposes of detoxification in the organism, biotransformation should avoid deleterious effects at the same time as the toxic compound is being unavailable. Whilst in the context of bioremediation, biotransformation aims to eliminate the compound from the environment (Cunningham et al., 1995). The Glutathione *S*-transferases (GSTs) are biotransformation enzymes of phase II and can be induced or inhibited after exposure to certain contaminants (Hayes et al., 2005). The GSTs catalyze the conjugation reaction of reduced glutathione (GSH) with electrophilic substrates. Conjugation products are more polar and less toxic (Marrs, 1996). The enzymes of different plant species are used in the bioremediation of several pollutants, reducing the concentrations of these compounds in the environment, allowing the plants to be used in bioremediation (Schröder, 2008). In this way, it is intended to relate the activity of the GSTs contained in plants with its detoxifying capacity.

Environmental detoxification using plants is addressed to the concept of "green liver" which was introduced in the last century and since then has been studied. Briefly, the concept is based on the fact that plants and animals have similar mechanisms of metabolism of xenobiotics (Sanderman, 1994). However, unlike animals, instead of processing and

excreting, plants have stages involving processing, deposition and translocation, usually in the vacuoles. The metabolism of xenobiotic compounds usually involves distinct stages, commonly referred to as phase I, phase II and phase III (Sandermann, 1992). Phase I of biotransformation involves an initial oxidation of the xenobiotic by cytochrome P450 (CYP) while phase II often involves conjugation reactions catalyzed by GSTs. Differently from how it occurs in animals, in plants the last stage of the process (phase III) is when phytoestabilization occurs and the xenobiotic is deposited in the vacuole (Coleman et al., 1997).

Once GST activity could be directly proportional to the remedial capacity when xenobiotic is GST substrate, it is important to assess the basal activity of organisms studied in view of the intended purpose. One of the best known model of study to evaluate GST *in vitro* is kinetic assay using 1-chloro-2,4-dinitrobenzene (CDNB), a relatively good substrate for interacting with several GST isoforms (Habig and Jakoby, 1981). This model has been used on investigations of xenobiotics biotransformation. Schrenk et al. (1998) have conducted a study relating GST activity measured, through CDNB methodology, to phytoremediation at environmental level, so the activity of the GSTs measured in certain plant can be related with its detoxifying capacity at the environmental level.

It has been established that is crucial to know glutathione conjugation, because this will determine the final fate of the compound in phytoremediation (Schröder, 2007). Since GST is indispensable to metabolism of several compounds (Edwards et al., 2000), substances considered as environmental contaminants can be used in kinetics assays to clarify *in vitro* their interaction to GSTs. There was a study assessing the effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants, the activity of GST systems was inhibited in the presence of the compound. Results of *in vitro* assays added to HPLC substrate decay analisys led authors to conclude the existence of a detoxication pathway for tested compound in aquatic plants (Pflugmacher et al., 1999)

A well-known issue which affects largely aquatic ambient is the environmental impact and toxicity of substances considered biocides: used in to prevent losses in agriculture and sometimes also to prevent bioincrustation in shipery (Castro et al, 2011). The biocides irgarol 1051 (irgarol), chlorothalonil, dichlofluanid, DCOIT and diuron are used as active ingredients in approved products intended to control organisms (Qian et al., 2013). Irgarol is a highly effective compound against freshwater and marine algae and is stated as phytotoxic (Dyer et al., 2016). Acting similar to Irgarol there is diuron which inhibits photosynthesis in organisms

(Giacomazzi and Cochet, 2004), both compounds are highly hazardous to plants in general (Castro et al, 2011). Chlorothalonil and dichlofluanid are protective fungicides used in agriculture with a wide range of action against a big number of organisms, and since 80's have been used as antifouling active principals (Castro et al, 2011; Qian et al., 2013). Chlorothalonil is toxic to fish and aquatic invertebrate, moreover it has been established that the biotransformation of the fungicide Chlorothalonil is glutathione-dependent (Kim et al., 2004). DCOIT is an incipient antifouling product having the long-term environmental impact poorly known (Cima et al., 2013).

Researching in vitro of GST activity in different organs and species of plants may reflect their phytoremediation potential, likewise is possible investigate differences in the biotransformation capacity of xenobiotics of interest. Thus, the present study was therefore aimed to investigate the (1) basal activity of GSTs from macrophytes distributed in different biotypes (2) *in vitro* inference of biotransformation of five antifouling compounds on plant GST cytosolic extract performing competitive kinetic assays with CDNB.

2. Material and methods

2.1. *Study area and sample collection*

The work was carried out in three shallow lakes (32.77766 S, 52.16875 O; 32.0739 S, 52.1654 O; 32.07112 S, 52.16408 O) of the campus of Federal University of Rio Grande (FURG) and Cassino beach (32.15592 S, 52.09978 O), in the municipality of Rio Grande, RS, Brazil. According to the classification of Strahler and Strahler (1997), the climate of the region is humid subtropical with soft climatic conditions and strong oceanic influence. Having vegetation predominantly shrubby, predominant ecosystems are lagoons and wetlands, sandy beaches, frontal and lacustrine dunes, coastal fields, restinga forests, buttresses and marshes (Burger and Ramos, 2006). Shallow lakes used in the study are all small artificial lakes, built about 20 years ago, having approximately 1 hectare of area and maximum depth of 1.5m (Marinho et al., 2009).

2.2 *Evaluation of GST activity in freshwater macrophytes*

Fourteen species of plants were collected (n=7 per specie) at a distance of approximately 4 meters between each individual and chosen according to their abundance in local ecosystem. Identification of the species was carried out using the Brazilian bibliography (Irgang and Gastal, 1996; Pott and Pott, 2000; Amaral et al., 2009), also the taxonomic

classification followed the one proposed by APG IV (2016). Plants from four ecotypes (i.e.: floating, submerged, emergent and amphibian) that were classified according to Pedralli (1990) were collected. The macrophytes *Nymphoides indica*, *Eichhornia crassipes*, *Salvinia auriculata* and *Pistia stratiotes* (fluctuant plants); *Sagittaria montevidensis*, *Typha domingensi*, *Hydrocotyle ranunculoides* (emergent plants); *Ludwigia repens*, *Nothoscordum gracile*, *Sarcocornia ambigua*, *Hydrocotyle bonariensis* and *Spartina alterniflora* (amphibian plants); *Appalanthe granatensis* and *Eggeria densa* (submerse plants) were collected.

The root, stem and leaf (1 g per organ) were dissected and used to prepare extracts of S9 fraction. The S9 fraction was prepared by homogenization and centrifugation at 9000 *x* g. For homogenization, tissue was macerated with a sodium phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 14mM DTT (dithiothreitol) and 1mM EDTA (ethylenediamine tetra acetic acid) macerated in ice using mortar and pestle (4mL of buffer per gram of tissue) according to Pflugmacher et al. (2007). Samples were stored at -80°C for subsequent analysis of GST activity and proteins content.

Kinetic assays were performed based on that described by Habig and Jakoby (1981). Briefly, in a microplate, kinetic assay consisted in adding 235 µL of reaction phosphate buffer 100mM pH 7 containing CDNB at 50mM (30°C), 20 µL cytosolic extract and 20 µL GSH 25mM in each well. Each assay was performed in triplicate, immediately reading of absorbance performed in spectrophotometer (ELx808 Absorbance Reader) at a wavelength of 340nm for three minutes. Formation of the GS-DNB conjugate indicates the presence of the enzyme, generating data of variation of absorbance per minute and thus quantifying the enzymatic activity. GS-DNB conjugate has a strong absorption at 340 nm with molar extinction coefficient (ε) = 9600 $M^{-1}.cm^{-1}$. This absorption variation in spectrophotometer is directly proportional at sample activity (Habig and Jakoby, 1981). The absorbance variation was determined per minute (Δ ABS/min) and enzyme activity calculated (GST/mg protein) with following equation: (Δ ABS (average) * sample dilution) / (9.6 * sample volume (mL) * sample protein concentration ($mg.mL^{-1}$)). Protein concentration of samples, necessary to estimate GST activity was determined using biuret based method for total protein (Total proteins; Labtest Kit, Minas Gerais, Brazil).

2.3 *Competitive kinetic test with compounds chlorothalonil, dichlofluanid, diuron, irgarol and DCOIT to estimate biotransformation potential*

The root of the plant *Nothoscordum gracile*, which showed the highest GST activity among all the tested plants (see result section), was chosen to assess competitive assays. In

these assays the activity of the enzyme was evaluated in absence and in the presence of biocides chlorothalonil, DCOIT, dichlofluanid, diuron, and irgarol (purity> 99%; Sigma-Aldrich, USA). This approach utilized CDNB and each biocide as competitive substrates. These assays consisted of addition of 225 µL of reaction buffer 100 mM containing CDNB at 50 mM (30 °C), 20 µL cytosolic extract, 10 µL of tested biocide (concentration range 10:400 µM) and 20 µL GSH 25mM in each well of microplate. Each trial was performed in triplicate. Control kinetic assay had the same components, except biocides which were substituted with DMSO. GST activity analysis was performed similarly to item 2.2. Inhibitory effect of each on of GST activity was estimate with following equation: GST activity inhibition = 100 - GST activity (in percentage). At end, the reduction of GST activity with CDNB in the presence of a given biocide is estimated, thus greater the decrease in activity, the greater the effect of the competing compound, and possibly the greater competition and affinity for the active site of GST.

Considering the need to compare the inhibitory effect of different substances on a vegetal source biological sample, the value of IC_{20} was convened (Sebaugh, 2011). In the case of interest, IC_{20} is the inhibitor concentration required to decrease 20% of the enzyme activity presented. Kinetics assays were performed using four concentrations of each compound. Obtained data was used to plot the percentage of inhibition of as a function of the concentration of biocide tested and adjustment of a second order equation to the values obtained (with $R^2 \approx 0.99$). Once estimated the IC_{20} of all the compounds tested, it was able to compare them with each other. The percentage of GST activity inhibition took as reference the concentration of each compound tested that inhibited 20% of GST activity (IC_{20}) compared to its respective control assay.

Kinetic assays with CDNB to infer inhibition type of each contaminant were performed to develop Lineweaver Burk graphics and confer the inhibitory effect caused by each compound on GST activity (Table 1 and Figure 1 of supplementary material). This is a necessary step to define that the inhibition is competitive, allowing us to perform this type of assay. For that, nine different concentrations of CDNB (10, 20, 30, 40, 50, 60, 90, 120 and 150 mM) and three concentrations of each compound (100, 200, 400 µM) dissolved in dimethyl sulfoxide (DMSO) were prepared to determine inhibitory concentrations (i.e.: IC_{20}). The GST activity assay was performed similarly to item 2.2. Lineweaver-Burk graph was plotted to visualize the intersection of GST activity with and without the presence of contaminant and determine parameters Vmax and K_M. This method, linearizes equation of

Michaelis-Menten in form 1/Vo and 1/[S], where the slope of the line is K_M/Vmax and intersection in axis 1/Vo is 1/Vmax; while that intersection in axis 1/[S] of the extrapolated line is $1/K_M$ (Nelson and Cox, 2000).

2.4 *Statistical Analyses*

Statistical analyses were performed using R program, version 2.9.0 (R Development Core Team, 2009). To check statistical similarity of GST activity in plants' species all data was log-transformed and ANOVA assumptions were verified. The mean GST activity value of root, stem and leaf were tested by analysis of variance (ANOVA one way) followed with comparisons of the means by Tukey's HSD test with a significance level of 5%.

3. Results

Statistical differences in basal activity of GST among species were found (Fig. 1). In stem and leaf, *Nothoscordum gracile* and *Spartina alterniflora* presented the highest GST activities (31.2±6.1 and 25.1±1.2 mU.mg protein^{-1} in stem, 16±3 and 18.5±3 mU.mg protein^{-1} in leaf, respectively) in comparison with all other species ($p < 0.05$) (Figure 1B and 1C). In the root, *Nothoscordum gracile* presented the highest activity (46.3±8.7 mU.mg protein^{-1}) in relation to all other species ($p < 0.05$) (Figure 1A). In the root, *Spartina alterniflora* and *Sarcocornia ambigua*, despite having less activity than *Nothoscordum Sarcocornia ambigua*, had higher activity than all other species (respectively 13.1±2.6 and 11.2±1.4 mU.mg protein^{-1}; $p < 0.05$) (Figure 1A).

Considering all species, the mean GST activity value of root, stem and leaf were respectively 7.4±11.7, 7.4±9.4 and 5.6±5.2 mU.mg protein^{-1}. The mean values of root and stem activity are statistically similar to each other ($p > 0.05$) and they are different from mean leaf values which were minor (Figure 1). *Nothoscordum gracile* figures as the plant with the best performance, since its fold values for roots are six times greater than the second higher root activity. This plant is amphibian specie which has high GST activity especially in roots.

For the competitive kinetics assays between the biocides and the CDNB substrate, the lowest concentration values required to cause 20% inhibition (IC 20) were caused by chlorothalonil and DCOIT compounds (11.1 and 10.6 μM, respectively), followed by dichlofluanid (38.6 μM). The IC_{20} value for diuron were the highest (353.1 μM), represented by approximately 30 times greater than the IC_{20} of chlorothalonil and DCOIT, and about 10 times greater than IC_{20} of dichlofluanid. The compound irgarol did not cause any inhibition in

GST activity at the concentrations tested. Inhibition of GST to CDNB activity caused by the concentration of 100nM biocide was higher for chlorothalonil, DCOIT and dichlofluanid compounds (46.5, 49.0 and 45.1 % respectively) than for diuron (6.5 %) and was not detectable for irgarol (Table 1).

Kinetic parameters obtained show that these compounds do not affect V_{max} but increases the K_m of GST. This suggests Chlorotalonil and Dichlofluanid decrease the availability of GST to conjugate CDNB and that higher concentrations of CDNB would be required to achieve a determinated rate ($V_{max}/2$) in the presence of competitor than in their absence. Results of Lineweaver-Burk plot, indicates changes in the line slope corresponding to GST activity in the absence or presence of contaminants classified as competitive inhibitors (Figure 2). Intercepts in Y and X, when X and Y are zero, respectively, allow determining kinetic parameters shown. Linear model used for estimation of these kinetic parameters was predictive (R^2 close to 1) and significant ($p < 0.05$) (Table 1).

4. Discussion

Wetlands of the extreme south of the country have high biodiversity of aquatic macrophytes (Irgang and Gastal, 1996), therefore they can shelter species still little studied that have high potential of bioremediation (Schröder, 2007). The present study estimates and compares the GST basal activity in root, stem and leaf organs from different species of aquatic macrophytes present in wetlands. These water-rich ecosystems are especially vulnerable to the biocides tested, once some of them are used both in agriculture and ship industry. The basal GST activity found suggests that some plants would possess great capacity to remediate compounds, such as chlorothalonil, dichlofluanide and DCOIT via GST.

The macrophyte *Nothoscordum gracile* showed the highest GST activity and also have exciting characteristics that make them feasible for remediation of aquatic environments, which, according to Amaral et al. (2009), is abundant in the South American native ecosystem and can be easily cultivated for bioremediation. The genus *Nothoscordum* was being formed initially by 13 species; five excluded from the genus *Allium* most of them in South America (Stearn, 1986). The name of this genus was formed by the words nothos (false) and scordon (garlic), indicating the close relation with Allium. *Nothoscordum* is in the family *Alliaceae*, subfamily *Gilliesioideae*, same as garlic (APG IV, 2016). Plants from this family are known for the presence of bulb roots (Stearn, 1986); anatomy of this kind of root is very propitious

when it comes to the construction of an efficient phytoremediation system as long as bulb roots are very resistant to weather action and also has increased reproduction capacity (Souza, 2008). In a study with garlic Jigang et al. (2001) shown the bulb plant can accumulate substantial amounts of cadmium, they report that aquacultured garlic structures may possess the potential to provide an effective method for the removal of substance from contaminated waters. Garlic bulbs showed an efficient action in various conditions, which includes various sources of contamination, such as sewage and water used for irrigation until industrial wastes, making the bulb plants a good model to remove xenobiotics from aqueous streams and soils. If organic contaminants would be remediated as well as metals, *Nothoscordum gracile* may act similar in an equivalent phytoremediation system, this point would make *Nothoscordum gracile* bulb an advantageous structure.

It can be defined whether the inhibition in the enzymatic activity by the compounds is competitive, non-competitive or mixed in GST of plants. This classification takes into account the interaction of the inhibitor with the enzyme. Competitive inhibition may certify that the compound is a potential substrate to be biotransformed via GST. Classification was an important step to then use the inhibition values IC20 and inhibition percentage in order to estimate the biotransformation potential of the contaminants. Increased levels of chlorotalonil, DCOIT and dichlofluanid decreased the GST activity for CDNB *in vitro*, suggesting competition with CDNB by the active site of GST. Inhibition type exerted by them on GST activity to CDNB substrate was competitive inhibition. In the other hand, biotransformation capacity of Diuron could not be fully explained as long as their inhibition was mixed type, which can be seen due mismatched V_{max} at kinetics and due pattern shown in Lineweaver-Burk plots. Diuron has shown an evident uncompetitive mixed pattern and a high IC_{20} (353.1 μM), so the substance perform a low affinity with the plants' GSTs.

In this study, we attempted to quantify, through IC_{20} values and inhibition percentage, how much GST are involved in the biotransformation of five biocides by analyzing *in vitro* competitive patterns with the GST substrate CDNB. Although is known GST plays an important role in biotransformation of innumerous xenobiotics, there is little information about GSTs in the metabolism of dichlofluanid, diuron, Irgarol and DCOIT except for chlorothalonil which is a well-known GST substrate (Kim et al., 2004). Biocides with shorter half-lives, such as chlorotalonil (Guardiola et al., 2012) and dichlofluanid (Avelelas et al., 2017), have been regarded as eco-friendly alternatives, being even allowed in most European countries. Chlorothalonil and dichlofluanid are both belonging to the same chemical class of

organochlorines, so inhibition pattern in competitive assays tended to be similar. Despite the similar inhibition percentage, these substances presented a mismatched IC_{20}, being IC_{20} of chlorothalonil three times smaller than dichlofluanid. This result could suggest that chlorothalonil has a higher affinity to GSTs binding pocket domain, compared to dichlofluanid, and then chlorothalonil could be easily biotransformated by plant´s GSTs.

While chlorothalonil and dichlofluanid are still eco-acceptable, DCOIT, diuron, irgarol were already prohibited or restricted, mainly due its toxicity for non-target species as long as persistence in the aquatic environment (Price and Readman, 2013). Our results shown that DCOIT has the highest affinity to GSTs of all biocides, its IC_{20} value, although similar to chlorothalonil, is lower than the other three. GST kinetics of DCOIT molecule has never been studied before as long as this compound is an incipient product in shipping industry (Chen et al., 2016). In the other hand, diuron has shown a high IC_{20}, which suggests that the substance performs a low affinity with the plants' GSTs. In a study about the protective effective that plants like *Lemna minor* (common duckweed) possesses against diuron molecules, GST enzymes were measured, at final was observed GSTs had been poorly stimulated by the herbicide and even inhibited (Teisseire and Vernet, 2000). It reinforces our results which demonstrated diuron having low affinity with GSTs, suggesting that this compound is poorly metabolized via GST.

According to results, irgarol haven't inhibited GST activity, thus did not affect CDNB conjugation *in vitro*. This compound had previously been reported to not be well metabolized in plants (Mohr et al., 2009) what could be an answer to the absence of inhibition in assays using GST from plants. There are evidences of bioaccumulation of not phytoestabilized irgarol molecules in vegetation of places where the pollutant was applied (Dyer et al., 2006; Fernandez and Gardinali, 2016).

Once GST from vegetal sources interact well with some compounds tested, macrophytes should be exploited to phytoremediation applications, since they are an eminent source of detoxication enzymes required. The model plant used here (*Nothoscordum gracile*) showed in *in vitro* results to be highly recommended to be implemented in a phytoremediation system based on GST activity which intends to remediate some biocides with high affinity here studied, such as chlorothalonil, dichlofluanid and DCOIT. Compounds classified as competitor inhibitors in Lineweaver-Burk plots could be related with better bioremediation by GSTs performance.

5. Conclusion

Comparative measurements of detoxication enzymes in *Nothoscordum gracile* clearly demonstrated that activity is highest in roots, organ that may be exposed to higher pollution in the ground. The compounds tested diverges as regards inhibition type, being classified as competitive and mixed, besides that there is a compound which did not competed with CDNB. In the context of phytoremediation, the metabolism of phase II is indispensable to environment remediation, as long as phytoestabilization step occurs in sequence. The results obtained contribute a further specification to comparative studies between different plant species. The model plant *Nothoscordum gracile* is able to compose a phytoremediation system, since the GSTs present in roots of the vegetable metabolize a wide range of compounds considered environmental contaminants.

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Figures:

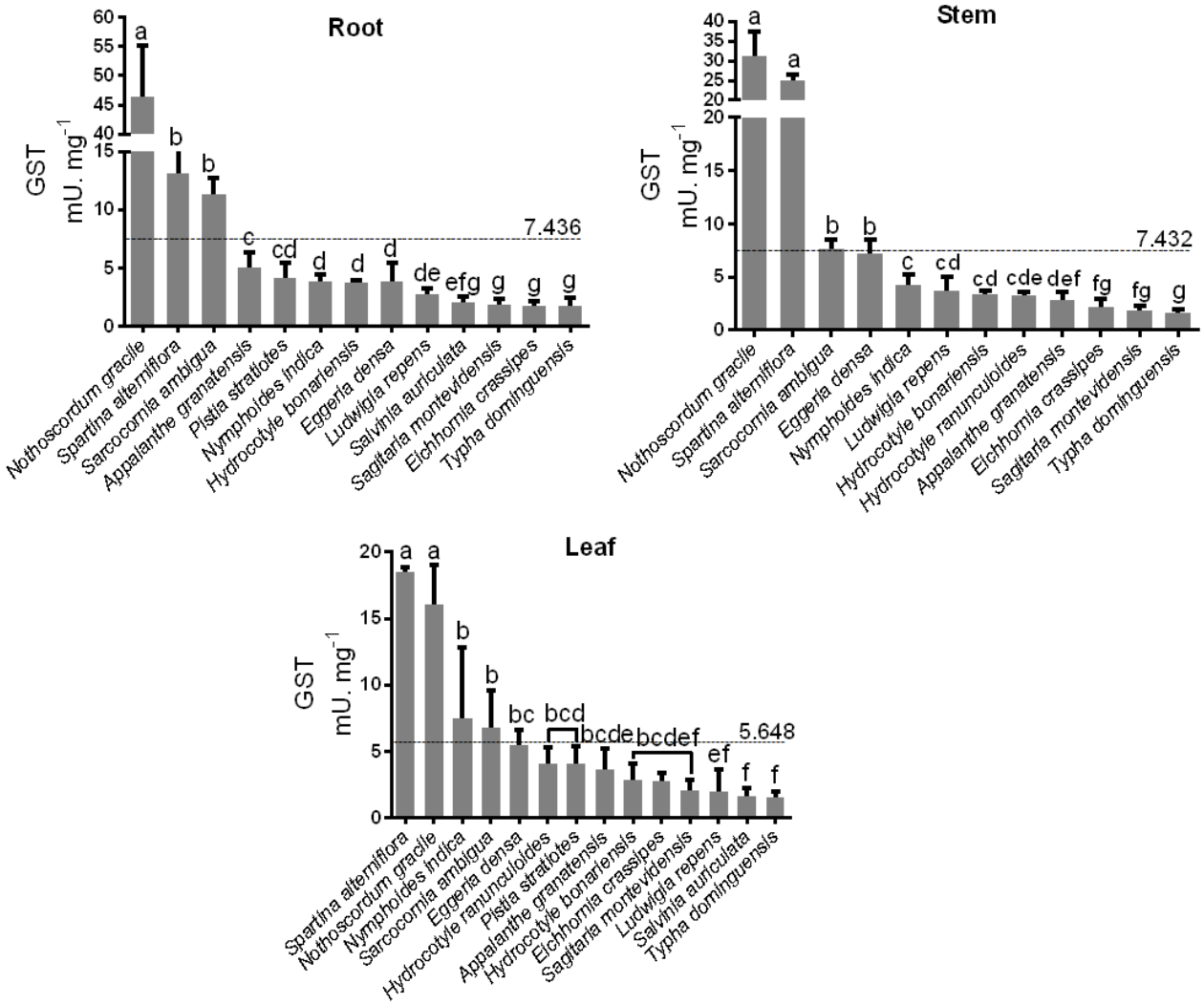

Fig. 1 Basal GST activity of roots, steam and leaves of aquatic macrophytes. Values means ± SD (n = 7). The different letters represent significant differences among groups (Two-way ANOVA followed by Tukey-HSD; $p < 0.05$). Dashed line refers to the average value of the activities of the given organ.

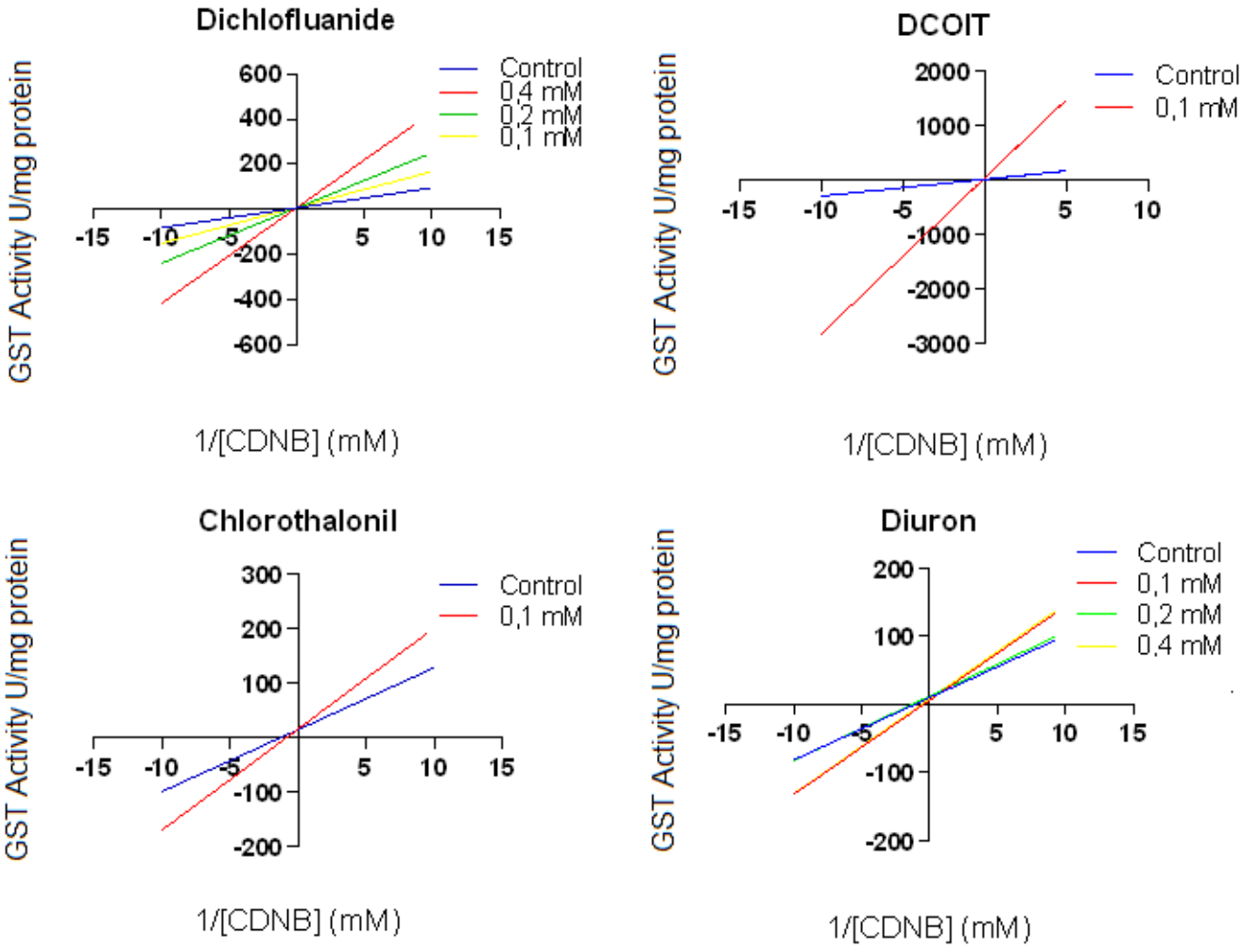

Fig. 2 Lineweaver-Burk plot showing inclination changes in the line that represents GST activity using CDNB (010, 20, 30, 40, 50, 60, 90, 120 and 150 mM) as substrate in the presence, or absence, of each compound using the cytosolic extract of *Nothoscordum gracile* roots.

Tables:

Table 1 - Kinetic parameters of GST *Nothoscordum gracile* roots cytosolic fraction in presence and absence of each contaminant, showing IC_{20} index, concentration tested and inhibition percentage. Kinetics results in presence and absence of contaminant, showing Michaelis constant, maximal rate value of reaction and linear equation validation. (**) Irgarol has not competed with GST

Contaminant	IC_{20}	Conc.	%	K_M	Vmax	Linear Equation	
	(μM)	(μM)	Inib.	(mM/min)	(mM/min)	R^2	Validation
Chlorotalonil	11.1	0	-	0.720	0.060	0.97	*
		100	46.5	1.110	0.059	0.99	*
DCOIT	10.6	0	-	0.933	0.032	0.99	*
		100	49.0	5.167	0.017	0.94	*
Dichlofluanid	38.6	0	-	1.220	0.140	0.99	*
		100	45.1	1.670	0.148	0.99	*
Diuron	353.1	0	-	1.111	0.111	0.99	*
		100	6.53	1.156	0.126	0.97	*
Irgarol	**	0	-	1.436	0.155	0.99	*
		100	**	**	**	**	**

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os resultados deste trabalho demonstram o potencial das GSTs de origem vegetal, colocando as macrófitas como espécies aptas a sistemas de remediação baseados em detoxificação via GSTs. Desta forma, sugerimos que estudos futuros abordem outros organismos que possuem GSTs tais como microalgas, além de considerar outros cenários de impactos ambientais. A capacidade da GST para biotransformar os biocidas testados observada pelos padrões de atividades basais GST estimadas nos ensaios *in vitro*, sugere como uma abordagem que pode ser adequada para estudar a interação das enzimas com os xenobióticos de interesse.